CYCLIC AMP AND COLD ACCLIMATION

Thesis presented by

MICHELLE LETARTE-MUIRHEAD

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

June 1972

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Jean Himms-Hagen for her constant encouragement and guidance throughout this work.

I wish to thank the National Research Council for the Centennial Scholarship which supported me during my studies.

SUMMARY

Cold acclimation is known to be characterized by an increased calorigenic response to catecholamines which is observed when cold acclimated rats are exposed to cold or when they are infused with noradrenaline. Although it is known that stimulation of adenyl cyclase is involved in many metabolic effects of catecholamines it is not known whether such a stimulation is an essential part of the calorigenic effect of catecholamines nor is it known whether the enhanced calorigenic effect of noradrenaline in cold acclimated rats is associated with a change in the adenyl cyclase system. The aim of the work described in this thesis was to see if any change in the amount and/or properties of the adenyl cyclase system in tissues of cold acclimated rats could account for the enhancement of the calorigenic response to catecholamines in these animals.

The activities of adenyl cyclase and phosphodiesterase were measured in brown adipose tissue and skeletal muscle, tissues believed important in the process of nonshivering thermogenesis, in warm acclimated rats and in cold acclimated rats during and after the development of the adaptation to cold. A possible change in the sensitivity of the adenyl cyclase to catecholamines was assessed from dose-response curves for the stimulation by noradrenaline and adrenaline of adenyl cyclase of brown adipose tissue and skeletal muscle. In addition, in order to further assess the role of cyclic AMP in mediating the calorigenic response to noradrenaline in warm acclimated rats and the enhanced

calorigenic response to noradrenaline in cold acclimated rats the concentration of cyclic AMP itself was measured in various tissues of rats exposed to cold or receiving an infusion of noradrenaline.

No evidence was obtained for any increase in the amount of catecholamine-sensitive adenyl cyclase in the brown adipose tissue or for any increase in the sensitivity to catecholamines of the adenyl cyclase of brown adipose tissue of cold acclimated rats. On the contrary, the specific activity of the noradrenaline-stimulated and of the adrenaline-stimulated adenyl cyclase was reduced by cold acclimation. Unlike the catecholamine-stimulated adenyl cyclase the fluoride-stimulated enzyme did undergo rapid changes in response to cold exposure and evidence was obtained for the existence of an extra adenyl cyclase, not sensitive to catecholamines, which appeared within two days of exposure to cold, persisted throughout cold acclimation and practically disappeared within one day when the cold acclimated rats were returned to room temperature. The appearance of this extra adenyl cyclase activity does not seem to be due to synthesis of new protein since doses of cycloheximide and actinomycin D which inhibited the usual cold-induced growth of the tissue did not block the appearance of the extra adenyl cyclase activity. No hormone could be found which would stimulate the extra adenyl cyclase activity in the brown adipose tissue of the cold exposed or cold acclimated rat. Phosphodiesterase activity, measured at millimolar substrate concentration, was unchanged in the brown adipose tissue during cold acclimation.

There was no difference in the amount or in the catecholamine sensitivity between the adenyl cyclase of skeletal muscle of warm acclimated rats and that of cold acclimated rats. However, during the first few days of exposure to cold there was a transient increase in the specific activity of the noradrenaline-stimulated, the adrenaline-stimulated and the fluoride-stimulated adenyl cyclase of skeletal muscle and an increased sensitivity of the adenyl cyclase to stimulation by adrenaline. Phosphodiesterase activity of skeletal muscle was not altered by acclimation to cold.

The concentration of cyclic AMP was higher in the brown adipose tissue and plasma of cold acclimated rats, regardless of whether they were in the cold or in the warm, and cold acclimated rats in the cold excreted more cyclic AMP in their urine. The increased amount of cyclic AMP excreted by the cold acclimated rats could be derived entirely from the brown adipose tissue although possible contribution by other tissues cannot be exluded. The concentration of cyclic AMP was not altered in other tissues (liver, muscle, white adipose tissue) of cold acclimated rats. Infusion of noradrenaline raised the concentration of cyclic AMP in skeletal muscle to the same level in cold acclimated rats as in warm acclimated rats. An increase in the cyclic AMP level also occurred in response to noradrenaline infusion in the plasma and liver of warm acclimated rats, to a much lesser extent in the plasma of cold acclimated rats and not at all in liver of cold acclimated rats.

CONCLUSIONS

1. No change in the amount, properties and operation of the catecholamine-sensitive adenyl cyclase system of the tissues important

in nonshivering thermogenesis (brown adipose tissue, skeletal muscle) occurs in association with the development of the enhanced calorigenic response to noradrenaline in cold acclimated rats. Thus the enhancement of this response brought about by the acclimation to cold cannot be attributed to a change in the system involved in the initial interaction of noradrenaline with the tissues involved.

2. A transient increase in amount and sensitivity to adrenaline of the adenyl cyclase of skeletal muscle appears to occur in association with shivering rather than with nonshivering thermogenesis. An increased defatiguing effect of adrenaline on the shivering muscles may occur at this time.

3. The increases in cyclic AMP concentration in the brown adipose tissue and the plasma of cold acclimated rats are associated with the state of adaptation rather than with the acute stimulatory effect of cold since they persist in cold acclimated rats after their return to the warm. The appearance of an extra adenyl cyclase and the rise in cyclic AMP concentration of the brown adipose tissue during acclimation to cold may be associated with a special function, other than heat production, for this tissue in the development and maintenance of the altered state of adaptation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACTH: adrenocorticotropic hormone

BAT: brown adipose tissue

cyclic AMP: adenosine 3',5'-monophosphate

FSH: follicle stimulating hormone

GH: growth hormone

ICSH: interstitial cell stimulating hormone

LH: luteinizing hormone

β-LPH: β-lipotropic hormone

MSH: melanocyte stimulating hormone

PEP: phosphoenolpyruvi acid (tricyclohexylamine salt)

TRH: thyrotropine releasing hormone

TSH: thyrotropine (thyroid stimulating hormone)

CHAPTER I: GENERAL INTRODUCTION

A) Introduction and statement of the problem

When a rat is exposed to cold, a muscular activity, shivering, is responsible for the heat production necessary for the survival of the animal. If the rat is allowed to live continuously in the cold, the shivering decreases and finally disappears after about four weeks (Hart et al., 1956), and a different process called nonshivering thermogenesis accounts for the heat production.* If shivering is prevented by tubocurarine (Hsieh et al., 1957) warm acclimated rats die in hypothermia whereas cold acclimated rats maintain their body temperature. Cold acclimation is thus a gradual process requiring approximately four weeks during which there is a gradual increase in cold resistance and in food consumption, an elevation of metabolic rate and of peripheral temperature, and a decrease in shivering thermogenesis accompanied by an increase in nonshivering thermogenesis.

Nonshivering thermogenesis means the production of heat other than by shivering or muscular movements. It is an adaptive process which occurs only in certain newborn species (rabbit, guinea pig, rat, human), in hibernating mammals

---

* Rats which have adapted to cold (4°C) in this way will be referred to as cold acclimated rats; rats which have not lived in the cold and are kept at controlled room temperature (28°C) will be referred to as warm acclimated rats.

(bat, hamster, ground squirrel), and in certain species that have acclimated to cold (see review by Himms-Hagen, 1970). It does not occur in those same species when they are adult or not hibernating or not acclimated to cold and this is why it is called an adaptive process. It is also a facultative process which does not occur continuously but can be turned on and off in accordance with the requirements of the animal.

It is believed that the switching on and off of nonshivering thermogenesis is due to the liberation of noradrenaline from sympathetic nerve endings. Infusion of noradrenaline into animals acclimated to cold can mimick this switching on and off of heat production caused by exposure to cold. This effect of noradrenaline of increasing both heat production and oxygen uptake of animals is called the calorigenic effect of noradrenaline. At the cellular level, an increased heat production is associated with an increased rate of oxidation and this would involve in the whole animal an increase in the oxygen consumption. The metabolic rate is expressed as consumption of oxygen per unit of surface area per minute, and this is why the terms increased oxygen consumption, increased metabolic rate and increased heat production are used interchangeably. In cold acclimated rats, there is an increased calorigenic effect of noradrenaline which is believed to be a basic factor in the development of cold acclimation (Hsieh and Carlson, 1957). A rat that has lived in the cold for four to six weeks can increase its metabolic rate during infusion of noradrenaline (at room temperature) to a level that approaches its maximal capacity for oxygen consumption

while the warm acclimated rat that has lived at 28°C for the same period of time can only slightly increase its metabolic rate during noradrenaline infusion.(Depocas, 1960b).

But there is an increase in heat production in both the warm and the cold acclimated rats when they are exposed to cold. The increase is due to shivering in the warm acclimated rats and to a nonshivering process in the cold acclimated rats. In both groups there is an increased oxidation of substrates in response to a higher demand for energy and the difference between the two groups does not reside in the way they metabolize the substrates responsible for the production of heat. The difference between these two groups resides in the mechanism by which the increased oxidation can be triggered. In the warm acclimated animals, increased ADP production in skeletal muscle due to shivering can increase the rate of oxidative phosphorylation in the muscle. In the cold acclimated animals, the exact nature of the triggering mechanism is unknown but the primary event is believed to be the release of noradrenaline from sympathetic nerve endings (Himms-Hagen, 1967). Cold acclimated rats differ from warm acclimated rats in their capacity to respond calorigenically to noradrenaline. Cold acclimation is accompanied by a large enhancement of the calorigenic response to noradrenaline.

The biochemical basis of acclimation to cold is unknown. The biochemical basis of the calorigenic effect of noradrenaline which occurs in warm acclimated rats to a limited extent and in cold acclimated rats to a larger extent, is also unknown.

However, all of those metabolic effects of the catecholamines which have been elucidated are known to be mediated by cyclic AMP and it is possible that the enhancement of the calorigenic response to catecholamines in cold acclimated rats might be due to an alteration of the adenyl cyclase system of the tissues involved in the nonshivering thermogenesis process.

The aim of this work is to see if there is any change in the amount and/or properties of the adenyl cyclase system in tissues of cold acclimated rats in order to see if the enhancement of the calorigenic response to catecholamines might be due to a change in the adenyl cyclase system.

B) Existence of nonshivering thermogenesis

There are two distinct mechanisms for increasing heat production: shivering thermogenesis and nonshivering thermogenesis. When warm acclimated rats are exposed to cold, there is an increase in the electrical activity of their skeletal muscles (Sellers et al., 1954; Héroux et al., 1956). The marked and continuous increase in electrical activity (which is referred to as shivering and can be recorded with an electromyograph) observed in these animals precedes by 15 or 20 minutes the rise in oxygen uptake which occurs when rats are exposed to cold. So the heat production in warm acclimated rats exposed to cold is due to a muscular activity, shivering. Cold acclimated rats show no change in the electromyogram when exposed to cold (6°C) implying that no shivering is present in those animals (Héroux et al., 1956). But the cold acclimated rats show a larger increase in metabolic rate upon exposure to

cold than do warm acclimated rats, and the mechanism responsible for this heat production, because it involves no shivering, is called nonshivering thermogenesis.

The disappearance of shivering in rats exposed to cold is proportional to the period of exposure to cold. During the first week, the electrical activity remains elevated and then slowly begins to fall, and in four weeks, it has reached the level observed in rats maintained at 30°C (Hart et al., 1956). So nonshivering thermogenesis is a gradual process requiring approximately four weeks during which there is a gradual decrease in shivering and a gradual increase in heat production by means other than muscular activity.

It is possible to prevent shivering by treating rats with tubocurarine, a compound which can block muscular activity. When curarized cold acclimated rats are exposed to cold, they are still able to increase their heat production while curarized warm acclimated rats are unable to do so and show a decrease in their body temperature (Cottle and Carlson, 1956; Hsieh and Carlson, 1957). In other words shivering, which is essential for heat production in warm acclimated rats exposed to cold, is replaced by another mechanism of heat production in cold acclimated rats during cold exposure, a mechanism called nonshivering thermogenesis.

C) Involvement of the sympathetic nervous system in acclimation to cold

1) *acute exposure to cold*

One of the ways to see the involvement of the sympa-

thetic nervous system during acute exposure to cold is to measure the levels of noradrenaline and adrenaline and their metabolites in the urine of animals exposed to cold. It was first reported by Leduc (1961) that cold exposure caused an increase in the excretion of noradrenaline and adrenaline in warm acclimated rats. Since then, the increased urinary excretion of adrenaline and noradrenaline and their metabolites (metanephrine, normetanephrine, MHPG (3-methoxy-4-hydroxy-phenylglycol) and MHMA (3-methoxy-4-hydroxy-mandelic acid) has been shown in different species of animals exposed to cold (see review by Himms-Hagen, 1972b). A value which might be called the minimum rate of catecholamine production has been calculated by Himms-Hagen (1972b) from the values of the excretion of catecholamines and their metabolites; this value is approximately 12µg/kg/hr for warm acclimated rats at room temperature and 45.5µg/kg/hr for warm acclimated rats exposed to cold (Himms-Hagen, 1972b). Thus, there is an increased excretion of catecholamines during acute exposure to cold.

Another approach to the study of the involvement of the sympathetic nervous system in cold exposure, is to measure the turnover rate of catecholamines in tissues of animals exposed to cold. The turnover rate of noradrenaline is increased in the heart of cold exposed mice (Oliverio and Stjarne, 1965) and rats (Bhagat and Friedman, 1969). There is a depletion of adrenaline and noradrenaline content of brain stem and heart of cold exposed rats (Gordon et al., 1966). There is also depletion of the noradrenaline content of liver, muscle,

spleen and brown adipose tissue in cold exposed animals (see review by Himms-Hagen, 1972b).

So, it is possible that noradrenaline turns over in nerve endings at an accelerated rate in animals acutely exposed to cold. In cold exposed animals, there is an increase in the excretion and probably in the synthesis and most likely in the secretion of catecholamines, phenomena which favor the participation of the sympathetic nervous system in acute exposure to cold.

2) acclimation to cold

The involvement of the sympathetic nervous system in cold acclimation is also shown by an increased excretion and probably by an increased synthesis and an increased secretion of catecholamines in cold acclimated animals.

The excretion of catecholamines and their metabolites is less in cold acclimated rats than in acutely cold exposed rats. In rats that have lived in the cold for four weeks, the excretion of adrenaline has returned to the low level observed in warm acclimated rats; the excretion of noradrenaline is also less than during the first week of exposure to cold but it remains elevated as long as the rats continue to live in the cold (up to six months) (Leduc, 1961; Leblanc and Nadeau, 1961). The excretion of MHPG remains at a high level in cold acclimated rats although it is less than after 24 hours of exposure to cold (Shum et al., 1969). The excretion of normetanephrine reaches a maximum after two weeks of exposure to cold and decreases thereafter (Shum et al., 1969).

There are very few measurements which would imply a higher synthesis of catecholamines in cold acclimated animals. The noradrenaline content of the tissues of cold acclimated rats is the same or higher than in warm acclimated rats (Leduc, 1961), and the turnover of noradrenaline in the brown adipose tissue of cold acclimated rats is increased (Cottle et al., 1967).

Although more measurements of the turnover rate of catecholamines in different tissues of cold acclimated animals should be done, it can be postulated that there is increased synthesis of catecholamines in cold acclimated animals; there is increased excretion and most likely increased secretion of catecholamines in cold acclimated animals. The sympathetic nervous system is then probably involved in the acclimation to cold.

3) mimicking effects of the administration of adrenaline and noradrenaline

Cold acclimated rats show an enhanced calorigenic response to administered catecholamines. Hsieh and Carlson (1957) first showed that an intramuscular injection of noradrenaline caused an increase in oxygen uptake in both warm and cold acclimated rats, although the increase was much larger in cold acclimated than in warm acclimated rats. The intravenous administration of noradrenaline (Depocas, 1960b) or adrenaline (Himms-Hagen, 1969) causes a much larger increase in oxygen consumption than does intramuscular or subcutaneous administration. The calorigenic response to intravenously administered catecholamines is greatly enhanced by cold accli-

mation (Depocas, 1960b; Himms-Hagen, 1969). The calorigenic response to catecholamines increases slowly with the time of exposure to cold and is maximum after four weeks (Depocas, 1960b; Mejsnar and Jansky, 1971). As the calorigenic response to catecholamines increases, the shivering decreases and it can be said that nonshivering thermogenesis is closely associated with an enhanced calorigenic response to catecholamines. Both noradrenaline and adrenaline are involved in nonshivering thermogenesis and the whole sympathetic nervous system, including the adrenal medulla, participates in the process of nonshivering thermogenesis.

Another line of evidence concerning the role of the sympathetic nervous system in cold acclimation, comes from the possibility to simulate a state of cold acclimation in warm acclimated rats by injecting them daily, for approximately six weeks, with small doses of noradrenaline. Rats injected daily for six weeks with noradrenaline have an enhanced calorigenic response to catecholamines; they can survive exposure to lower temperatures for longer periods of time than control rats and the growth of their brown adipose tissue is promoted (Leblanc and Pouliot, 1964; Leblanc and Villemaire, 1970).

So, not only can rats progressively increase their calorigenic response to catecholamines with prolonged exposure to cold but they can reach a state similar to that of cold acclimation if they are injected daily for six weeks with noradrenaline at room temperature. The observations that cold

acclimated rats respond more to administration of catecholamines and that a state similar to cold acclimation can be induced by repeated administration of noradrenaline, demonstrates the importance of the sympathetic nervous system in cold acclimation.

4) inhibition of the activity of the sympathetic nervous system

It is possible to inhibit part of the functioning sympathetic nervous system by surgical, immunological or pharmacological means. In order to see the role of adrenaline in cold acclimation, rats can be demedullated (by removal of the adrenal medulla which is responsible for the production of adrenaline). Young rats lacking their adrenal medulla do not maintain their body temperature and do not survive very long when exposed to cold (see Himms-Hagen, 1972b). But older rats when demedullated can survive in the cold for several months. However, if rats already cold acclimated are demedullated, and then reexposed to cold, they show a much smaller increase in metabolic rate and become somewhat hypothermic (Cottle and Carlson, 1956). So the adrenal medulla and consequently adrenaline, seem to play an important role in the heat production in young rats and in cold acclimated rats; but, in older rats, the sympathetic nervous system can compensate for the removal of the adrenal medulla by increasing the role of some of its other constitutive parts in order to maintain survival of the older animals (see review by Himms-Hagen, 1972b).

Immunosympathectomy (which results in the lack of adrenergic nerve endings in some tissues such as heart and blood vessels of skeletal muscle but not in tissues such as intestine and reproductive organs) does not alter the ability of rats to survive in the cold. This appears to be due to the remnant of the sympathetic nervous system still functioning in these rats since their excretion of noradrenaline is not diminished by the intervention (see Himms-Hagen, 1967). So the immunological techniques are not very useful in the study of the involvement of the sympathetic nervous system in cold acclimation since it is almost impossible to render the sympathetic nervous system partially inactive because of the compensation by the other parts of the system.

Administration of the ganglionic blocking agent hexamethonium, and of the adrenolytic agent piperoxane, prevents the rise in oxygen uptake normally observed when cold acclimated rats are exposed to cold. These rats were curarized to prevent any heat production by muscular activity. The effects of hexamethonium could be reversed by injecting the rats intramuscularly with adrenaline or noradrenaline (Hsieh and Carlson, 1957). So, blocking the sympathetic nervous system activity, prevents the normal manifestations of cold acclimation (higher calorigenic response to catecholamines) while treatment of rats with adrenaline and or noradrenaline can restore the state of acclimation to cold to its full expression.

When the sympathetic nervous system can be successfully inhibited, the ability of animals to acclimate to cold is impaired, and this emphasizes the involvement of the sympathetic nervous system in the process of nonshivering thermogenesis responsible for the acclimation of animals to cold.

D) Sites of nonshivering thermogenesis

Nonshivering thermogenesis then seems to be a process mostly due to the action of noradrenaline liberated from nerve endings in the different tissues of the animal acclimated to cold. The location of the heat production, or in other words, the location of the nonshivering thermogenesis process, in the different tissues of the cold acclimated animals is a difficult problem to study. Different approaches have been taken, most of them not leading to any conclusive evidence.

The respiration of tissue preparations in vitro does not correspond to the oxygen consumption of the tissue in the intact animal and it is impossible to estimate the relative contribution of different tissues to the total heat production of the animal by comparing their rates of in vitro metabolism.

The best way to assess the heat production of a tissue in an intact animal is by measuring its oxygen consumption but there are problems in assessing this parameter in the intact animal. Blood flow measurements to different tissues of both cold and warm acclimated rats have been obtained (Jansky and Hart, 1968). But measurement of the blood flow to a tissue is only the expression of the total amount of blood

and of the total amount of oxygen entering the tissue. The tissue does not usually consume all the oxygen contained in the blood entering the tissue and only when the arterio-venous difference is obtained for a tissue is it possible to estimate the amount of oxygen consumed by the tissue. Blood flow measurements must then be accompanied by a measurement of the arterio-venous difference across the tissues studied in order to indicate how much oxygen a tissue is taking in and consuming, and comparisons of the arterio-venous differences and of the blood flow measurements of the tissues of the warm and the cold acclimated rats must be done, in order to assess the importance of the different tissues during cold acclimation. Direct measurements of the oxygen consumption of individual tissues (by arterio-venous differences and blood flow measurements) have been done for different tissues in different species (see review by Jansky, 1965) and extrapolations of these values have been used to measure the percentage of oxygen consumed by different tissues of warm acclimated rats (Jansky, 1966). The carcass (including skeletal muscles, bone and skin) consumed 63.5% of the total oxygen consumed by the rat; the liver, 20.4%; the heart, 2.5%; the kidneys, 6.6%; the brain, 2.8%; the lungs, 0.6% (Jansky, 1966). Most of the oxygen consumed by an animal is consumed by the skeletal muscles and the liver, and the possible contribution of these tissues to the increased metabolic rate observed during non-shivering thermogenesis has been studied.

Measurements of cytochrome oxidase activity of different tissues have also been used to estimate the participation

of different tissues to nonshivering thermogenesis. Jansky (1966) made the assumption that the total oxidative metabolism of all the tissues of an animal could not be greater than the sum of the activities of cytochrome oxidase, the terminal enzyme of the oxidation chain, and he arrived at a fairly good agreement between the cytochrome oxidase activity of different tissues (which they called the metabolic capacity of the tissues) and the maximal metabolism of the same tissues measured in vivo (from blood flow measurements and arterio-venous differences) (Jansky, 1965). But it is possible (Himms-Hagen et al., 1972) to obtain values of cytochrome oxidase activity much higher than those obtained by Jansky and co-workers (Jansky, 1966; Jansky et al., 1969), which would give values higher than maximal for the metabolism of the whole animal. So, measuring the capacity of a system to consume oxygen (cytochrome oxidase activity) does not give a measurement of the amount of oxygen actually consumed by the system.

It is possible to measure the contribution of a tissue to nonshivering thermogenesis by observing the effects of its removal on the oxygen consumption of the whole animal. This method also presents problems; removal of a vital organ can have effects other than direct ones on the oxygen consumption of an animal (the general metabolism of the animal can be impaired so much that the animal will not survive more than a few hours after the treatment). Removal of a tissue such as brown adipose tissue is also problematic because of the

diffuse distribution of the tissue. Only the interscapular brown fat pad can be removed easily but the remnant of the brown adipose tissue can overgrow to compensate for the lack of the interscapular pad (Himms-Hagen et al., 1972). However, use of this technique has brought some useful information concerning the role of the brown adipose tissue (Himms-Hagen, 1969; Himms-Hagen et al., 1972) and of the skeletal muscle (Depocas, 1960a) in nonshivering thermogenesis.

Two lines of evidence point to the non-significant contribution of the liver to the process of nonshivering thermogenesis. First, evisceration of cold acclimated rats (which consists in occluding the blood supply to the liver and the intestine) does not significantly affect the increased oxygen consumption observed after noradrenaline infusion (Depocas, 1960b). Secondly, no change in the liver oxygen consumption is observed after perfusion of the tissue with noradrenaline, although the glucose output of the liver is increased considerably (Jansky et al., 1964). So the liver cannot respond calorigenically to the catecholamines and this makes it a very unlikely site of direct heat production in the cold acclimated rats.

Skeletal muscle contributes to a large extent to the production of heat during cold acclimation. The evisceration of cold acclimated rats showed the non-participation of the liver in nonshivering thermogenesis but it also strongly suggested the important contribution of skeletal muscle to the increased heat production (Depocas, 1960b). Direct measurement

of the oxygen consumption (using arterio-venous difference and blood flow measurement) of leg muscles have been done in situ, in anesthetized and curarized cold acclimated rats before and during cold exposure and before and during noradrenaline infusion (Jansky and Hart, 1963). The oxygen consumption of the leg muscles doubled during noradrenaline infusion or exposure to cold. The oxygen consumption in the whole animal also doubled during noradrenaline infusion or cold exposure. From these results, the contribution of skeletal muscle to the process of nonshivering thermogenesis was estimated. The total amount of oxygen consumed by the rat muscles, assuming that muscles represent 50% of the body mass and assuming that all muscles consume the same amount of oxygen per unit of weight, only represented 12% of the total oxygen consumed by the rat. But the quantitative comparison of the response of the leg muscle and of the whole rat, cannot be carried too far because the leg muscle may not be responding maximally under these experimental conditions, and it may be responding less than other muscles of the body. But, if it is assumed that all cells of the body have the same average metabolic rate which is represented by that of the leg muscle, then the relative contribution of the muscle can be estimated in another way. As was said before, muscle accounts for 50% of the total mass of the rat (which makes it a very good potential tissue for nonshivering thermogenesis as far as the mass of the tissue is concerned), and in the experiment being described, 50% of the total body mass has doubled its oxygen consumption while the whole body has also doubled its oxygen

consumption; it can then be calculated that the relative contribution of muscle to the heat production is 50%. The assumptions made are unlikely to be true but the fact that muscle can double its oxygen consumption in cold acclimated rats exposed to cold or receiving noradrenaline at room temperature (Jansky and Hart, 1963) (Mejsnar, 1971), and the fact that it represents approximately 50% of the total body mass of the rat, makes it a very likely important contributor to the process of nonshivering thermogenesis.

There has been recently a lot of controversy about the significant contribution of brown adipose tissue to heat production during the process of nonshivering thermogenesis. The properties, distribution and heat production of the brown adipose tissue have been reviewed (Johansson, 1959; Smith and Horwitz, 1969; Himms-Hagen, 1970), and will only be summarized here in order to ascertain how brown adipose tissue could contribute significantly to nonshivering thermogenesis. The rate of oxygen uptake per unit of mass, either in vivo or in vitro, is very high in the brown adipose tissue. The cells carry a very good potential for metabolic power, both absolutely and relative to other cell types. The cells contain a large number of mitochondria which under the electron microscope appear to be heavily laminated with cristae (Smith and Horwitz, 1969). The oxygen uptake of isolated cells, slices or homogenates from rat interscapular brown adipose tissue can be stimulated by noradrenaline to levels corresponding to those observed in the whole rat (Himms-Hagen, 1970). When normal rats are exposed to cold, the brown adipose tissue

hypertrophies and there is proliferation of mitochondria and more specifically of mitochondrial inner membrane with increases in activity of the respiratory chain enzymes of the inner membrane.(Skala et al., 1970b). The brown adipose tissue, and particularly the interscapular brown adipose tissue, is the warmest region of the body when nonshivering thermogenesis occurs and under those circumstances the oxygen uptake, the blood flow and the temperature of the tissue are increased (Himms-Hagen, 1970). Brown adipose tissue has a high content and turnover of catecholamines, and the turnover of noradrenaline is significantly increased during cold stress (Smith and Horwitz, 1969).

The distribution of the brown adipose tissue is diffuse. In the adult rat, the areas where brown adipose tissue generally occurs are:

i) a pair of brown adipose tissue pads located deep in the middorsal superior cervical region

ii) the interscapular region with ventrolateral extensions which protect the vessels of the axillary region

iii) the thorax where the tissue overlays the aorta and azygous vein as well as the sympathetic chain

iv) the extensions along the aorta and spreading out medio dorsally at the level of the kidneys where it often covers the converging iliacs and renal veins (Smith and Roberts, 1964).

The brown adipose tissue is then strategically located around major blood vessels and nerve tracts in the thoracic and cervical regions. It also protects the spinal cord all

the way down to the kidneys by providing a local heat source. The increase in brown adipose tissue mass observed with cold exposure is common to all of the major sites and the condition can lead to a doubling of the total tissue mass. It can be said that in the acutely exposed animal, the central core is protected by virtue of some closely applied heating by the brown adipose tissue.

So, the high metabolic rate of the brown adipose tissue and its capacity to respond to catecholamines as well as its strategic location would make it likely to play an important role in the increased heat production observed during cold acclimation. But, in the white rat, for example, the brown adipose tissue represents only 1% of the total body mass and this is in disfavour of an important contribution by the brown fat to the process of nonshivering thermogenesis. The contribution of the brown adipose tissue to the process of nonshivering thermogenesis has been estimated to be from 6% (Jansky and Hart, 1968) to 8% (Imai et al., 1968) by calculations based on blood flow measurements and heat production by the brown adipose tissue. Removal of the interscapular brown adipose tissue causes a decrease in the oxygen uptake of the cold acclimated rat receiving an infusion of noradrenaline. Extrapolation to the total mass of the brown adipose tissue, brings the contribution of the brown adipose tissue to 10 or 12% of the nonshivering thermogenesis (Himms-Hagen, 1969). But four days after the removal of the interscapular brown adipose tissue, the calorigenic response of the rat to noradre-

naline and adrenaline has decreased considerably compared to that of the sham operated rats. The magnitude of the decrease in the response to catecholamines is too large to be accounted for by removal of the oxygen-consuming tissue itself and it has been postulated that the brown adipose tissue has some function other than heat production during cold acclimation. It has been postulated that the brown adipose tissue secretes a "cold-acclimation factor" which can modify the ability of other tissues to respond calorigenically to catecholamines (Himms-Hagen, 1969). So although the direct contribution of the brown adipose tissue to the increased heat production in cold acclimated rats is only 10%, the tissue may have an important indirect contribution to the process of nonshivering thermogenesis by modifying the ability of other tissues to respond calorigenically to catecholamines.

E) Metabolic effects of catecholamines

Catecholamines have numerous effects on metabolism which are generally described as their metabolic effects. It is far beyond the scope of this presentation to discuss these numerous effects of the catecholamines. The subject has been discussed extensively by Himms-Hagen (1972a). Catecholamines increase the breakdown of triglycerides mainly in adipose tissue but also in skeletal muscle, heart, liver and brown adipose tissue. Some of the consequences of the lipolytic effect of catecholamines are the increased concentrations of FFA, glycerol and ketone bodies in the blood and the increased oxidation of free fatty acids. The primary direct effect of catecholamines

on carbohydrate metabolism is to modify the activities of glycogen synthetase and glycogen phosphorylase in liver and other tissues as well. The principal result of this action is the increased breakdown of glycogen and an increase in the blood glucose level. The latter is further amplified by the decrease in the production of insulin by the pancreas caused by the catecholamines. Direct effects of catecholamines on carbohydrate metabolism occur in liver, striated and smooth muscles, heart and adipose tissue (see review by Himms-Hagen, 1967). The consequences of the metabolic effects of catecholamines on the whole animal are a shift in the energy reserves, mainly glycogen from the liver and triglycerides from white adipose tissue, for use by other tissues. The sum of these metabolic effects can only account for a small portion, quantitatively, of the calorigenic effect of catecholamines in warm as well as cold acclimated animals (Himms-Hagen, 1967) (Himms-Hagen, 1972a).

F) Role of cyclic AMP in mediating the metabolic effects of catecholamines

All of those effects of the catecholamines which have been elucidated have been shown to involve the adenyl cyclase system (see review by Robison et al., 1971). Cyclic AMP was discovered by Sutherland and Rall in 1957. Its role in mediating hormonal action has now been established in numerous systems and the word cyclic AMP has become synomymous with intracellular messenger. Cyclic AMP is the second messenger for the

production of one or more of the effects of the following hormones: catecholamines, ACTH, glucagon, LH, TSH, MSH, TRH, prostaglandins, thyroid and parathyroid hormones, vasopressin, histamine and serotonin (Robison et al., 1971).

The chain of events by which a given hormone can cause an intracellular effect is the following:

a) The hormone interacts with its target tissue at the receptor site (broadly defined as that part of the cell membrane with which the hormone interacts specifically to produce a response).

b) The receptor is in close contact with adenyl cyclase, plasma membrane bound enzyme which catalyzes the formation of cyclic AMP from ATP. The interaction of the hormone with its receptor causes activation or inhibition of adenyl cyclase which in turn leads to increased or decreased intracellular level of cyclic AMP.

c) Cyclic AMP can then have one of many different effects depending on the tissue concerned. Most, if not all of the effects of cyclic AMP, can be attributed to the activation of some enzyme, usually a protein kinase.

d) Cyclic AMP is then hydrolyzed to AMP by an enzyme called cyclic AMP-phosphodiesterase, a soluble enzyme first discovered by Sutherland and Rall (1958).

The level of cyclic AMP represents a balance between the activities of adenyl cyclase and phosphodiesterase. A small stimulation of adenyl cyclase by a given hormone can cause important changes in cellular activity; cyclic AMP is said to be a very sensitive regulator of cell function (Robison et al., 1971).

Although it is known that stimulation of adenyl cyclase is involved in the effects of catecholamines on carbohydrate and lipid metabolism, it is not known whether such a stimulation is an essential part of the calorigenic effect of catecholamines and the role of cyclic AMP in the calorigenic effect of catecholamines has been little studied. Strubelt (1968) injected high doses of cyclic AMP (50 and 100 mg/kg, intravenously) in the whole rat and did not observe any effect on the oxygen consumption. No conclusion can be drawn from this negative result because of the low permeability of cells to cyclic AMP and also because injecting large doses of an intracellular mediator of the actions of many hormones on different tissues can cause numerous effects unrelated to the calorigenic effects of catecholamines. Strubelt and Siegers (1969) injected an inhibitor of cyclic AMP phosphodiesterase in order to cause an accumulation of cyclic AMP in the cells and to see if this would lead to an increased calorigenic response in the whole rat. Theophylline and caffeine (60mg/kg) could increase oxygen consumption by 65% in awake rats, and by 38% in anesthetized rats. But both theophylline and caffeine are well known to cause a release of catecholamines from nerve endings. In fact, the calorigenic effect of 6.6mg/kg of theophylline was completely abolished by pretreatment of rats with reserpine (which can cause depletion of noradrenaline from nerve endings) but the response to 60mg/kg of theophylline was not blocked by reserpine but was blocked completely by the β-blocking agent propanolol (Strubelt and Siegers, 1969). Calorigenic effects of small doses of methyl xanthines were entirely due

to their effect on the release of catecholamines from nerve endings but the effects of larger doses were possibly due not only to indirect but also to direct effects of the methyl xanthines, i.e. on the accumulation of cyclic AMP in the cells and not only on the release of catecholamines from nerve endings (Strubelt and Siegers, 1969). No conclusions can be drawn from those results concerning the role of cyclic AMP in the calorigenic response to catecholamines.

G) Possible role of cyclic AMP in nonshivering thermogenesis and in cold acclimation

It is possible that the enhancement of the calorigenic response to catecholamines observed in cold acclimated rats might be due to an alteration of the adenyl cyclase system of the tissues involved in the nonshivering thermogenesis process. In the present study, in order to assess the role of cyclic AMP in acclimation to cold, the activities of adenyl cyclase and cyclic AMP phosphodiesterase were measured in brown adipose tissue and skeletal muscle, tissues believed important in the process of nonshivering thermogenesis. Such measurements have been made during the development of the adaptation to cold to see if any changes in the amount and/or properties of these enzymes occurs in association with the development of the capacity for nonshivering thermogenesis. This higher capacity of the cold acclimated rats to respond calorigenically to catecholamines could be due to an increase in sensitivity to noradrenaline and adrenaline of the adenyl cyclase system. The sensitivity of the enzyme to adrenaline or noradrenaline can be

measured, in vitro, by doing a dose-response curve for those two catecholamines. A change in sensitivity of the adenyl cyclase to adrenaline or noradrenaline would give rise to a shift in the dose-response curve. Dose-response curves for the stimulation of adenyl cyclase by noradrenaline and by adrenaline were done in the brown adipose tissue and the skeletal muscle of warm and cold acclimated rats.

The activity of an enzyme only measures the capacity of a system to carry on a given reaction. In order to assess the amount of a given metabolic intermediate at any one time, the actual level of the compound must be measured in vivo under the experimental conditions being studied. So, to further assess the role of cyclic AMP in acclimation to cold, the levels of cyclic AMP were measured in brown adipose tissue and skeletal muscle and also in white adipose tissue, liver, plasma and urine of warm and cold acclimated rats. If cyclic AMP is involved in the acclimation to cold, higher levels of cyclic AMP should be found especially in the tissues known to be important in the process of nonshivering thermogenesis. The possible role of cyclic AMP in the mediation of the calorigenic effect of noradrenaline in the warm and the cold acclimated rats was studied by measuring levels of cyclic AMP in different tissues of warm and cold acclimated rats after infusion of concentrations of noradrenaline known to increase the oxygen consumption of the animals.

H) Summary

Cold acclimation is characterized by an increased calorigenic response to catecholamines which is observed when cold

acclimated animals are exposed to cold or infused with catecholamines.

Cyclic AMP is the intracellular mediator of the action of many hormones on many tissues including those of catecholamines on different tissues.

The purpose of this study is to see if the adenyl cyclase system is involved in cold acclimation. The activities of adenyl cyclase and cyclic AMP phosphodiesterase were measured in brown adipose tissue and skeletal muscle (tissues believed important in the acclimation to cold) of warm and cold acclimated rats. Such measurements were also made during the development of the adaptation to cold to see if any changes in the amount and/or properties of these enzymes occur in association with the development of the capacity for nonshivering thermogenesis.

The increased calorigenic response to catecholamines observed with nonshivering thermogenesis could be due to an increase in sensitivity of adenyl cyclase to catecholamines. Dose-response curves for catecholamines of adenyl cyclase of brown adipose tissue and skeletal muscle were done with tissues from warm and cold acclimated rats.

Levels of cyclic AMP were measured in different tissues of warm and cold acclimated rats, in order to see if in the intact animal, cold acclimation had caused an increase in the concentration of the cyclic nucleotide. To see if cyclic AMP is involved in the mediation of the calorigenic effect of noradrenaline, tissue levels of the cyclic nucleotide were measured after noradrenaline infusion, in warm and cold acclimated rats.

CHAPTER 2: ADENYL CYCLASE IN TISSUES

Section I: Survey of adenyl cyclase

A) Distribution of adenyl cyclase

1) *tissue distribution of adenyl cyclase*

Adenyl cyclase is present in every mammalian tissue that has been studied. Catecholamines can stimulate adenyl cyclase in many mammalian tissues including liver, spleen, kidney, cardiac, smooth and skeletal muscles, brain, pineal gland, white adipose tissue, pancreas, leucocytes, parotid gland, lung (see review by Robison et al., 1971), and brown adipose tissue (Skala et al., 1970a; Forn et al., 1970a). Adenyl cyclase is stimulated by ACTH in adrenal cortex and adipose tissue, and by glucagon in liver, pancreas, adipose tissue and heart. Adenyl cyclase is stimulated by parathyroid hormone in kidney and bone, by TRH in anterior pituitary, by TSH in thyroid gland and by thyroxine in heart and white adipose tissue. Adenyl cyclase is also stimulated by vasopressin in various epithelial tissues, by LH in ovarian and testicular tissues, by histamine in the brain and by prostaglandins in various tissues (see review by Robison et al., 1971). Adenyl cyclase is then widely distributed in mammalian tissues where it is stimulated by various hormones depending on the tissue considered.

Adenyl cyclase is also present in species other than mammals. It is present in avian skeletal muscle (Sutherland et al., 1962)

and erythrocytes (Davoren and Sutherland, 1963) where it is stimulated by catecholamines; in amphibian erythrocytes where again it is stimulated by catecholamines (Rosen and Rosen, 1969). Adenyl cyclase is also present in fish, insects and segmented worms (Sutherland et al., 1962). In the liver fluke Fasciola hepatica adenyl cyclase is stimulated by serotonin (Mansour et al., 1960). In bacteria, adenyl cyclase is not, as may be expected, stimulated by hormones; but it can be dependent or not on pyruvate for activation according to the type of bacteria studied (Hirata and Hayaishi, 1967; Khandelwal and Hamilton, 1971; Tao and Lipmann, 1969).

2) subcellular distribution of adenyl cyclase

Adenyl cyclase appears to be a constituent of the plasma membrane in those cell types in which its subcellular distribution has been studied. Sutherland and coworkers (1962) reported that particulate fractions from a number of tissues were able to synthesize cyclic AMP when they were incubated with ATP and Mg^{++}. The adenyl cyclase of rat liver homogenate sedimented at 1,000g, in the so-called "nuclear" fraction which may have contained both nuclei and cell membranes. It was concluded that these two cell components were the principal candidates for the location of adenyl cyclase (Sutherland et al., 1962). Under certain conditions of homogenization, nuclei and cell membranes can both be sedimented together by centrifugation at low speed but can be separated effectively in certain preparations by utilizing a dispersion procedure (such as pressure homogenizer) which extensively fragments the membranes but

does little damage to the nuclei. Davoren and Sutherland (1963) used this technique with pigeon erythrocytes and rat liver preparations and showed that adenyl cyclase activity under these conditions was located in the cell membrane. The different subcellular fractions were identified by electron microscopy studies.

The activity of adenyl cyclase can sediment with different subcellular fractions depending on the tissue studied and on the degree of homogenization. But electron microscopy studies or enzyme marker studies should be done before assigning the activity of the enzyme to a particular organelle. In rat cerebral cortex, for example, most of the adenyl cyclase activity sediments with the mitochondrial fraction but electron microscopy studies have shown that this fraction contains mainly synaptic membranes (De Robertis et al., 1967). In all cases well studied, adenyl cyclase has been found associated with the cell membrane (Davoren and Sutherland, 1963; De Robertis et al., 1967; Skala et al., 1970a; Seraydarian and Mommaerts, 1965).

3) adenyl cyclase in brown adipose tissue

During the course of this study, two papers have been published on adenyl cyclase in the brown adipose tissue. Forn and coworkers (1970a) have shown that denervation does not affect the stimulation of adenyl cyclase from brown fat cells homogenates by noradrenaline and NaF. Skala and coworkers (1970a) have measured adenyl cyclase activity in homogenates of BAT* from young rats and have observed that adenyl cyclase could be

---

BAT: brown adipose tissue (see list of abbreviations)

stimulated by noradrenaline and NaF and that most of the activity was associated with the 100,000g pellet and supernatant, as well as with the 650g pellet. Parallel studies of the subcellular distribution of the plasma membrane marker 5'-nucleotidase showed a similar pattern of sedimentation and it was concluded that the plasma membrane had been broken in small fragments during homogenization and that adenyl cyclase was in fact as expected in the plasma membrane of brown fat cells (Skala et al., 1970a).

A variety of hormones are known to stimulate lipolysis in BAT slices. Joel (1965) has reported the stimulation by glucagon, ACTH, TSH, noradrenaline and adrenaline of lipolysis and oxygen consumption of slices of BAT. Beviz and coworkers (1971) have shown that noradrenaline, ACTH and serotonin can stimulate O_2 consumption and the production of lactate and glycerol in BAT slices. These effects were blocked by the β-blocking agent MJ 1999 (Beviz et al., 1968). Skala and coworkers (1970a) could not observe any significant effect on adenyl cyclase from BAT homogenate of ACTH, beta-MSH, alpha-MSH, GH, TSH, secretin, glucagon, beta-LPH, ICSH, vasopressin, thyroxin, insulin, prostaglandin E_1, oxytocin or dexamethasone. This could mean that adenyl cyclase is selectively stimulated by catecholamines but it is also possible that homogenization damages the receptors for some of these hormones, especially those able to affect the respiration and the lipolysis of BAT. In isolated brown fat cells, only catecholamines stimulated the oxygen uptake while serotonin, insulin, glucagon, ACTH and prostaglandin E_1 were

without effect (Girardier and Seydoux, 1971). So, only in slices and not in homogenates or in isolated fat cells of BAT, have hormones other than catecholamines been able to stimulate respiration or lipolysis and it is possible that the positive effects observed with the tissue slices are due to an indirect effect of these hormones on the cells via the release of catecholamines from the nerve endings (Girardier and Seydoux, 1971).

It is quite clear that in the brown adipose tissue cyclic AMP mediates the calorigenic effect of the catecholamines in the warm acclimated rat. Noradrenaline can increase the concentration of cyclic AMP in incubated slices of brown adipose tissue; addition of cyclic AMP or the derivative dibutyryl-cyclic AMP or theophylline to BAT slices can increase the oxygen consumption and the release of glycerol, lactate and free fatty acids from the tissue (Beviz et al., 1968; Beviz et al., 1971; Reed and Fain, 1968). Cyclic AMP and theophylline, in combination, have a potentiating action on the oxygen consumption of BAT slices and an additive effect on lactate and glycerol production (Beviz et al., 1968).

Thus, in considering the mechanism of the calorigenic effect of the catecholamines in the intact animal, it is possible, for at least brown adipose tissue, to assume that stimulation of adenyl cyclase is a necessary step in the process.

4) adenyl cyclase in skeletal muscle

Adenyl cyclase in skeletal muscle has not been charac-

terized very well. The subcellular distribution of adenyl cyclase has however been studied in skeletal muscle (Sutherland et al., 1962; Rabinowitz et al., 1965; Seraydarian and Mommaerts, 1965). The enzymatic activity of adenyl cyclase has been associated with the mitochondrial and the microsomal fractions and it is not known if this activity derives from fragments of the sarcoplasmic reticulum or from fragments of the T system which is regarded as a continuation of the plasma cell membrane (Franzini-Armstrong, 1964). Although this constitutes all the work reported on skeletal muscle adenyl cyclase, more evidence has been obtained concerning the increase in cyclic AMP levels caused by adrenaline in skeletal muscle.

Adrenaline injected in the heart of a cat caused an increase in the level of cyclic AMP in the anterior tibialis and soleus muscles (Butcher et al., 1965). Intracardial or intraperitoneal injection of adrenaline caused a rise in the level of cyclic AMP in the rat gastrocnemius (Posner et al., 1962), and intravenous injection of adrenaline provoked an increase in the level of cyclic AMP in the mouse gastrocnemius (Lyon and Mayer, 1969). In all of those experiments, the muscles to be studied were exposed before the injection and were quickly frozen within one minute after the injection. In vitro studies were also done with rat diaphragm. Addition of adrenaline to incubated rat diaphragm caused an increase in the cyclic AMP content of the tissue (Goldberg et al., 1967; Lundholm et al., 1967; Craig et al., 1969).

Although a stimulation of the activity of adenyl cyclase by adrenaline has not been measured directly, it is quite certain that the increase in cyclic AMP in the tissue caused by adrenaline is due to a stimulation of adenyl cyclase activity.

5) variation in the amount of adenyl cyclase

Since in the present work a change in the amount and/or properties of adenyl cyclase is being sought in order to explain a change in response to catecholamines, a survey will be presented of these instances in which changes in amount and properties of adenyl cyclase have been observed. Several examples may be cited of variation of the amount and/or properties of adenyl cyclase with the stage of development of the animal or with changes in environmental stimuli.

Metamorphosis of the tadpole to the frog is associated with an increase in the specific activity of the red cell adenyl cyclase and with the appearance of sensitivity to stimulation by catecholamines (Rosen and Rosen, 1968; Rosen and Erlichman, 1969).

The mammalian pineal gland is another tissue in which the amount of adenyl cyclase can vary; when rats are kept in continuous light or when the pineal gland is denervated (both situations being associated with low or absent sympathetic stimulation of the gland) the amount of adenyl cyclase in the gland rises (Weiss, 1969). Response of adenyl cyclase to noradrenaline and NaF increases with age in the rat pineal gland (Weiss, 1971).

Denervation of the heart does not alter adenyl cyclase activity at a time when the tissue shows increased responsiveness to catecholamines (Sobel et al., 1968). Hearts from hyperthyroid cats contain normal amounts of fluoride-stimulated adenyl cyclase but contain more catecholamine-stimulated adenyl cyclase than normal hearts (Sobel et al., 1969a; Levey et al., 1969a). Hearts from hypothyroid cats have less fluoride-stimulated adenyl cyclase than hearts from euthyroid cats but respond normally to catecholamines (Levey et al., 1969b). Guinea pig failing hearts have less fluoride-stimulated adenyl cyclase than normal hearts (Sobel et al., 1969b). Adenyl cyclase from failing human hearts has lost the ability to be stimulated by glucagon (Goldstein et al., 1971).

White adipose tissue from hyperthyroid rats has more fluoride-stimulated adenyl cyclase and more catecholamine-stimulated adenyl cyclase than the tissue from euthyroid rats (Krishna et al., 1968b). White adipose tissue from hypothyroid rats has less fluoride-stimulated adenyl cyclase than normal and also has a reduced lipolytic response to catecholamines (Krishna et al., 1968b). White adipose tissue from fasting rats has less noradrenaline-stimulated adenyl cyclase than tissue from fed rats (Brodie et al., 1969); white adipose tissue from cold acclimated rats has more basal and more noradrenaline-stimulated adenyl cyclase than tissue from warm acclimated rats (Therriault et al., 1969). White fat cell adenyl cyclase from adrenalectomized or hypophysectomized rats is much less activated by ACTH than that from normal rats (Braun and Hechter,

1970). Adenyl cyclase decreases with age in rat white adipose tissue (Forn et al., 1970b).

Glucagon and adrenaline responsive adenyl cyclase activities decline with age in rat liver but are always higher in the female than in the male rat. The glucagon and adrenaline responsive components of liver adenyl cyclase vary independently as a function of age, sex and steroid hormone levels in the rat (Bitensky et al., 1970). Cholera toxin activates the basal adenyl cyclase in mouse liver to the level of the adrenaline-stimulated activity; adrenaline can then no longer stimulate adenyl cyclase in vitro while glucagon has not lost its ability to stimulate the enzyme (Gorman and Bitensky, 1972).

The activity of rat cerebral and brain stem adenyl cyclase (basal and fluoride-stimulated) increases rapidly in the first two weeks after birth, then reaches a maximum at twenty days and then declines during several weeks; in contrast, cerebellar adenyl cyclase activity increases very slowly reaching a maximum after 32 days (Weiss, 1971).

Testicular adenyl cyclase activity can be stimulated by FSH in rats less than 21 days old and in adult hypophysectomized rats but very little stimulation of adenyl cyclase by FSH is observed in testicles of mature intact rats (Kuehl et al., 1970). There is more stimulation of adenyl cyclase by LH in immature ovaries from young female rabbits than in mature ovaries (Smith and Major, 1971).

When salivary glands are stimulated to excessive secre-

tion by isopropylnoradrenaline they lose much of the adenyl cyclase activity (Schramm and Naim, 1970). There is more adenyl cyclase in the isolated islets of Langerhans of obese-hyperglycemic newborn mice than in normal newborn mice (Atkins and Matty, 1971).

Denervation of brown adipose tissue does not alter adenyl cyclase activity at a time when the tissue shows increased responsiveness to catecholamines (Forn et al., 1970b). The fluoride-stimulated adenyl cyclase activity of BAT reaches a maximum three days before birth, is minimum at birth and then slowly increases to reach again the maximum after ten days (Counis and Raulin, 1970).

So, the amount and/or properties of adenyl cyclase in different tissues can vary with changes in environmental stimuli or in the stage of development of the animal. It is then possible that a change in the temperature of acclimation of rats (from 28°C to 4°C) might induce changes in the amount and/or properties of adenyl cyclase in different tissues.

B) Purification and properties of adenyl cyclase

1) purification of adenyl cyclase

A lot of difficulty has been encountered in the attempts to purify adenyl cyclase. The fact that adenyl cyclase is a plasma membrane enzyme, probably of lipoprotein nature, has rendered difficult the solubilization and purification procedures. Sutherland and Rall (1960) have shown that adenyl cyclase is relatively labile and that it behaves much like a lipoprotein.

Attempts to purify the enzyme in higher organisms have not been very successful: a two or three fold purification of the 2000g precipitate from brain homogenate was obtained while a 15 fold purification of the 600g precipitate from liver homogenate was obtained (Sutherland et al., 1962). Rosen and Rosen (1969) were able to purify the frog erythrocyte adenyl cyclase 150 to 200 fold. The fact that the enzyme is particulate and present in the plasma membrane is responsible for the difficulties encountered in purifying the enzyme. The bacterial enzyme is not as tightly bound to the membrane and for that reason, it has been possible to solubilize it and to purify it more. In Escherichia coli, the enzyme was purified 100 fold and required Mg^{++} for activity and was inhibited by fluoride and pyrophosphate and was not stimulated by pyruvate (Tao and Lipmann, 1969). Hirata and Hayaishi (1967) purified adenyl cyclase from Brevibacterium liquefaciens about 100 fold. In addition to Mg^{++}, this enzyme preparation requires pyruvate for activity (or another α-keto-monocarboxylic acid). Khandelwal and Hamilton (1971) have purified adenyl cyclase from Streptococcus salivarius and have obtained up to 3200 fold purification. The enzyme was completely devoid of ATPase, GTPase, cyclic AMP phosphodiesterase and pyrophosphatase and was always recovered from the Sephadex G-200 column in three distinct peaks (I, II and III). Enzyme III was the most homogeneous containing only two contaminating bands on disc gel electrophoresis. The enzyme was Mg^{++} or Mn^{++} dependent and was still stimulated by NaF. The possibility that the three peaks represent three isozymes was

discussed by the authors (Khandelwal and Hamilton, 1971). The differences in the activation by NaF, in the rates of the reverse reaction and in the degree of NaF inhibition of the reverse reaction favor that hypothesis. But it is always possible that contamination in peaks I and II (which were purified 267 and 975 fold respectively compared to 3200 fold for peak III) may be altering the kinetic parameters. The bacterial enzymes do not seem to have uniform properties from one species to the other and the fact that they cannot be stimulated by hormones suggest that they may be somewhat different from the mammalian enzyme. So the purification of adenyl cyclase from higher organisms will be essential before one can explain the mechanism of the reaction and of the stimulation of that reaction by hormones and NaF.

2) properties of adenyl cyclase

a) <u>stimulation by hormones</u>

The mechanism by which a given hormone stimulates adenyl cyclase is still unknown, and will probably remain unknown until the purification of adenyl cyclase and/or of the receptor for some hormone is achieved. Adenyl cyclase behaves as a lipoprotein in most tissues and may be associated with the lipid matrix of the cell membrane (Sutherland et al., 1962; Davoren and Sutherland, 1963). Robison and coworkers (1967a) have proposed a model for adenyl cyclase in which the molecule is composed of at least two distinct subunits, a regulatory subunit facing the extracellular fluid and a catalytic subunit, the active center of which is in contact with the interior of the cell. According to this model, the hormone would be expected

to interact with the regulatory subunit which in turn might influence the configuration of the catalytic subunit. In other words, the receptor would be a part of the regulatory or discriminator subunit of adenyl cyclase and would represent the variable component of the adenyl cyclase system, varying to some extent from tissue to tissue while the catalytic subunit might be expected to be fundamentally similar in all tissues (Bär and Hechter, 1969).

There is of course some disagreement with this model. Marinetti and coworkers (1969) believe that the adrenaline receptor in rat liver is a protein different from adenyl cyclase. Their evidence is based on the following results: i) the binding of labelled adrenaline to isolated rat liver plasma membranes precedes by ten minutes the activation of adenyl cyclase (Marinetti et al., 1969). ii) Heating the membrane preparation for one minute at 90°C greatly inhibits the adenyl cyclase activity without affecting the binding of adrenaline to the membrane preparation (Tomasi et al., 1970). iii) 10^{-4}M para-chloromercuribenzoic acid increases adenyl cyclase activity while decreasing the binding of adrenaline (Tomasi et al., 1970). iv) Adrenaline is bound to a fast moving protein fraction on Sephadex G-200 which can be separated from the fraction containing the bulk of the adenyl cyclase activity (Tomasi et al., 1970). But the question of whether the receptors are part of the adenyl cyclase system or not cannot be answered, since there is no conclusive evidence one way or the other and the answer must await the purification of mammalian adenyl cyclase.

In cells in which two or more structurally different hormones are known to be capable of stimulating adenyl cyclase, the question arises as to whether all the hormones stimulate the same enzyme or whether there is a different adenyl cyclase for each hormone. For rat adipocytes, the available evidence (Rodbell, 1967; Birnbaumer and Rodbell, 1969) suggests that all the hormones (catecholamines, ACTH, thyroxine, serotonin, LH and prolactin) stimulate the same enzyme since the activity of adenyl cyclase in the presence of two hormones only reaches the activity produced by the more effective of the two (Butcher et al., 1968). The failure to produce additive effects on cyclic AMP levels in fat cells when various lipolytic hormones are tested in combination, suggests that multiple forms of hormone-sensitive adenyl cyclase are unlikely to exist. A single catalytic subunit could be associated in the cell membrane with either a single discriminator or regulatory subunit possessing different sites for different hormones or multiple discriminator subunits each of which interacts with a different hormone with the same catalytic subunit (Bär and Hechter, 1969).

In the liver, the situation is different and rather confusing. Bitensky and coworkers (1968) have been able to selectively destroy the adrenaline stimulated adenyl cyclase activity and to increase the glucagon sensitive adenyl cyclase activity by repeated washings or serial dilution of liver homogenates. The adrenaline responsive component of adenyl cyclase is also more easily damaged by heating or sonication. If glucagon and adrenaline are added in combination at saturating

concentration, the rate of cyclic AMP formation is equal to the total sum of the rates obtained by the individual hormones. Bitensky and coworkers (1970) have shown that the activities of the glucagon and adrenaline responsive adenyl cyclase can vary independently as a function of age, sex and steroid hormone levels in the rat. From those results, the authors have concluded that there were two different adenyl cyclases in rat liver, one responding to glucagon and the other responding to adrenaline. But it is possible that the greater lability of the adrenaline responsive adenyl cyclase is due to removal by washing or dilution of some factor such as Ca^{++} which would be essential for stimulation of the adrenaline component of adenyl cyclase. Marinetti and coworkers (1969) have found that a minimum concentration of 10^{-5}M Ca^{++} was necessary for stimulation of liver adenyl cyclase by adrenaline, while stimulation by glucagon was reduced by Ca^{++}. It is also possible that adenyl cyclase in different cells of the liver may have different hormonal sensitivities. A recently devised cytochemical technique (Reik et al., 1970) has shown that isopropylnoradrenaline stimulated adenyl cyclase is present in the plasma membrane of parenchymal cells but not in the plasma membrane of reticuloendothelial cells while the glucagon stimulated adenyl cyclase is present mainly in the reticuloendothelial cells. The fluoride stimulated adenyl cyclase is localized in both cell types. Although these results seem to suggest that there is more than one type of adenyl cyclase in rat liver, they do not permit one to distinguish between either more than one type of adenyl cyclase or more than one type of discriminator subunit on the

same catalytic subunit, since different discriminator subunits could have different lability and ion requirement which would lead to an apparent separation of different adenyl cyclases (Bär and Hechter, 1969). The fact that a given type of cell does not respond to stimulation by a given hormone could mean that the discriminatory subunit for that hormone is not a constitutive part of the adenyl cyclase present in that cell type. The situation would then be very similar to the one in white adipose tissue where different discriminator subunits are attached to the same catalytic subunit. It may in fact be that the catalytic subunit of adenyl cyclase is always very similar but that the discriminator subunits are different in different tissues according to the hormone by which they can be modified.

b) the adenyl cyclase reaction

The α-phosphate of ATP is the precursor of the phosphate of cyclic AMP and the β and γ phosphate residues of ATP appear as inorganic pyrophosphate. Adenosine 3',5'-phosphate-P^{32} is formed by adenyl cyclase in the presence of $AP^{32}PP$ while little radioactive cyclic AMP is formed in the presence of $APP^{32}P^{32}$ (Rall and Sutherland, 1962).

c) cofactor requirements

Excluding some preparations of the bacterial enzyme for which pyruvate is an essential cofactor (Hirata and Hayaishi, 1967), the only known cofactor for the adenyl cyclase reaction is Mg^{++} (in some cases Mg^{++} can be effectively replaced by Mn^{++}) (Rall et al., 1957). Ca^{++} is usually inhibitory although it has been reported that calf brain is stimulated by Ca^{++} (Bradham et al., 1970) and that adrenaline requires Ca^{++} to stimulate

liver adenyl cyclase (Marinetti et al., 1969); ACTH seems to require Ca^{++} in order to stimulate adenyl cyclase in isolated white fat cells and in adrenal cortex (Bär and Hechter, 1969).

d) pH optimum

The pH optimum for the formation of cyclic AMP in most preparations is broad and lies between pH 7.2 and 8.2 (Sutherland et al., 1962).

e) stimulation by fluoride

Fluoride ion can stimulate the activity of adenyl cyclase in most broken cell preparations that have been studied (Robison et al., 1971). The mechanism of this stimulation is unknown. The maximum velocity of the adenyl cyclase reaction is usually markedly increased by fluoride with little or no change in the apparent affinity of the enzyme for ATP. The optimal concentration of NaF may vary but is generally between 5 and 10 mM (Robison et al., 1971). Fluoride has not been shown to stimulate the formation of cyclic AMP in intact tissue. For example, incubation with 5mM NaF stimulated brain homogenate adenyl cyclase markedly but did not increase the level of cyclic AMP in slices of the same brain (Robison et al., 1970). Similar observations have been made with a variety of other tissues (Robison et al., 1970). An interesting finding is that of Schmidt and coworkers (1970) who observed that in the first few days following birth, the adenyl cyclase of washed particulate preparation of rat brain was insensitive to stimulation by NaF. At some point between the fifth and the ninth day after birth, adenyl cyclase activity measured in the absence of NaF

began to decline, reaching very low levels by the twentieth day, at which point the apparent stimulation by NaF was very striking. It would appear that this effect represents the reversal by NaF of an inhibitory influence which is absent in the newborn rat brain. This effect of NaF may develop differently in different organs and in different species since in preparations from newborn rat liver, fluoride produced a substantial stimulatory effect even at birth (Wicks, 1969). Although its mechanism of stimulation of adenyl cyclase is unknown, NaF has been used extensively in the study of different adenyl cyclase preparations and the NaF-stimulated activity is very often referred to as the maximum activity of adenyl cyclase. The basal level of adenyl cyclase in tissues is usually very low and the use of NaF has permitted to characterize better the different adenyl cyclase systems.

C) Methods of estimation of adenyl cyclase

Rall and Sutherland (1958) designed the first method of determination of adenyl cyclase activity. The method is based on the transformation of liver phosphorylase b into liver phosphorylase a by cyclic AMP formed by adenyl cyclase. The hydrolysis of glycogen by phosphorylase a can be assayed by the formation of inorganic phosphate or by the intensity of the polysaccharide-iodine color. The amount of cyclic AMP formed is then proportional to the amount of inorganic phosphate formed or to the intensity of the polysaccharide-iodine color, assuming that appropriate blanks are taken into account (Rall and Sutherland, 1958).

The more recent methods of estimation of adenyl cyclase are based on the formation of labelled cyclic AMP from labelled ATP and on the separation by chromatographic means of the labelled cyclic AMP from other nucleotides. ATP-U-C^{14} has been used as the substrate in many systems and the C^{14}-cyclic AMP formed has been isolated by either thin-layer chromatography on silica gel (Jungas, 1966), or descending paper chromatography (Streeto and Reddy, 1967; Williams et al., 1968). ATP-P^{32} has been used as a substrate (Rabinowitz et al., 1965) in a system using descending paper chromatography for separation of cyclic AMP-P^{32} from other nucleotides.

Krishna and coworkers (1968a) proposed a simple and sensitive method for the assay of adenyl cyclase in tissue homogenates. Tritiated cyclic AMP formed from tritiated ATP is isolated by chromatography on Dowex 50-H^{+} columns followed by precipitation of all nucleotides except cyclic AMP by zinc sulfate-barium hydroxide treatment. The recovery of the procedure is measured by the addition of C^{14}-cyclic AMP to the medium after the incubation is terminated. The method is sensitive enough to detect a 0.005% conversion of ATP to cyclic AMP and is more rapid than the paper chromatography systems (Brodie et al., 1966; Krishna et al., 1968a; Krishna and Birnbaumer, 1969).

Another method which has the advantage of being able to measure adenyl cyclase activity directly in the cells has been designed by Kuo and Dill (1968). Fat pads are preincubated with adenosine-8-C^{14} and glucose prior to the isolation of the

fat cells by digestion with collagenase, according to the method of Rodbell (1964). After the digestion is completed, the dispersed adipocytes are washed of the excess of adenosine-C^{14} and resuspended before the incubation. The cyclic AMP-C^{14} formed during the incubation is isolated by the $BaSO_4$ treatment (Krishna et al., 1968), without chromatography on the Dowex-H^+ columns. This modification of the method was introduced by Kuo and Dill (1968) and is only applicable in systems where the amount of labelled ATP is limited. In order to decrease the non-enzymatic formation of labelled cyclic AMP during the isolation procedure, a concentration of cold ATP which could give approximately a fifty-fold dilution of the labelled ATP was added to the incubation medium, after the reaction was terminated (Rodbell, 1967). The $BaSO_4$ supernatants were examined chromatographically to ensure that the radioactivity present in those fractions was associated exclusively with cyclic AMP (Kuo and DeRenzo, 1968). This method offers a very good way of studying adenyl cyclase directly in the cells without having to homogenize the tissue, a procedure which may cause the loss of hormonal sensitivity of the preparations. One of the inconveniences of this method is that the concentration of substrate is unknown and that it may not be optimal during the incubation.

Section II: Experimental: adenyl cyclase in brown adipose tissue

A) Materials and methods

1) *animals*

Male white Sprague-Dawley rats weighing 120 grams, obtained from the Holtman Company (Madison, Wisconsin), were kept at room temperature (25-28°C) in individual wire mesh cages until they weighed 150 grams. Some of the rats were then killed, they are referred to as being killed on day zero. Other rats were either placed at 4°C for up to eight weeks (cold exposed or cold acclimated depending on the period of exposure to cold) or kept at room temperature for the same length of time (warm acclimated rats). Rats were killed by decapitation within five minutes of their removal from the temperature of acclimation with the exception of some rats which, after being kept in the cold for eight weeks, were transferred to room temperature for 24 hours before they were killed (referred to as cold acclimated rats in the warm).

2) treatment with cycloheximide and actinomycin D

In the experiments in which cycloheximide was administered, rats weighing 150 grams were divided in four groups, two receiving NaCl 0.9% and the other two receiving cycloheximide in saline solution. Two hours after the first injection, one control and one treated group were transferred to 4°C while the other two groups were kept at room temperature. In the first series of experiments, the rats received a total of three injec-

tions over a period of 48 hours. The first injection was a dose of 1.5 mg/kg of body weight while the subsequent injections were 0.75 mg/kg, given intraperitoneally. In the second set of experiments with cycloheximide, the rats received 0.8 mg/kg per day for a total of five injections (96 hours). The rats were weighed before every injection and the amount of cycloheximide or saline to be given was calculated. The dilution of the cycloheximide solution was such that the animals would receive approximately 0.5 ml per injection. All rats were killed two hours after the last injection.

In the experiments using actinomycin D, the rats (at 150 grams) were divided in six groups, two saline-treated and four actinomycin D-treated, two different doses of actinomycin D being used (50 and 12.5 µg/kg, twice a day). The rats were placed either at $4^{\circ}C$ or $28^{\circ}C$ one and a half hour after the first injection and were killed one and a half hour after the last injection. In the first set of experiments, the animals received a total of five injections over a period of 48 hours. In the second series of experiments, the animals were supposed to receive a total of nine injections over a period of 96 hours. But in the groups treated with 50 µg/kg twice a day, signs of the toxicity of the drug started to appear on the third day and in some cases the injections were discontinued to ensure that the animals would stay alive for the time of the experiment. In the group kept at room temperature, the rats received five, six, seven, eight and nine injections respectively but the results being of the same order of magnitude were compiled together. In the cold exposed group, treated with the high dose

of actinomycin D, only two rats survived, one having received nine and the other one five injections. Their results were also compiled together. Rats were weighed before every injection and the volume of the solution of either saline or actinomycin D (50 µg or 12.5 µg/kg) was calculated. Animals were receiving approximately 0.5 ml per injection. When signs of toxicity started to appear, in addition to the brown adipose tissue, the following tissues were removed and weighed after the rats were killed: liver, kidneys, heart, lungs and spleen.

3) preparation of the brown adipose tissue

The interscapular brown adipose tissue was removed immediately after the rat was killed and placed in ice-cold 0.25 M sucrose; it was then cleaned of adhering white and connective tissues, minced with scissors, homogenized in a glass homogenizer in 0.25 M sucrose, filtered through glass wool and diluted to a final concentration of 40 mg of tissue per ml of homogenate.

4) assay of adenyl cyclase

Adenyl cyclase activity was measured by the method of Krishna, Weiss and Brodie (1968) with some modifications, introduced to ensure optimal conditions for the brown adipose tissue enzyme and for recovery of contaminant-free cyclic AMP.

In this method, tritiated ATP is the substrate and the labelled cyclic AMP formed during the reaction is isolated by chromatography on Dowex 50-H^+ columns followed by treatment with zinc sulfate and barium hydroxide. ATP, ADP, 5'AMP, adenine and inorganic phosphate are quantitatively precipitated by this

treatment whereas more than 99.9% of the cyclic AMP remains in the supernatant fluid (Krishna et al., 1968a). The following compounds are also precipitated by zinc sulfate and barium hydroxide: xanthosine, xanthine, 2'AMP, guanine, guanosine, cyclic GMP, GTP, hypoxanthine, inosine, ITP, theophylline, NADP and NADPH (Krishna et al., 1968). This procedure in combination with chromatography on Dowex 50-H^+ gives a very good separation of cyclic AMP from ATP and other products formed during the reaction with crude enzyme assays. Krishna and coworkers (1968) have demonstrated the purity of cyclic AMP in the supernatant fraction after the zinc sulfate barium hydroxide treatment by ion-exchange and paper chromatography, by crystallization to constant specific activity, and by electrophoresis.

The separation of cyclic AMP from other nucleotides by chromatography on Dowex 50-H^+ was first demonstrated by Sutherland and Rall (1957b). Figure IA illustrates the separation on a Dowex 50-H^+ column of the products of incubation of H^3-ATP with 5 mg of brain homogenate. No labelled cyclic AMP was added after the reaction was terminated but unlabelled cyclic AMP was added to decrease the nonenzymatic formation of tritiated cyclic AMP. ATP and ADP are eluted in fractions 1-3; the broad peak (fractions 8-15) consists of AMP. The formation of ADP and AMP during the reaction is due to the action of ATPase and phosphatases. The cyclic AMP formed during the reaction is eluted in fractions, 4, 5, and 6, and was identified by its absorbance at 260 nanometers. The peak of tritiated cyclic AMP is not visible on the figure IA due to the fact that the per-

Figure 1A: Separation on a Dowex 50-X8 column of the products of incubation of H^3-ATP with 5 mg of brain homogenate.

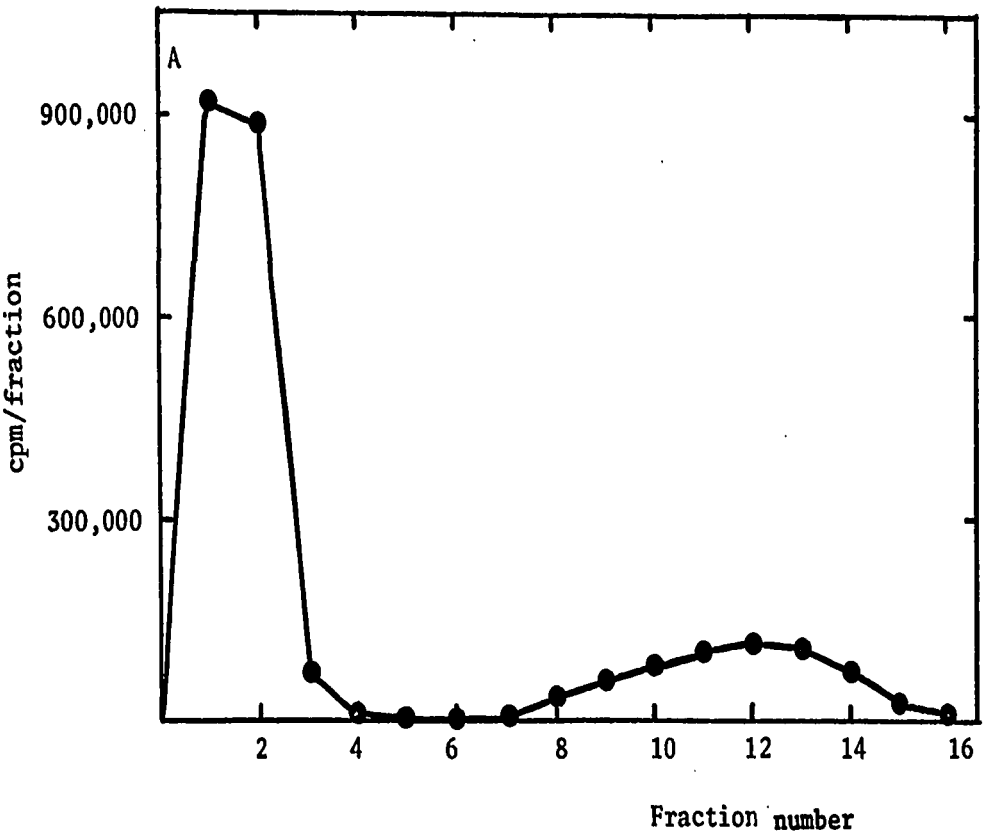
The incubation medium contained 2mM ATP, 16mM Mg , 10mM theophylline, 10mM NaF in Tris buffer pH 7.4 (40mM). The reaction was carried out for 15 minutes at 30°C and was stopped by boiling for three minutes. The mixture was centrifuged and the supernatant was applied to a Dowex 50-X8 (hydrogen form) column, 0.5cm by 5.5cm in dimensions. Nucleotides were eluted from the column with glass distilled water and 1 ml fractions were collected and added to 13 ml of modified Bray solution (as described in the text) and counted for radioactivity.

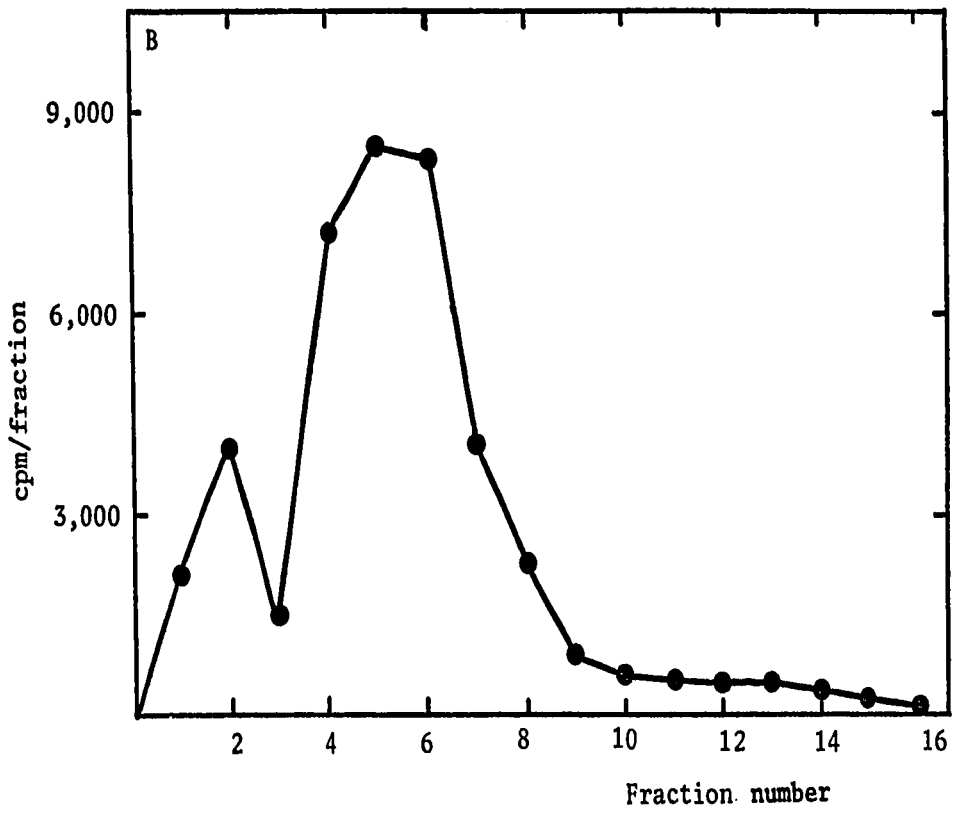
No labelled cyclic AMP was added after the reaction was terminated but unlabelled cyclic AMP was added to decrease the nonenzymatic destruction of H^3-cyclic AMP.

The first peak (fractions 1 - 3) consists of ATP and ADP; the broad peak (fractions 8 - 15) consists of AMP. Cyclic AMP would be present in fractions 4 - 6 (see Figure 1B). Note that this separation of cyclic AMP by column chromatography is followed by a further purification of the pooled fractions 4 - 6 (see text for details).

Figure 1B: Elution of C^{14}-cyclic AMP in fractions 4 - 6 from a Dowex 50-X8 column.

Unlabelled ATP was incubated with 5 mg of brain homogenate. The incubation medium was as described for Figure 1A. After the reaction was terminated C^{14}-cyclic AMP plus some unlabelled cyclic AMP was added. Most of the labelled material was eluted in fractions 4 - 6.


A
900,000
600,000
300,000
cpm/fraction
2
4
6
8
10
12
14
16
Fraction number


B
9,000
6,000
3,000
cpm/fraction
2
4
6
8
10
12
14
16
Fraction number

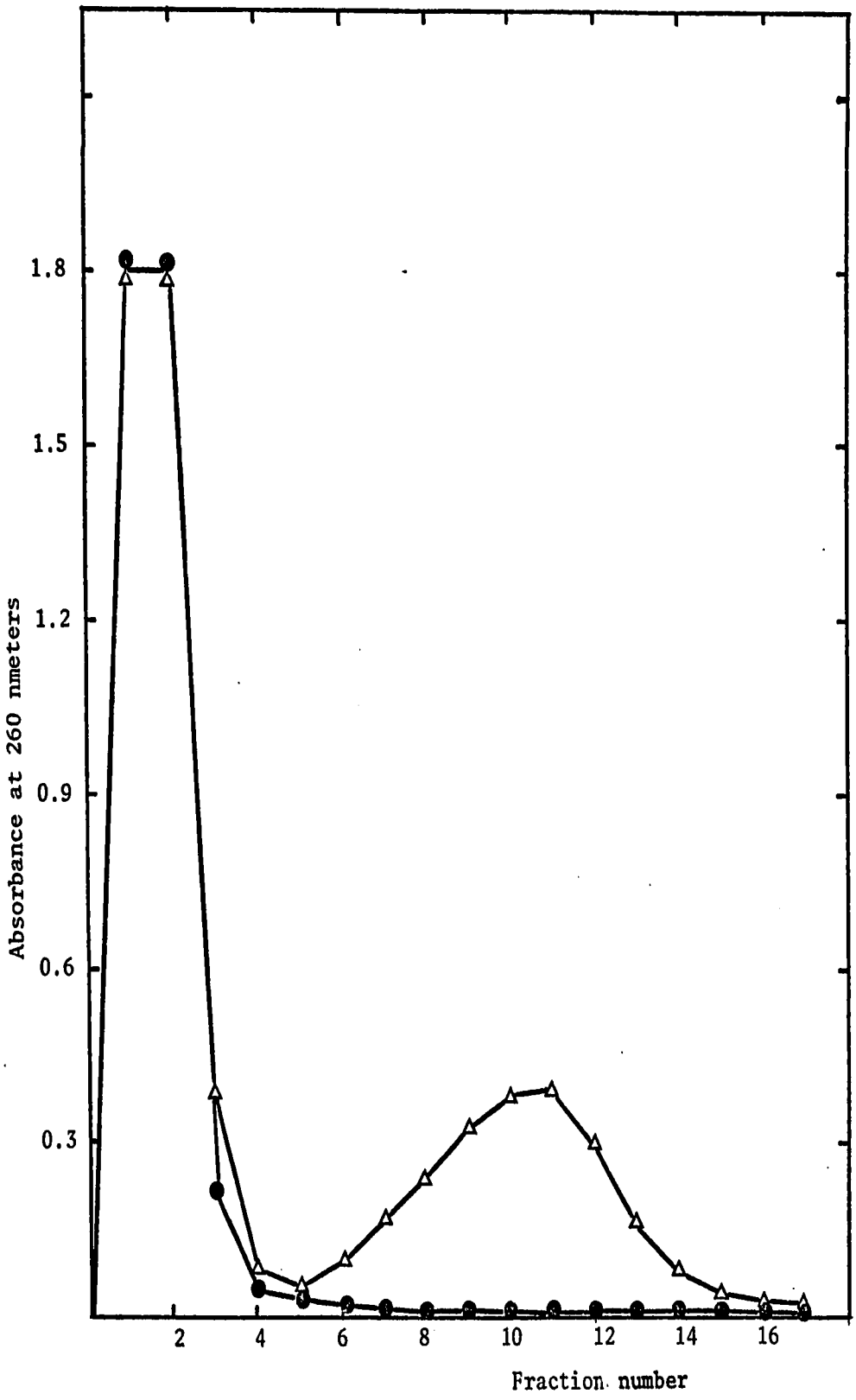
centage of conversion of ATP to cyclic AMP by adenyl cyclase is very small (1% or less). There is nevertheless 20,000 cpm in fractions 4 to 6. Note that this separation of cyclic AMP by column chromatography is followed by a further purification of the pooled fractions (4-6) by $BaSO_4$ treatment. Figure IB illustrates the elution of cyclic AMP in fractions 4 to 6 on the Dowex 50-H^+ column. C^{14}-cyclic AMP plus some unlabelled cyclic AMP were added after the reaction was terminated. The medium contained cold ATP but no tritiated ATP. The nucleotide content of every fraction was estimated by its absorbance at 260 nanometers and the radioactivity of every fraction was counted.

A modification of the Krishna method (Krishna et al., 1968a) was introduced to maintain the concentration of ATP constant during the reaction. ATPase activity is very high in homogenates and the addition of an ATP-regenerating system is essential to maintain the concentration of ATP, the substrate of the adenyl cyclase reaction, constant during the reaction (Williams et al., 1968). Figure 2 illustrates the prevention of the accumulation of AMP (fractions 6 to 15) by the addition to the incubation medium of 20mM phosphoenolpyruvic acid (tri-cyclohexylamine salt) and of one unit of pyruvate kinase (type I). The formation of ADP from ATP which takes place in the absence of the ATP-regenerating system cannot be seen in this chromatographic system because both ATP and ADP are eluted in fractions I and 2. The ADP produced is further hydrolysed to AMP which can be detected because it is eluted later on the Dowex

Figure 2: Prevention of the accumulation of AMP by including an ATP-regenerating system in the reaction mixture.

The constituents of the incubation medium are the same as described in figure 1. H^3-ATP and C^{14}-cyclic AMP were present but no unlabelled cyclic AMP was added after the reaction was terminated. In addition, an ATP-regenerating system was added to the incubation mixture designated by closed circles (● - ●). The ATP-regenerating system consisted of 20mM phosphoenolpyruvic acid (tricyclohexylamine salt) and of one unit of pyruvate kinase, type I. (One unit will convert one µmole of phosphoenolpyruvate per minute, at pH 7.6, at 37°C).

The nucleotide content of the different fractions is measured by their absorbance at 260 nanometers.


Absorbance at 260 nmeters
1.8
1.5
1.2
0.9
0.6
0.3
2
4
6
8
10
12
14
16
Fraction number

50-X-8 column; the prevention of the accumulation of AMP by the ATP-regenerating system can be used as a fair measurement of the efficacy of the system to maintain the level of ATP constant. The reaction catalyzed by pyruvate kinase (ADP + phosphoenolpyruvate → ATP + pyruvate) is such that the ADP formed by ATPase action is reconverted to ATP. The concentration of substrate for the adenyl cyclase can thus be maintained constant during the incubation.

With the brown adipose tissue preparation, the incubation was carried out at 30°C in 0.04M tris (hydroxymethyl) aminomethane-HCl buffer, pH 7.4, containing 10 mM theophylline to inhibit the hydrolysis of cyclic AMP by phosphodiesterase. The medium also contained optimal concentrations of enzyme, ATP, $MgSO_4$, and NaF or noradrenaline when necessary. The labelled substrate, ^{3}H-ATP, had a final specific activity of 2mCi/mmole (it was purchased at a specific activity between 5 and 15 Ci/mmole). The ATP-regenerating system used with the BAT preparation consisted of 13 mM phosphoenolpyruvic acid, tricyclohexylamine salt, and 2 μmolar units of pyruvate kinase (pyruvate kinase, type I, from rabbit skeletal muscle; one μmolar unit will convert one mole of phosphoenolpyruvate to pyruvate per min at pH 7.6 and 37°). The reaction was initiated by the addition of 0.1 ml of homogenate, which brought the final volume to 0.4 ml, and the samples were incubated at 30°C for 15 minutes. Carrier cyclic AMP was then added (0.1 ml of a solution containing 0.3 mg of ^{14}C-cyclic AMP with a specific activity of 3.75 μCi/mmole; (^{14}C-cyclic AMP was obtained at a specific activity of 50 mCi/mmole) and the reaction terminated by immersing

the tubes in a boiling water bath for 3 minutes. The tubes were centrifuged and the supernatant fluids chromatographed on 0.5 x 5.5 cm columns of the cation exchange resin, AG 50W - X8, 100 - 200 mesh, hydrogen form. Nucleotides were eluted from the columns with water and 10 fractions of 1 ml each were collected. Nucleotide content was determined by measuring its absorbance at 260 nanometers. The three fractions containing cyclic AMP were pooled and treated with zinc sulfate and barium hydroxide. 0.3 ml of $ZnSO_4$ 0.25M and 0.3 ml of $Ba(OH)_2$ 0.25M were added to the fractions containing the cyclic AMP, mixed and centrifuged. The supernatant was transferred to another tube by decantation and the treatment with barium hydroxide-zinc sulfate was repeated adding the $Ba(OH)_2$ first. An aliquot of 1.5 ml of the supernatant from this second precipitation was added to 13 ml of a phosphor mixture (5 grams 2,35-diphenyloxazole (PPO), 100 grams naphthalene in 1 liter of 1,4-dioxane) (modified Bray solution). Radioactivity was measured at room temperature in a Beckman LS-250 liquid scintillation counter with Automatic Quench Correction. Under these conditions the efficiency for counting tritium was 30% and the efficiency for counting carbon-14 was 65%; the contribution of carbon-14 in the tritium channel was 10%. Recovery of ^{3}H-cyclic AMP produced in the adenyl cyclase reaction was assessed from the recovery of the added ^{14}C-cyclic AMP. Activity was expressed as nanomoles of cyclic AMP produced per minute (see Appendix 1 for the method of calculation of the results).

When the effects of different agents on adenyl cyclase were studied, these were added to the incubation medium after they had been diluted to give the final concentration desired. Hormone solutions were made fresh immediately before the incubation. Noradrenaline, adrenaline, ACTH, dexamethasone, growth hormone and serotonin creatinine H_2SO_4 complex, were diluted in glass distilled water; corticosterone was diluted in 10% ethanol; insulin, glucagon, L-triiodothyronine were made up in 10^{-5}M NaOH.

5) protein estimation

Protein was estimated by the Lowry method (Lowry et al., 1951). Standard curves were performed with every set of determinations.

6) chemicals

- Adenosine-H^3(G)5'-triphosphate was obtained as the tetrasodium salt from New England Nuclear
- Adenosine-C^{14}(8) 3',5' cyclic monophosphate was purchased from Schwartz Bioresearch
- Dowex 50-X8, 100-200 mesh, hydrogen form was obtained from Bio-Rad
- Serotonin creatinine H_2SO_4 complex H_2O and theophylline were purchased from Calbiochem
- ATP, cyclic AMP, phosphoenolpyruvic acid tricyclohexylamine salt, pyruvate kinase (type I), noradrenaline, (L-arterenol bitartrate), cycloheximide, actinomycin D (prepared from Streptomyces Chrysomallus), growth hormone, insulin, ACTH, corticosterone, L-triiodothyronine, glucagon, dexamethasone

sucrose and calcium chloride dihydrate, Trizma-HCl and Trizma-base were obtained from Sigma

- Adrenaline (L-epinephrine bitartrate was obtained from Winthrop laboratories
- Sodium fluoride, $Ba(OH)_2.8H_2O$, $ZnSO_4.7H_2O$, 2,5-diphenyl oxazole (PPO), naphthalene (liquid scintillation grade), 1,4-dioxane (liquid scintillation grade) were all "Baker Analyzed" Reagents obtained from Fisher Co.
- Triton X-100 was obtained from Harleco.

B) Properties

The following optimal concentrations of the different components required for the adenyl cyclase reaction have been found for brown adipose tissue homogenates.

1) *enzyme concentration*

The rate of reaction for basal, noradrenaline-stimulated and NaF-stimulated AC activities is linear for up to 5 mg of BAT (total amount in the incubation medium) during a 15 minute incubation period at 30°C (figure 3). Higher concentrations of enzyme were assayed in experiments other than those reported on figure 3 and did not give rise to a linear relationship. Four mg of BAT was the amount usually present in the incubation. The homogenate of BAT was diluted in 0.25 M sucrose to a concentration of 40 mg/ml and 0.1 ml of that solution was added to the incubation mixture to initiate the reaction.

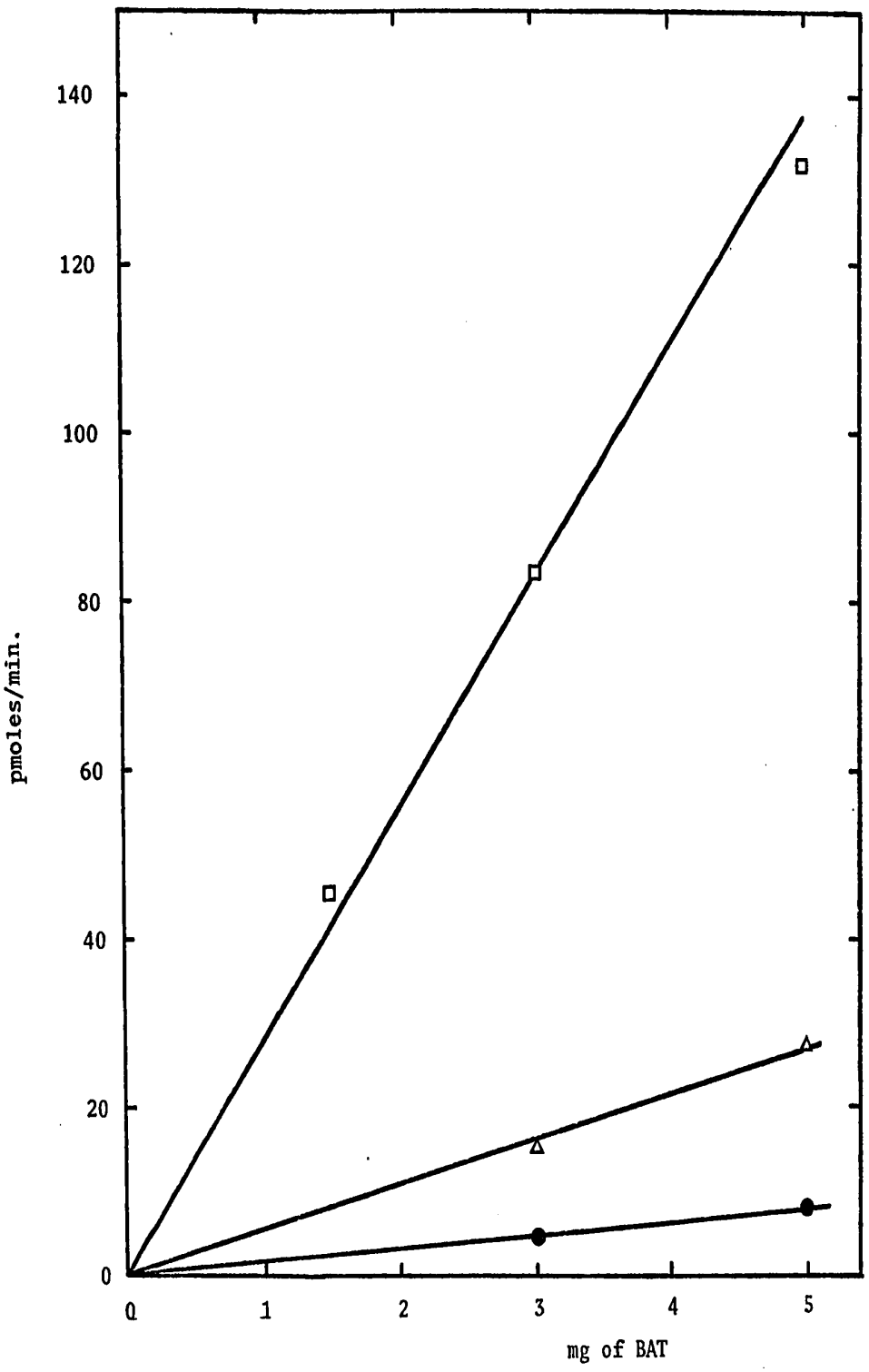
Figure 3: Brown adipose tissue adenyl cyclase; velocity versus enzyme concentration.

The incubation was carried out for 15 minutes at 30°C. The enzyme concentration is expressed here in terms of mg of brown adipose tissue present in the incubation medium. The latter contained, 1.5mM ATP, 16mM Mg^{++}, 13mM PEP, one unit of pyruvate kinase type I, 10mM theophylline in Tris buffer, pH 7.4 (40mM).

● - black circles represent the basal adenyl cyclase activity

Δ - open triangles represent noradrenaline-stimulated activity of adenyl cyclase (noradrenaline concentration 0.75mM)

□ - open squares represent the NaF-stimulated adenyl cyclase activity (NaF concentration 2.5mM).



2) fluoride concentration

The adenyl cyclase from brown adipose tissue is stimulated by fluoride. With all conditions optimized, NaF can increase the basal enzyme activity by a factor ranging from 4 to 15. The maximum stimulation of the enzyme from BAT by NaF is obtained with a concentration of 2.5mM and there is inhibition with higher concentrations (figure 4).

3) concentrations of adrenaline and noradrenaline

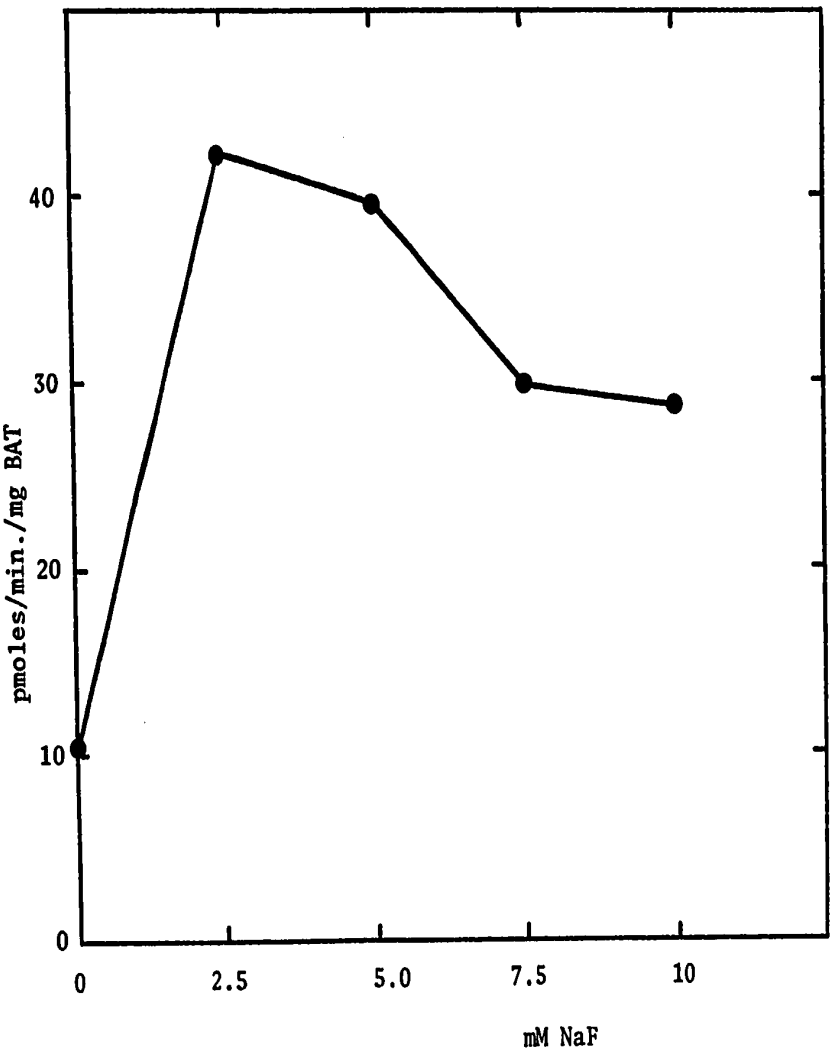
Dose-response curves to noradrenaline and adrenaline have been done with brown adipose tissue adenyl cyclase from warm and cold acclimated rats as well as from rats that have been exposed to cold for two days. Maximum stimulation of the enzyme was obtained with 0.75mM - 1.0mM noradrenaline or adrenaline in all three groups of rats. Figures 8 and 12 illustrate the dose-response curves for the stimulation of adenyl cyclase by noradrenaline and adrenaline in warm acclimated rats and in cold exposed rats (2 days).

The dose-response curves for the stimulation of adenyl cyclase by noradrenaline and adrenaline in cold acclimated rats are not illustrated but they are not different from those of the warm acclimated rats or from the cold exposed rats, represented in figures 8 and 12. Skala and coworkers (1970a) have obtained maximum stimulation of BAT adenyl cyclase in newborn rats with concentrations of noradrenaline and between 0.1 and 0.5mM. Forn and coworkers (1970) with the guinea pig BAT adenyl cyclase have observed that concentrations of noradrena-

Figure 4: Adenyl cyclase in brown adipose tissue; velocity versus fluoride concentration.

Five mg of brown adipose tissue were incubated with 2mM ATP, 8mM Mg^{++}, 10mM theophylline with the usual ATP-regenerating system in 40mM tris buffer, pH 7.4 for 15 minutes at 30°C.

Different amounts of sodium fluoride were added to the medium, from 0 to 10mM (final concentration in the incubation medium). Notice inhibition with concentrations higher than optimal.



line between 0.1 and 1mM were optimal. The basal adenyl cyclase activity was stimulated on the average 5 or 6 fold by noradrenaline and adrenaline.

4) concentrations of ATP and Mg^{++}

For the noradrenaline and the fluoride stimulated activities, 3mM ATP is the optimal substrate concentration and there is inhibition at higher substrate concentrations (figure 5A). The basal adenyl cyclase activity is maximum with 4mM ATP (figure 5A). Higher concentrations of ATP did not give rise to higher activity with the basal adenyl cyclase and were even inhibitory. With the optimal concentration of substrate the three adenyl cyclase activities were tested for optimal Mg^{++} concentration. The basal activity is maximum with 16 and 24mM Mg^{++} while the NaF activity was maximum at 16mM. The noradrenaline-stimulated activity was also maximum at 16mM Mg^{++} because higher concentrations, when tested gave rise to inhibition of stimulation (figure 5B).

The optimal final concentrations in the incubation medium were as follows: basal activity, 4mM ATP, and 24mM Mg^{++}; noradrenaline-stimulated activity, 3mM ATP, 16mM Mg^{++} and 0.75mM noradrenaline bitartrate; fluoride-stimulated activity, 3mM ATP, 16mM Mg^{++} and 2.5mM NaF. All subsequent experiments were done using these concentrations, unless otherwise specified.

Figure 5: Adenyl cyclase in brown adipose tissue; velocity versus ATP and Mg^{++} concentrations.

Four mg of brown adipose tissue were incubated for 15 minutes at $30^{o}C$. The constituents of the incubation medium were: 40mM Tris buffer pH 7.4, 10mM theophylline, 13mM phosphoenol-pyruvate and 1 unit of pyruvate kinase, type I.

● - black circles represent basal adenyl cyclase activity

Δ - open triangles represent noradrenaline (0.75mM) stimulated adenyl cyclase activity

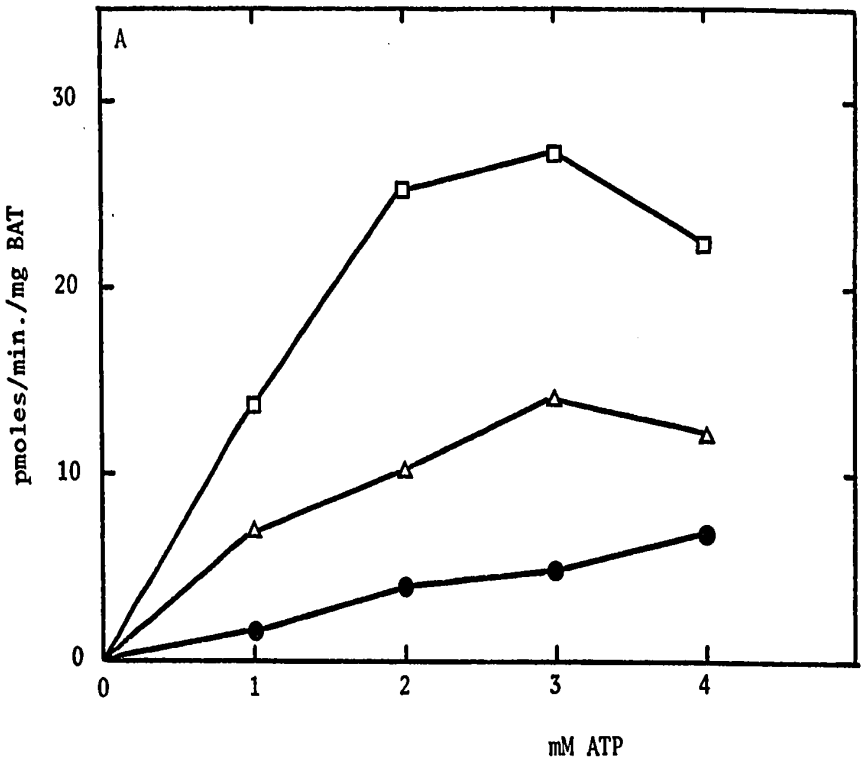
□ - open squares represent fluoride (2.5mM) stimulated adenyl cyclase activity.

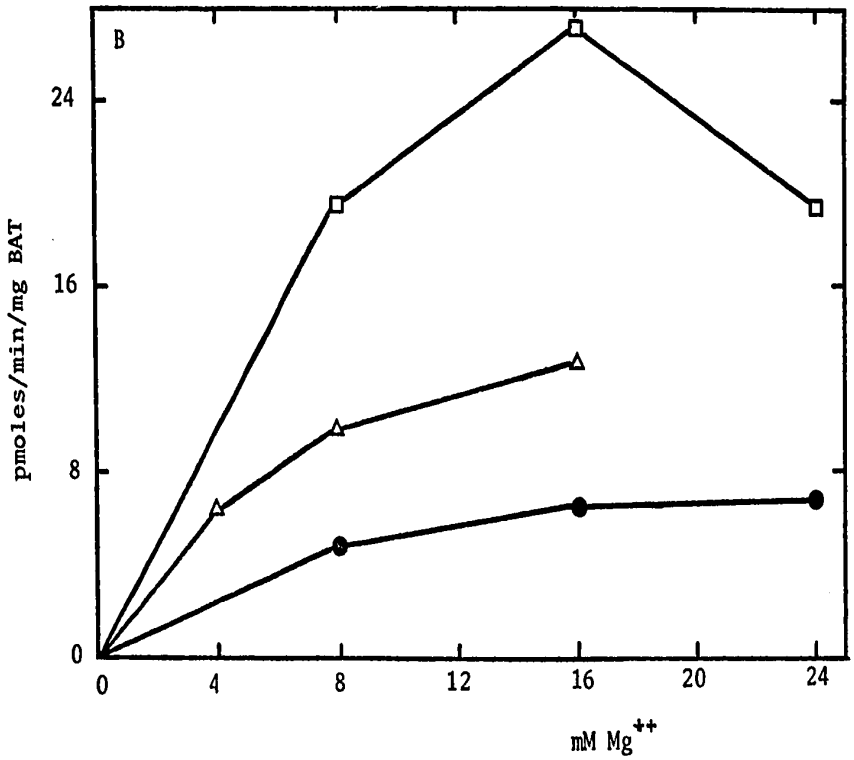
A) Velocity versus ATP concentration

For the basal adenyl cyclase activity, the Mg^{++} concentration was 24mM while for the noradrenaline and the fluoride stimulated activities, the Mg^{++} concentration was 16mM.

B) Velocity versus Mg^{++} concentration

For the basal activity, the ATP concentration was 4mM while for the noradrenaline and the fluoride stimulated activities, the substrate concentration was 3mM.





C) Effects of cold exposure and cold acclimation on adenyl cyclase activity in the brown fat

1) *wet weight and protein content of the interscapular brown adipose tissue*

The increase in growth of the brown adipose tissue, as measured by its wet weight and protein content, was apparent only after 4 days of cold-exposure (Fig. 6). After 8 weeks in the cold the protein content of the tissue in the cold-acclimated rats had increased by 450 per cent (there was a 200 per cent increase in protein content per unit of body weight). During this same time the protein content of the tissue in the rats living at room temperature increased by only 50 per cent (so that the protein content of brown adipose tissue perunit of body weight decreased by 27 per cent) (Table I). No change in protein content was observed after 1 or 2 days in the cold.

2) *adenyl cyclase activity*

The specific activity of noradrenaline-stimulated adenyl cyclase decreased after 2 days in the cold, at a time when no change in protein content had occurred, and remained at a low level throughout the 8 weeks of cold-acclimation (Fig. 7B), whereas the specific activity of noradrenaline-stimulated adenyl cyclase in rats that lived at room temperature during this same period was not significantly different from that of the younger rats at day zero (Table I). Note that the

Figure 6: Wet weight and protein content of interscapular brown adipose tissue of rats during acclimation to cold.

Values shown are means ± standard errors. Open columns represent wet weight and black columns represent total protein present in the interscapular brown fat pad. The number of animals in each group is as follows: days 1, 2, 4, 14 and 28, five rats each; day 0, seven rats; day 7, six rats; day 56, eight rats. These are the same rats as those used in the experiments illustrated in figures 7 and 9.

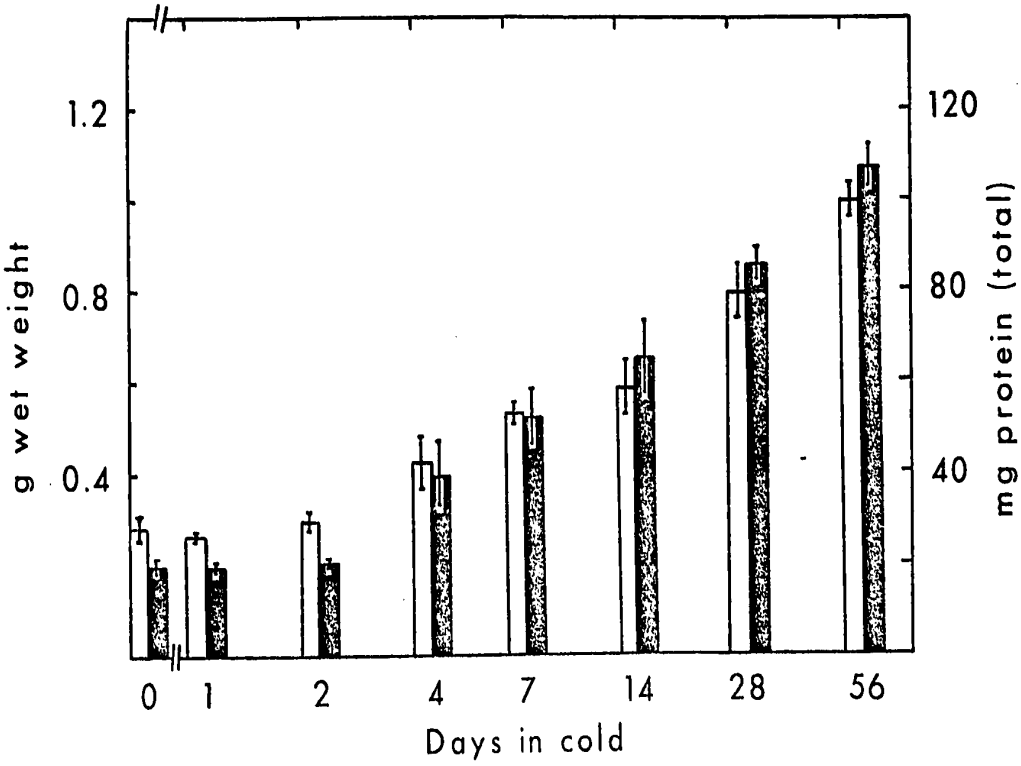


Figure 7: Changes in total activity and in specific activity of basal, noradrenaline-stimulated, and fluoride-stimulated adenyl cyclase in the interscapular brown adipose tissue of rats during acclimation to cold.

Sections A and B represent specific activities and sections C and D represent total activities. The following symbols are used:

- ■ fluoride-stimulated activity;
- ▲ noradrenaline-stimulated activity;
- ● basal activity.

Note that the scale on section A (specific activity) is different from the scale on section B and that the scale on section C (total activity) is different from the scale on section D. The numbers of animals are the same as in figure 6.

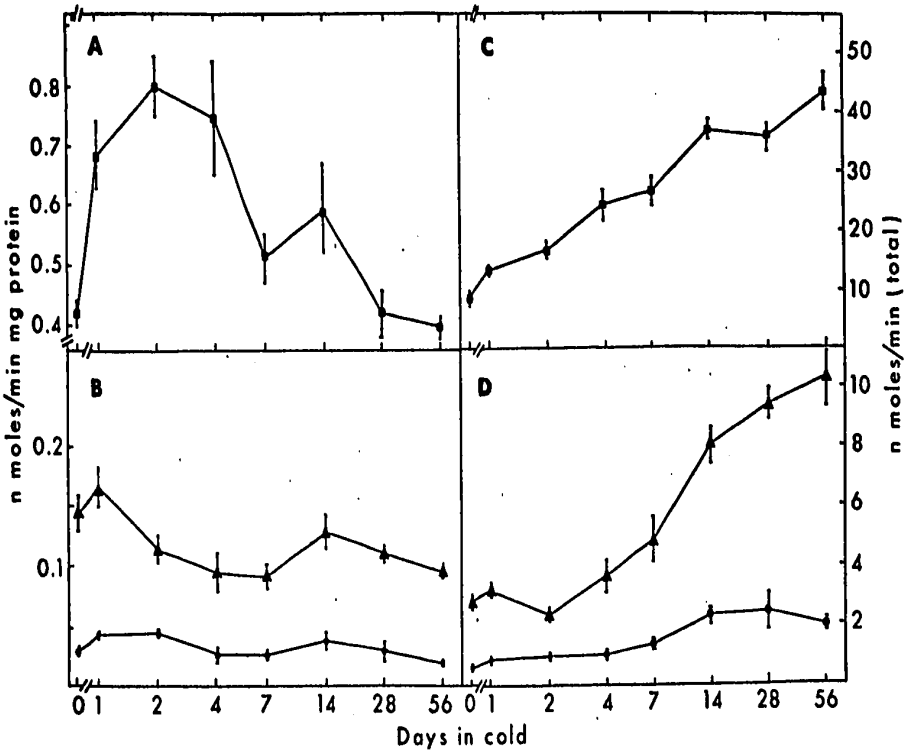


TABLE I

Noradrenaline-stimulated and fluoride-stimulated adenyl cyclase activities of interscapular brown adipose tissue in control rats and in cold acclimated rats, either in the cold or returned to the warm for one day.

Group of rats (no.)	Body weight	Brown adipose tissue weight	Total protein content	Adenyl cyclase activity					
				Basal	NA[b]	NaF	Basal	NA	NaF
	g	g	mg	nmoles / min . mg protein			nmoles / min by total organ		
Control: day zero	171[a]	0.282	19.5	0.030	0.145	0.421	0.569	2.76	8.38
(7)	±8	±0.027	±1.9	±0.004	±0.015	±0.023	±0.068	±0.27	±1.32
Control: 8 weeks	352	0.434	29.2	0.023	0.130	0.354	0.682	3.76	10.00
(4)	±3	±0.046	±4.0	±0.003	±0.008	±0.043	±0.190	±0.51	±0.98
Cold-acclimated:	313	0.997	107.0	0.018	0.095	0.397	1.95	10.26	42.71
8 weeks (8)	±11	±0.038	±5.0	±0.002	±0.006	±0.019	±0.21	±1.02	±3.34
Cold-acclimated:	303	0.821	82.7	0.023	0.129	0.408	1.99	10.80	33.58
8 weeks + 1 day at	±9	±0.018	±4.1	±0.003	±0.008	±0.012	±0.38	±1.12	±1.41
room temperature (6)									

[a] All value are means ± standard errors for the number of rats shown in brackets.

[b] NA = noradrenaline

specific activity of the noradrenaline-stimulated adenyl cyclase remained fairly constant during the period from 4 days in the cold to 8 weeks in the cold at a time when the protein content was increasing rapidly (Figs. 7B and 6); this reflects the parallel increases in total noradrenaline-stimulated adenyl cyclase activity of the tissue (Fig. 7D) and in protein content (Fig. 6).

The specific activity of the fluoride-stimulated adenyl cyclase activity, in contrast to the noradrenaline-stimulated activity, increased markedly during the first two days of cold-exposure (at a time during which no change in protein content occurred), then gradually decreased during the 8 weeks of acclimation to cold (Fig. 7A); after 8 weeks in the cold the specific activity of the fluoride-stimulated enzyme was not significantly different from that in a rat kept at room temperature during this same period (Table I). The total amount of fluoride-stimulated enzyme increased progressively during the 8 weeks in the cold (Fig. 2C) and from day 4 to week 8 bore a close relationship to the increase in total protein (Fig. 6).

The total basal adenyl cyclase activity, which is very low and difficult to measure with accuracy, also increased during the 8 weeks in the cold (Fig. 7D) more or less in parallel with the growth of the tissue.

The decrease in specific activity of the noradrenaline-stimulated adenyl cyclase might have been due to an altered sensitivity to noradrenaline and possible inhibition by excess noradrenaline. Therefore, dose-response curves were constructed

for preparations of brown adipose tissue from rats at day zero and from rats that had been 2 days in the cold. No change in sensitivity was noted (Fig. 8). It is apparent that high concentrations of noradrenaline do indeed inhibit activity but in both these preparations the concentration chosen for all the other experiments, 0.75mM, produced maximum activation.

3) proportion of total (fluoride-stimulated) adenyl cyclase susceptible to stimulation by noradrenaline

In this section two assumptions are made: (a) that fluoride-stimulated adenyl cyclase represents the total enzyme present; and (b) that noradrenaline can stimulate a constant proportion of the total activity of that adenyl cyclase with which the adrenoceptors are associated. The proportion of total adenyl cyclase stimulatable by noradrenaline remains fairly constant in rats living at room temperature (33% at zero time; 37.6% at 8 weeks; Table I). However, the proportion of fluoride-stimulated adenyl cyclase stimulatable by noradrenaline decreases markedly in two days of cold-exposure (to 13.5%; Fig. 7) and remains low throughout the 8 weeks in the cold (24% at 8 weeks; Table I). If it is assumed that noradrenaline still has access to 33% of that adenyl cyclase with which the adrenoceptors are associated it follows that a considerable amount of adenyl cyclase that is no longer accessible to noradrenaline has appeared during exposure to cold. This amount has been calculated as extra adenyl cyclase in the total tissue and is illustrated in Fig. 9. Starting from no extra enzyme at day zero the

Figure 8: Dose-response curve for the stimulation of adenyl cyclase activity by noradrenaline in control and in cold-exposed rats.

Each graph represents pooled values for two rats. In each case activities are expressed in terms of the maximum activity, shown here as 100%. The vertical line indicates the concentration of noradrenaline used in all other experiments (0.75mM). Section A: rats exposed to cold for 2 days; section B: control rats. Note that concentrations of noradrenaline higher than 1mM inhibit the enzyme.

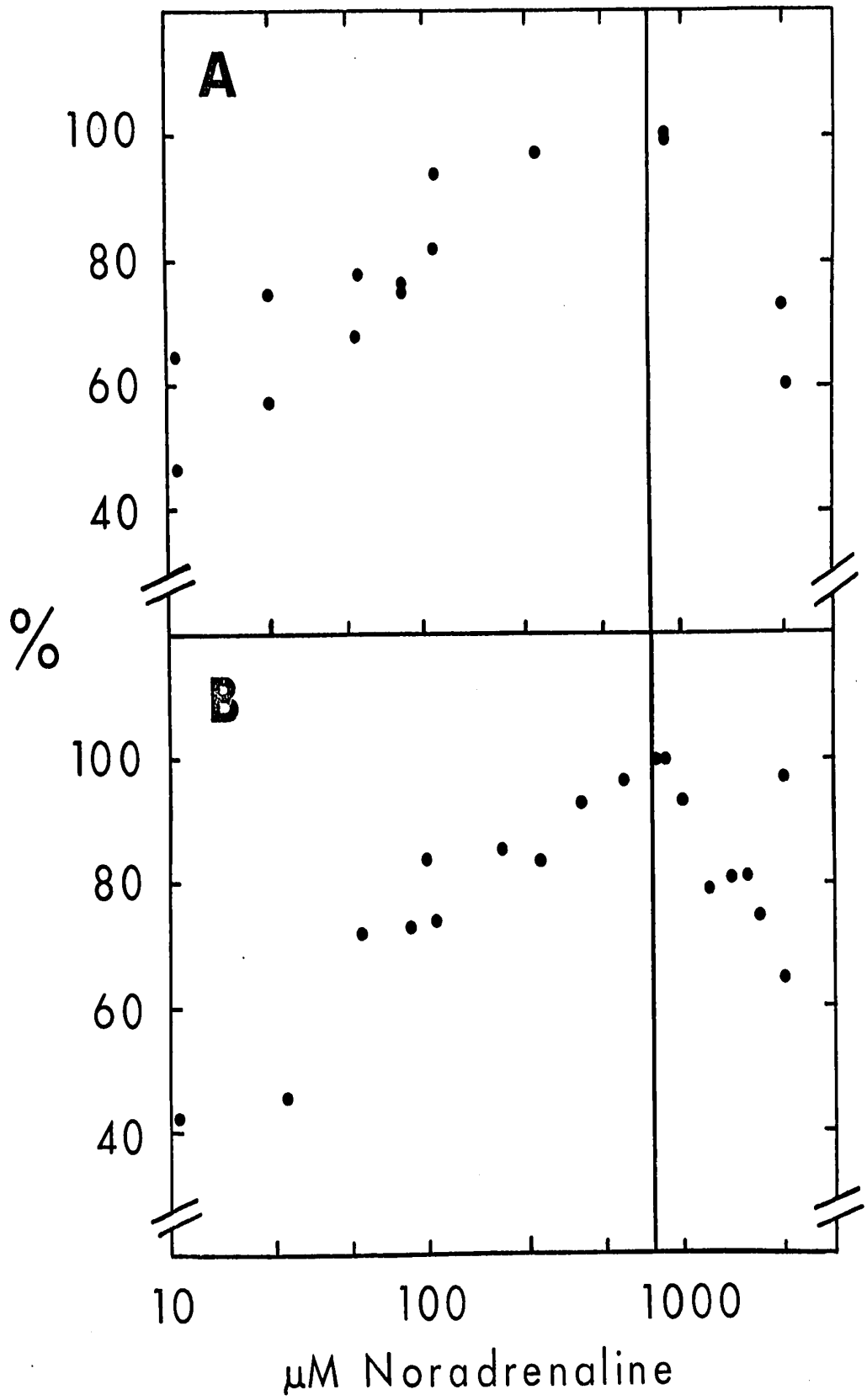
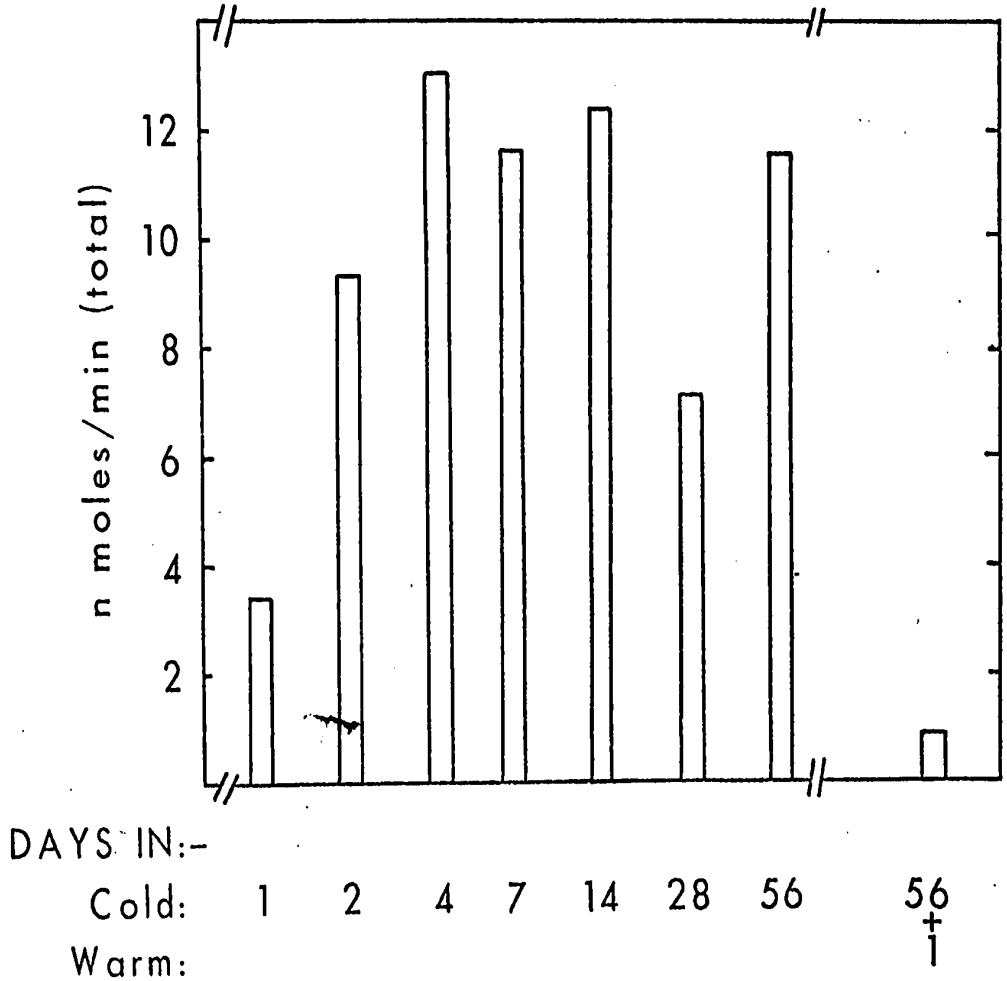


Figure 9: "Extra" fluoride-stimulated adenyl cyclase activity in interscapular brown adipose tissue of rats during acclimation to cold.

Two assumptions are made in the calculation of the values shown in this graph. (i) Fluoride-stimulated adenyl cyclase activity is a measure of the total amount of adenyl cyclase present. (ii) Noradrenaline stimulates a constant proportion of that adenyl cyclase with which the adrenoceptors are associated. The proportion observed in the tissue at day zero is 33% (Table 1). If total noradrenaline-stimulated adenyl cyclase activity (Fig. 7D) is taken as 33% throughout the 8 weeks of acclimation to cold and the 100% calculated from this value then subtracted from the observed total fluoride-stimulated adenyl cyclase activity (Fig. 7C), a difference is obtained; this difference is plotted in this figure and is referred to as "extra" adenyl cyclase activity.



amount of extra enzyme increases rapidly during the first 2 to 4 days of exposure to cold and thereafter remains fairly constant. When the rats that have been in the cold for 8 weeks are returned to room temperature for one day this extra enzyme disappears (Fig. 9). This decrease is due to a decrease in total fluoride-stimulated adenyl cyclase activity with no change in total noradrenaline-stimulated adenyl cyclase activity (Table I). Note that the specific activity of the noradrenaline-stimulated adenyl cyclase activity has increased to the control (8 week) level in these animals after one day in the warm whereas the specific activity of the fluoride-stimulated (i.e. total) adenyl cyclase activity has not changed. Thus, this extra activity appears within 2 days of cold exposure, persists throughout the 8 weeks of acclimation and practically disappears within 1 day when the rats are returned to room temperature.

D) Effects of cycloheximide and actinomycin D

The extra adenyl cyclase activity observed during cold exposure and all the way throughout cold acclimation could be due to the synthesis of a different adenyl cyclase (synthesis induced by exposure to cold of the animal) or to a stimulation of adenyl cyclase already present by a hormone other than noradrenaline. The first hypothesis, synthesis of a new adenyl cyclase, could be tested by treating rats with inhibitors of protein synthesis at the same time that they are submitted to cold stress. If cold exposure is responsible for the induction of synthesis of a new adenyl cyclase, then protein synthesis in-

hibitors should block the increase in adenyl cyclase observed after two days of exposure to cold.

Cycloheximide is a good inhibitor of protein synthesis which has been used in yeasts as well as in mammals. Appendix 2 summarizes the properties and usage of this compound. Actinomycin D is a potent inhibitor of RNA synthesis and, as an immediate consequence, a good inhibitor of protein synthesis (see appendix 2).

1) *cycloheximide*

a) <u>effects on body weight</u>

When rats are exposed to cold, they first lose weight but after a few days they increase their food consumption and start gaining weight again. After two days in the cold, the saline-treated rats had lost an average of fourteen grams compared to the saline-treated rats at room temperature. But, treatment with cycloheximide caused a larger decrease in body weight. The rats at room temperature receiving cycloheximide had lost an average of fifteen grams while the cold cycloheximide-treated rats had lost an average of five more grams than the saline-treated cold exposed rats. After four days in the cold in the second series of experiments, the warm cycloheximide-treated rats had lost an average of twelve grams while the cold cycloheximide treated rats had lost an average of twenty-four grams more than the saline-treated cold exposed rats. So, cycloheximide did slow down the growth of the animals.

b) effects on brown adipose tissue weight and protein content

As shown in the figures 10A and 10B, there was an increase in the weight and protein content of the BAT when rats were cold exposed for two days (saline-treated rats). Cycloheximide has completely inhibited the increase in BAT weight and protein content observed with cold exposure. So, cycloheximide has effectively blocked the growth of the brown adipose tissue in the cold exposed rats, after two days of treatment.

In the four days experiment, cycloheximide has significantly but not completely inhibited the larger increase in BAT wet weight ($p < 0.2$) and protein content ($p < 0.1$) observed with four days of cold exposure (Figures 10E and 10F). The weight of the BAT was slightly less in the cycloheximide-treated group at room temperature than in the saline-treated group at room temperature although the protein content of the BAT in the two groups was not different (Figures 10E and 10F). So cycloheximide has significantly inhibited the growth and the protein synthesis in the BAT of rats exposed to cold for four days.

c) effects on adenyl cyclase activity

After two days of treatment with cycloheximide, there was no significant difference for the noradrenaline-stimulated adenyl cyclase in any of the four groups of rats. The fluoride-stimulated adenyl cyclase showed the characteristic increase in total and specific activities observed after two days of exposure

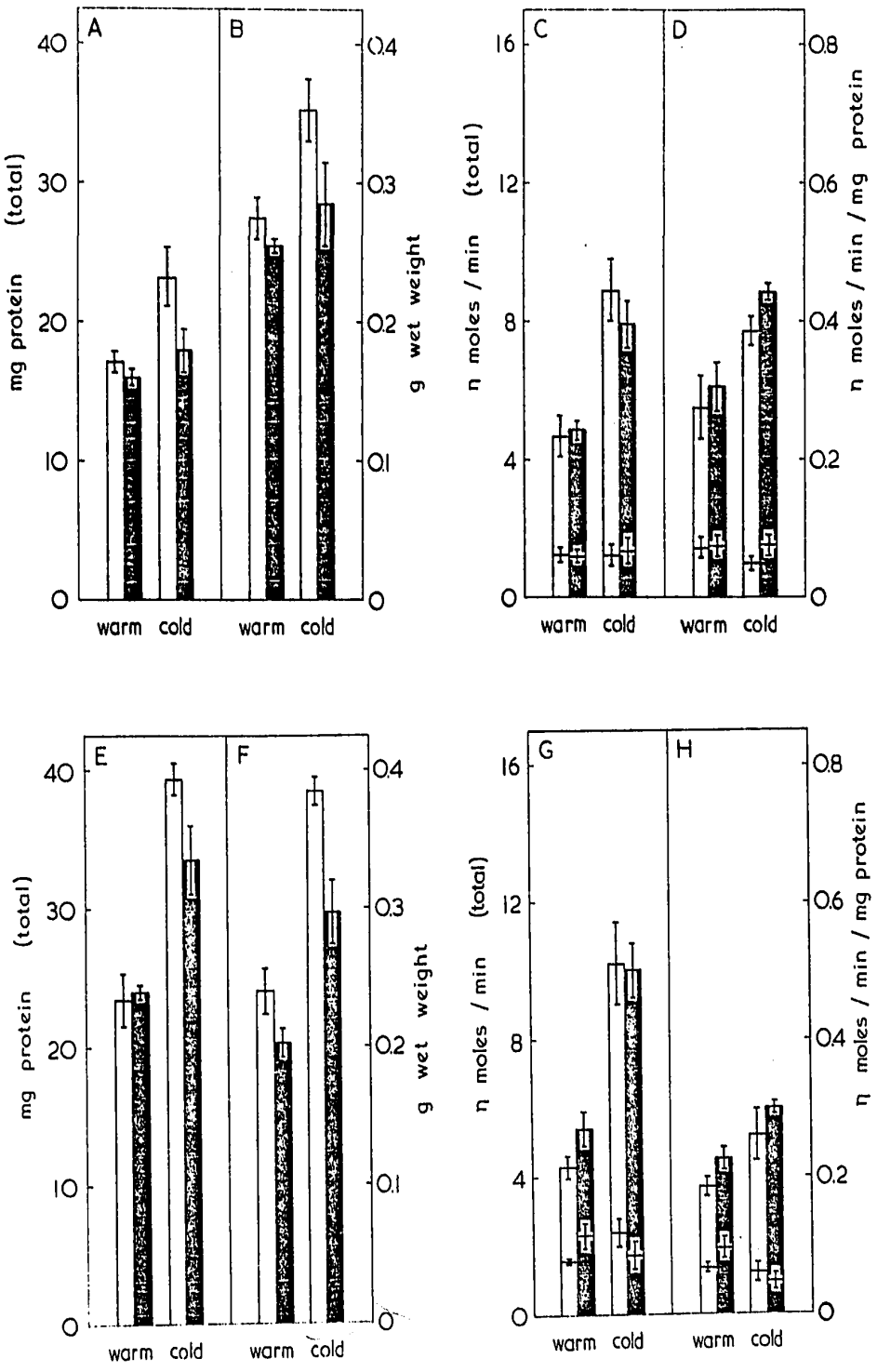
Figure 10: Effect of cycloheximide treatment on growth, protein content and adenyl cyclase activity in the interscapular brown adipose tissue of rats exposed to cold for two days.

Black bars represent cycloheximide-treated rats and open bars represent control (saline-treated) rats. Sections A, B, C and D are the results of experiments in which rats were treated for 2 days; the results of the experiments in which rats were treated for 4 days are in sections E, F, G and H. The rats were either in the warm or in the cold during the treatment, as shown.

From left to right the individual panels represent total protein content (A and E), wet weight (B and F), total activity of adenyl cyclase (C and G) and specific activity of adenyl cyclase (D and H). In those bars which represent adenyl cyclase activity the lower portion denotes the noradrenaline-stimulated activity and the total column denotes the fluoride-stimulated activity.

The effect of cold may be seen by comparing the right with the left bars within each panel. The effect of cycloheximide treatment may be seen by comparing the black bars with the open bars.

There are five rats in all groups except in the cycloheximide-treated cold-exposed, two day group in which there are four. The results are expressed as means ± standard errors.



to cold, in both the saline and the cycloheximide treated groups (Figures 10C and 10D). After four days of cold exposure and of treatment with cycloheximide, the results are essentially the same. The characteristic increase in the total and specific fluoride-stimulated adenyl cyclase activities (Figures 10G and 10H) in the cold exposed rats has not been inhibited by treatment with cycloheximide. The noradrenaline-stimulated enzyme showed some variations but there was no significant difference between the two cold exposed groups (Figures 10G and 10H).

Cycloheximide has then efficiently blocked the growth and protein synthesis of the brown adipose tissue in rats exposed to cold without inhibiting the increase in total and specific activities of the fluoride-stimulated adenyl cyclase caused by the exposure to cold.

2) actinomycin D

a) <u>effects on body weight</u>

Five injections of 12.5µg/kg of actinomycin D did not affect very much the growth of the rats either at 4°C or at 28°C. Five injections of 50µg/kg of the drug, caused a loss of an average of fifteen grams in body weight in the warm rats and a loss of an average of sixteen more grams in the cold treated rats than in the corresponding saline cold rats. In the four days experiment, nine injections of 12.5µg/kg caused a loss of an average of sixteen grams of body weight in rats at 28°C and a loss of an average of ten more grams in the treated rats at

Table 2

Toxicity of actinomycin D

Treatment	Warm Saline	Warm Actin. D 12.5µg/kg 9 injections	Warm Actin. D 50µg/kg 6 injections (average)	Cold Saline	Cold Actin. D 12.5µg/kg 9 injections	Cold Actin. D 50µg/kg 5 injections
Increase in body weight * during treatment	+26	+11	-33	-15	-25	-59
Number of rats per group	3	3	3	3	3	1
* Weight of tissues after treatment						
LIVER	9.72 ± 0.35	9.06 ± 1.68	5.66 ± 0.68	8.34 ± 0.93	9.50 ± 0.81	6.21
KIDNEYS	1.83 ± 0.11	1.55 ± 0.15	1.29 ± 0.10	1.72 ± 0.19	1.79 ± 0.17	1.56
HEART	0.80 ± 0.00	0.71 ± 0.04	0.57 ± 0.05	0.81 ± 0.06	0.80 ± 0.05	0.69
LUNGS	1.20 ± 0.08	1.17 ± 0.11	0.91 ± 0.12	1.14 ± 0.08	1.31 ± 0.07	0.90
SPLEEN	0.67 ± 0.05	0.54 ± 0.11	0.17 ± 0.08	0.67 ± 0.02	0.58 ± 0.08	0.20

* weights expressed in grams

4°C than in the control rats at that same temperature. Only one rat at 28°C and one rat at 4°C survived through nine injections of 50µg/kg of actinomycin D. The one at 28°C had lost thirty-seven grams by the end of the treatment while the one at 4°C had lost fifty grams, compared to the saline rats at 4°C which had lost an average of sixteen grams. All other rats treated with 50µg/kg of the drug, even if they received less than nine injections had also lost a lot of weight during the experiment.

b) toxicity of actinomycin D

All rats receiving from five to nine injections of 50µg/kg of actinomycin D showed signs of sickness. They all lost weight, their peritoneal cavity was full of fluid and their organs showed a decrease in weight. Table 2 shows that the three rats kept at 28°C which had received respectively five, six and seven injections have significantly less of all the organs weighed. The spleen was the most affected by the treatment and had gone from 0.67 g to 0.17 g. This would be in agreement with a very fast turnover of the cell components of that tissue (Leblond and Walker, 1956) or simply with the dissolution of the cells by actinomycin D (Harris, 1968). The different tissues are almost melting in the presence of the drug (Harris, 1968). The only cold exposed rat treated with 50µg/kg that could be dissected (four out of six rats died before the end of the experiment) had also less of all the tissues weighed (table 2). This remarkable toxicity of actinomycin D has been reported before (see appendix 2 for more details) and has made

difficult the use of the compound as a RNA or protein synthesis selective inhibitor. It is a too powerful tool in one sense because it will kill the animals at the same time that it will inhibit the RNA or the protein synthesis being studied.

c) effects on brown adipose tissue weight and protein content

The lower dose (12.5µg/kg) of actinomycin D, after two days of treatment has not affected the weight and protein content of the BAT in the rats kept at 28°C but has significantly ($p < 0.05$) blocked the increase in BAT wet weight and protein content observed with cold exposure (Figures 11A and 11B). The higher dose (50µg/kg) of the drug has inhibited growth and protein synthesis of the BAT in the warm acclimated rats as well as in the cold exposed rats. In other words, general protein synthesis has been blocked in the tissue and not only that which was induced by cold exposure (Figures 11A and 11B).

When the rats were treated for four days with 12.5µg/kg of the drug, there was no inhibition of the growth or of the protein synthesis in the BAT of the rats kept at either 4° or 28°C (Figures 11E and 11F). It seems that the animals are able to metabolize the drug because the effect that was obtained after two days with actinomycin D is not seen any more after treatment of the animals for two more days with the same concentration of the drug. But the high dose of actinomycin D had a tremendous blocking effect on the growth and the protein synthesis of the BAT either in warm acclimated or in cold exposed

rats. The rats were intoxicated but it is certain that protein synthesis in the BAT was blocked efficiently (Figures 11E and 11F).

d) effects on adenyl cyclase activity

There was no difference in the noradrenaline-stimulated total activities between the six groups of rats after two days of treatment (Figure 11C). The total NaF-stimulated activities increased with cold exposure and there was no difference between the three cold exposed groups, as well as between the three warm groups. After four days of treatment with actinomycin D, there was no difference between the three groups kept at 28°C for the total NaF-and noradrenaline-stimulated activities respectively. The cold exposed rats were not significantly different from each other as far as the NaF and the noradrenaline-stimulated total adenyl cyclase activities were concerned (Figure 11G). In other words, actinomycin D has not inhibited the increase in the total fluoride-stimulated adenyl cyclase activity observed with cold exposure.

The figures 11D and 11H illustrate that in all three groups of rats (saline, low actinomycin D concentration, high actinomycin D concentration) there was the typical increase in NaF specific activity of adenyl cyclase with cold exposure. The group treated with 50µg/kg showed a significantly higher activity with fluoride than the other two groups. The same effect is observed in the warm rats. But the total activity being the same in all three cold exposed groups, a decrease in total proteins of the tissue without a decrease in total adenyl cyclase activity would cause an increase in specific activity of adenyl

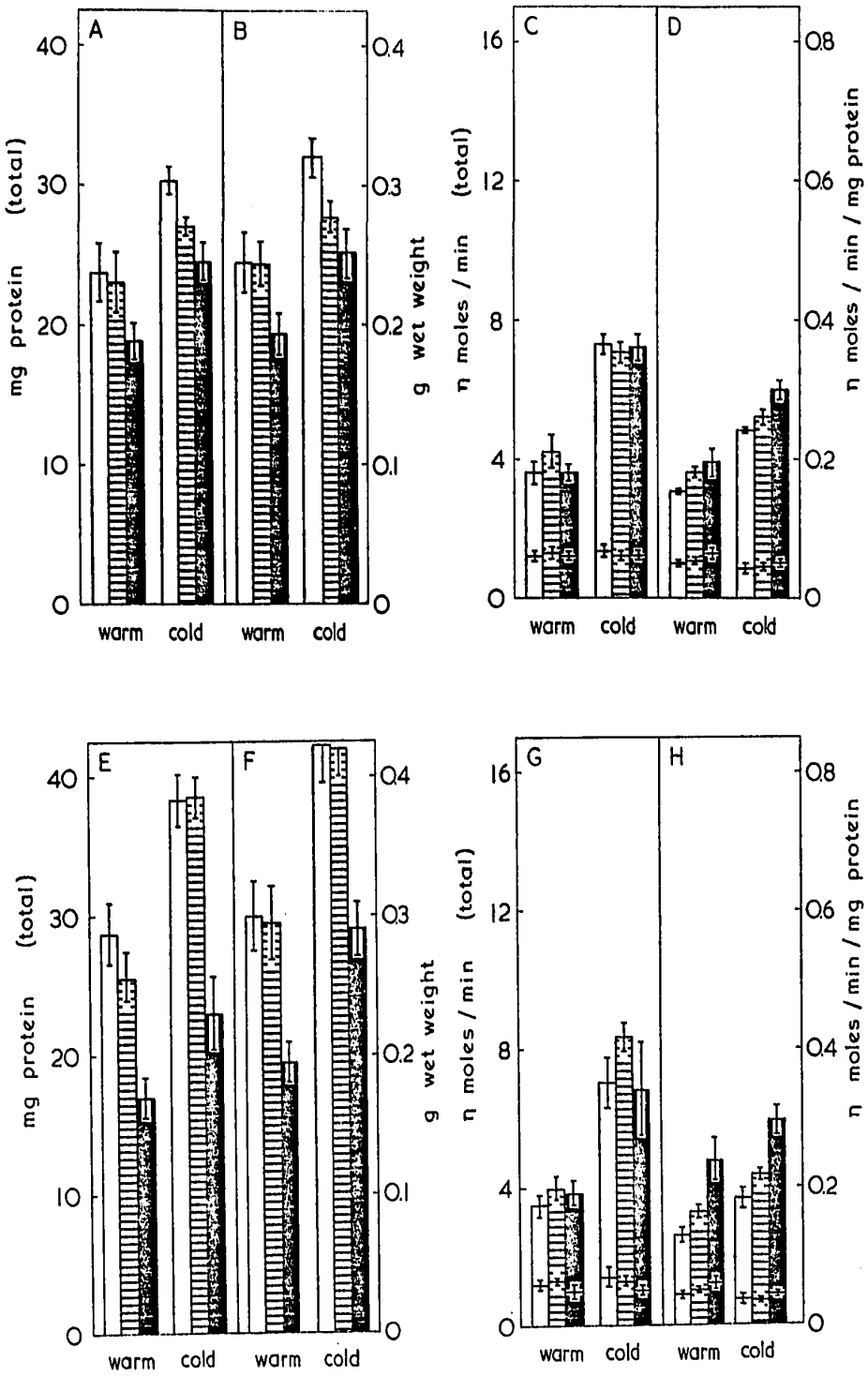
Figure 11: Effect of actinomycin D treatment on growth, protein content and adenyl cyclase activity in the inter-scapular brown adipose tissue of rats exposed to cold for two days.

White bars represent saline-treated rats, black bars represent rats treated with 50µg/kg (twice a day) of actinomycin D and striped bars represent rats treated with 12.5µg/kg (twice a day) of actinomycin D. Sections A, B, C and D are the results of experiments in which rats were treated for two days; the results of the experiments in which rats were treated for four days are in sections E, F, G and H. The rats were either in the warm or in the cold during the treatment, as shown.

From left to right, the individual panels represent total protein content (A and E), wet weight (B and F), total activity of adenyl cyclase (C and G) and specific activity of adenyl cyclase (D and H). In those bars which represent adenyl cyclase activity the lower portion denotes the noradrenaline-stimulated activity and the total column denotes the fluoride-stimulated activity.

The effect of cold may be seen by comparing the right with the left bars within each panel. The effect of actinomycin D (12.5µg/kg) treatment may be seen by comparing the striped bars with the white bars and the effect of actinomycin D (50µg/kg) treatment may be seen by comparing the black bars with the white bars.

There are five rats in all groups except in the actinomycin D (50µg/kg) treated cold-exposed rats, four day group in which there are two. The results are expressed as means ± standard errors.


A
B
C
D
E
F
G
H
mg protein (total)
g wet weight
η moles / min (total)
η moles / min / mg protein
warm
cold
warm
cold

cyclase. If the groups are compared two by two, [cold saline versus warm saline, cold actinomycin D (12.5µg/kg) versus warm actinomycin D (12.5µg/kg) and cold actinomycin D (50µg/kg) versus warm actinomycin D (50µg/kg)] in order to eliminate the effects of the treatment observed in the warm acclimated rats, it becomes clear that the increase in the NaF-stimulated adenyl cyclase activity observed with cold exposure as well as the decrease in the noradrenaline-stimulated adenyl cyclase observed with cold exposure have not been affected by treatment of the rats with actinomycin D for two or four days.

In other words, it has been possible to stop the growth and protein synthesis in the brown adipose tissue of rats exposed to cold with actinomycin D without affecting the increase in the NaF stimulated activity of the adenyl cyclase and the decrease in the noradrenaline-stimulated activity observed when rats are exposed to cold.

E) Further studies of the extra adenyl cyclase activity observed during cold exposure and cold acclimation

The extra brown adipose tissue adenyl cyclase which appears after two days of exposure to cold and persists throughout cold acclimation cannot be due to synthesis of a new adenyl cyclase induced by cold exposure since treatment of the rats with doses of cycloheximide and actinomycin D which inhibit the growth and the protein synthesis of the tissue cannot block the appearance of the extra adenyl cyclase activity with cold exposure. It is possible that this extra adenyl cyclase activity is due to a

modification of the enzyme already present. This modification could be, for example, the stimulation of the enzyme by a hormone different from noradrenaline to which the enzyme was not sensitive in the warm acclimated rat. Cold stimulus, if this was the case, could induce a change in the properties of adenyl cyclase. Agents other than hormones could also possibly modify adenyl cyclase activity in the cold exposed rat but not in the warm acclimated rat.. Some of these possibilities were tested in order to elucidate the reason for the increase in adenyl cyclase activity observed in the cold.

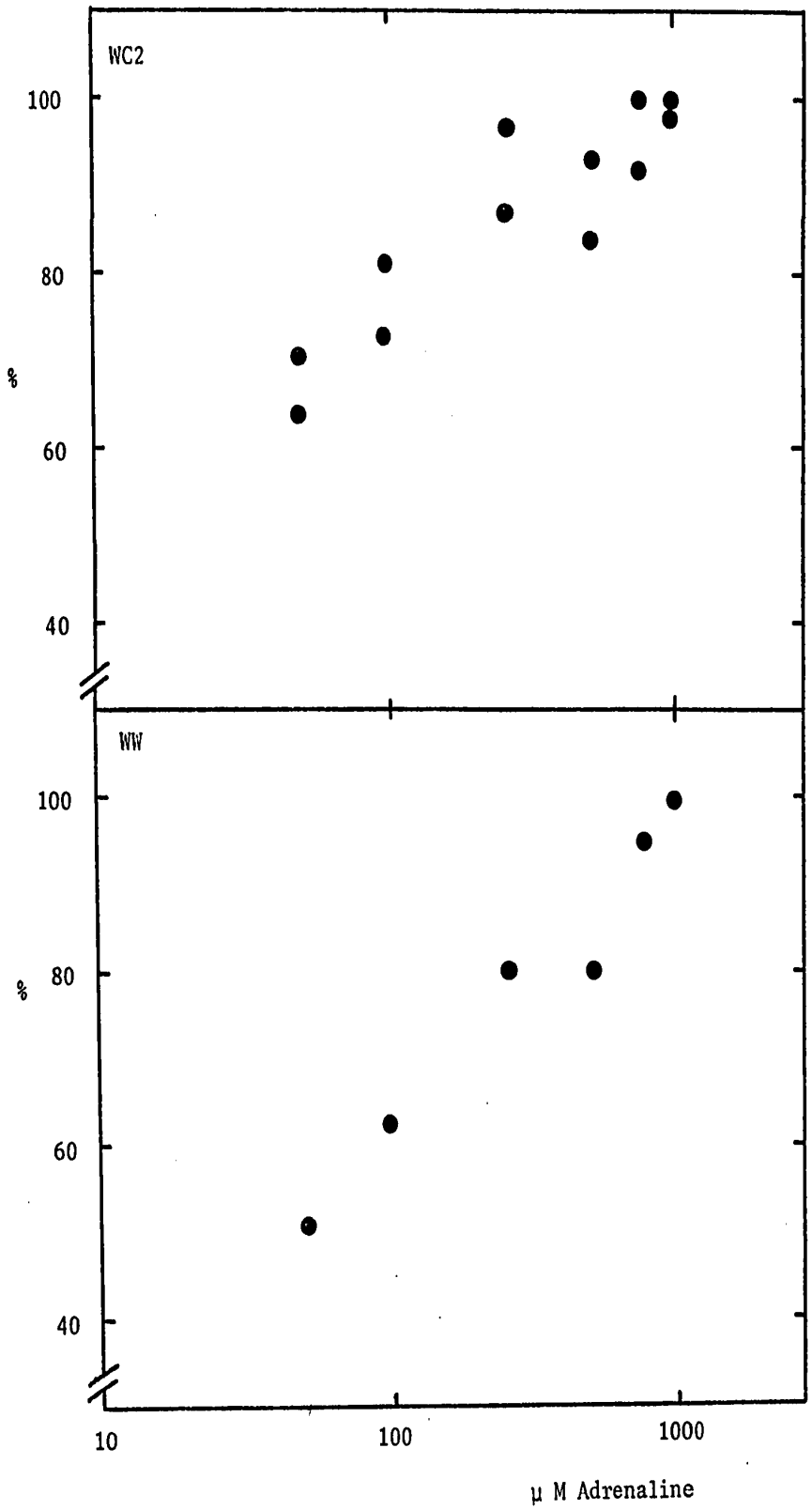
1) *Stimulation by adrenaline*

A change in the stimulation of adenyl cyclase by noradrenaline does not necessarily imply a change in the stimulation of the enzyme by adrenaline. Adenyl cyclase is stimulated to the same extent by the same concentration of adrenaline and noradrenaline in BAT of warm and cold acclimated rats. The dose-response curves to noradrenaline and adrenaline are almost identical and maximal stimulation is obtained in both cases with 0.75mM. After two days of exposure to cold, adenyl cyclase from BAT is much less stimulated by noradrenaline than in the warm acclimated rats but the sensitivity of the enzyme to noradrenaline is not changed so that the decrease in stimulation is not due to a shift in the dose-response curve (Figure 8). The sensitivity of BAT adenyl cyclase to adrenaline is not affected either by exposure to cold (Figure 12). The proportion of the total enzyme (NaF-stimulated) that is stimulated by adrenaline is 30% in warm acclimated rats and 15% in rats exposed to cold

Figure 12: Dose-response curve for the stimulation of adenyl cyclase activity by adrenaline in control and in cold exposed rats.

The top graph represents pooled values for two rats and the bottom graph represent values for one rat (means of duplicate determinations). In each case, activities are expressed in terms of the maximum activity, shown here as 100%.

The section designated by the symbol WC_2 represents the dose-response curve obtained for rats that have been exposed to cold for two days. The section designated by the symbol WW represents the dose-response curve for the control rats, i.e. the warm acclimated, day zero rats.



for two days. The extra enzyme activity was defined as that part of the NaF-stimulated activity which is not stimulated by noradrenaline and it is probably not stimulated either by adrenaline. The adrenaline-stimulated activity seems to vary in the same way as the noradrenaline-stimulated activity, since the dose-response curve to both hormones is the same and since both activities are decreased to approximately the same extent by exposure to cold.

2) stimulation by other hormones

Figure 13 shows that none of the following hormones is able to modify significantly adenyl cyclase from BAT homogenate of rats that have lived in the cold for two days: ACTH, 5 to 100µg/ml (figure 13B); dexamethasone, 1 to 10µg/ml (figure 13C); growth hormone, 5 to 100µg/ml, (figure 13D); corticosterone, 1 to 10µg/ml (figure 13E); L-triiodothyronine, 5 to 50µg/ml (figure 13F); serotonin, 1 to 10µg/ml (figure 13G); insulin, 10 to 100munits/ml, (figure 13H).

It was shown by Skala and coworkers (1970a) that adenyl cyclase from brown adipose tissue homogenate from young warm acclimated rats cannot be stimulated by any of the hormones tested here (see section 1A). So, the extra BAT adenyl cyclase activity observed when rats are exposed to cold cannot be explained by a stimulation of the enzyme by any of the hormones tested.

There is no stimulation of adenyl cyclase by ACTH when 1mM Ca^{++} is added to the incubation medium. There is no stimulation of adenyl cyclase by serotonin when 0.1mM pargyline (an

Figure 13: Stimulation of brown adipose tissue adenyl cyclase by hormones other than catecholamines.

The following hormones were tested:

section A, ACTH, 5 to 100μg/ml;

section B, glucagon, 5 to 100μg/ml;

section C, dexamethasone, 1 to 10μg/ml;

section D, growth hormone, 5 to 100μg/ml;

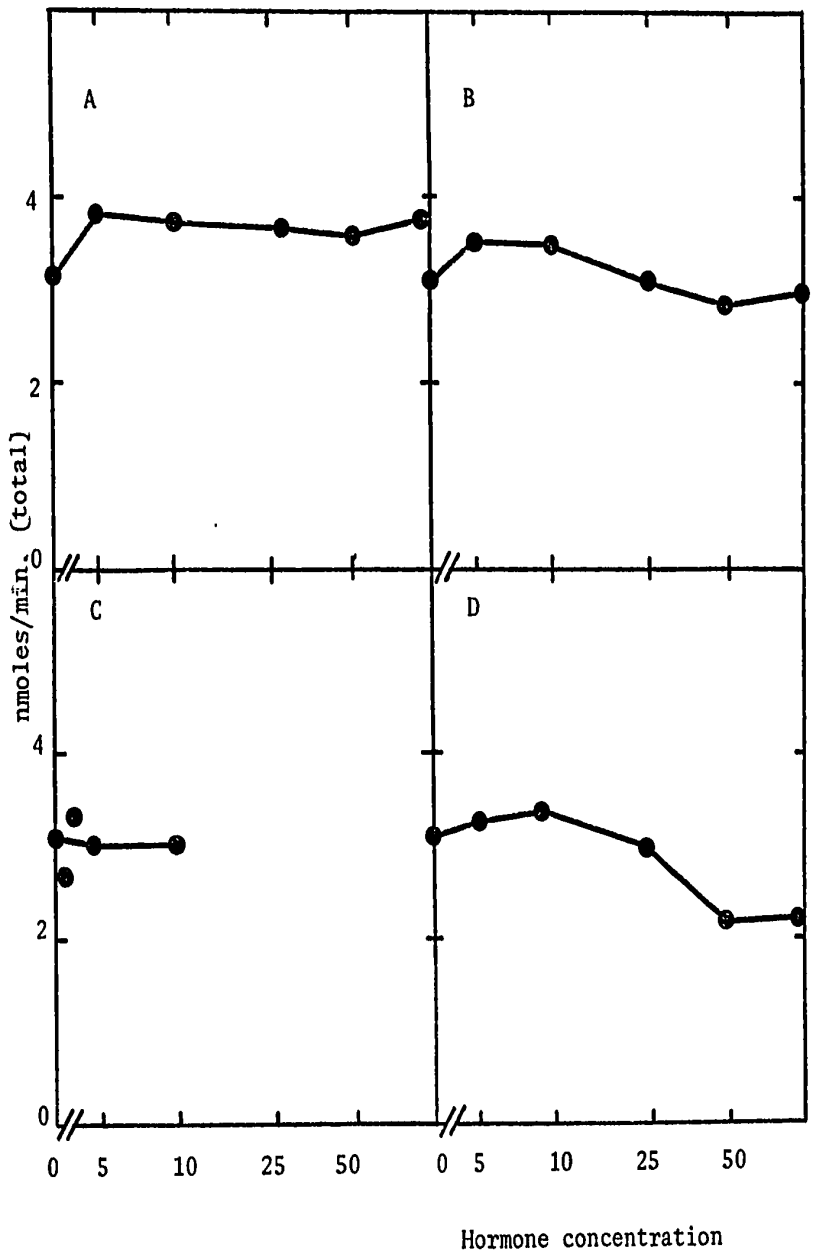
section E, corticosterone, 1 to 10μg/ml;

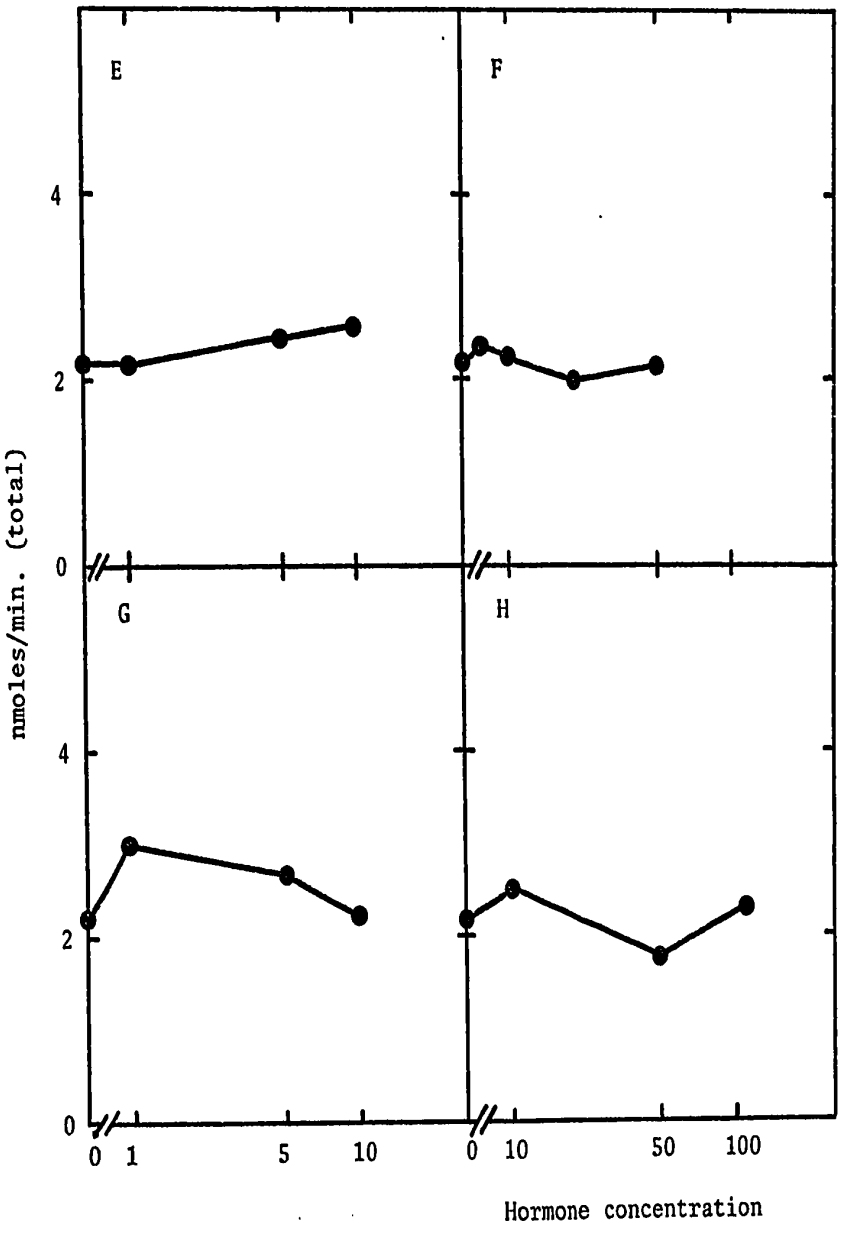
section F, L-triiodothyronine, 5 to 50μg/ml;

section G, serotonin, 1 to 10μg/ml;

section H, insulin, 10 to 100munits/ml.

Adenyl cyclase activity expressed in nanomoles per minute per total interscapular brown adipose tissue. Determinations were done in the homogenate from two pooled interscapular brown fat pads from rats that had been exposed to cold for two days.





inhibitor of monoamine oxidase) is added to the incubation medium.

3) effects of calcium

1mM Ca^{++} inhibited the basal, noradrenaline-stimulated, adrenaline-stimulated, and NaF-stimulated adenyl cyclase activities in the BAT of rats exposed to cold for two days. (Figure 14A). No stimulation of the noradrenaline and NaF-stimulated activities was obtained over a range of calcium concentrations from 0.05mM to 2.0mM, and in fact inhibition was obtained at every concentration tested (Figure 14B). The addition of EGTA [ethyleneglycol-bis (β-amino ethyl ether) N,N'-tetraacetic acid], a chelating agent which binds specifically Ca^{++} and removes all calcium from the incubation medium, had no effect on adenyl cyclase from BAT homogenate, at concentrations from 0.025 to 0.25mM. So, the amount of calcium present in the tissue homogenate is not sufficient to inhibit adenyl cyclase activity but addition of 2mM Ca^{++} can cause complete inhibition of adenyl cyclase activity.

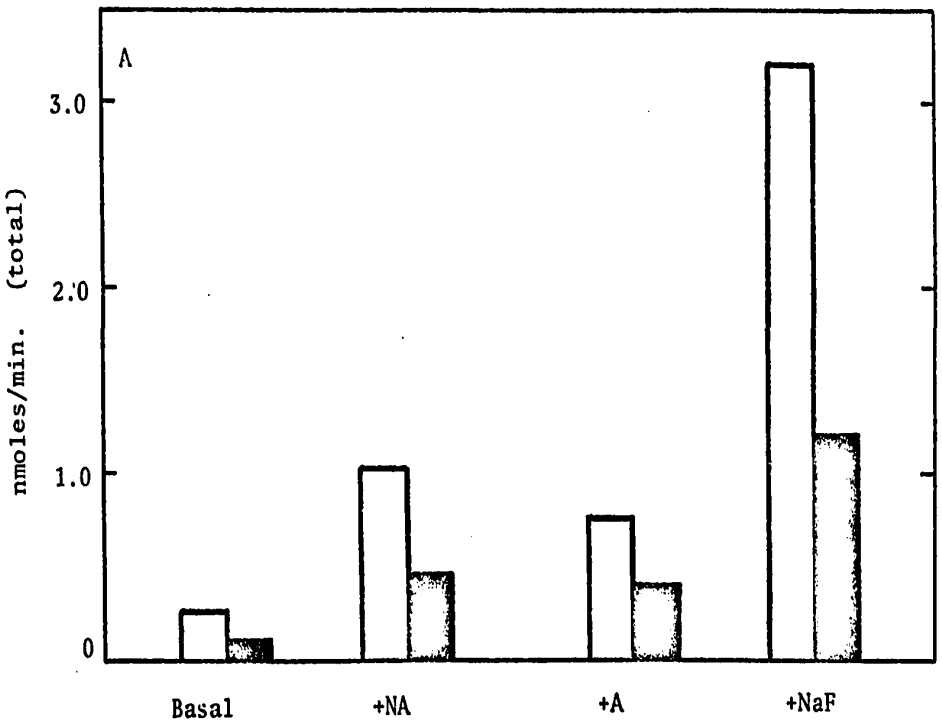
4) effects of triton X-100

Triton X-100 can stimulate adenyl cyclase activity from rat cerebral cortex homogenate (Perkins and Moore, 1971); concentrations between 0.01% and 0.2% in the incubation medium stimulated the basal adenyl cyclase more than 5mM NaF did, and stimulated the fluoride activity as well. In that case, it cannot be said that the fluoride-stimulated activity represented the total amount of enzyme present. In the BAT homogenate, the

Figure 14: Inhibition of brown adipose tissue adenyl cyclase activity by calcium.

A) Inhibition by 1mM calcium of the basal, the noradrenaline-stimulated, the adrenaline-stimulated and the fluoride-stimulated adenyl cyclase activities in brown adipose tissue from rats exposed to cold for two days. White columns represent the activity in the absence of Ca^{++} while the black columns represent the activities obtained when 1mM Ca^{++} is present in the incubation medium. Activities are expressed in nanomoles per minute per total interscapular brown fat pad.

B) Inhibition of the NA-stimulated (Δ) and the NaF-stimulated (□) adenyl cyclase activities by different amounts of Ca^{++}. The activities are expressed in percentage of the activity obtained when no Ca^{++} is present in the medium. The concentrations of Ca^{++} used vary between 0.05 and 2mM.



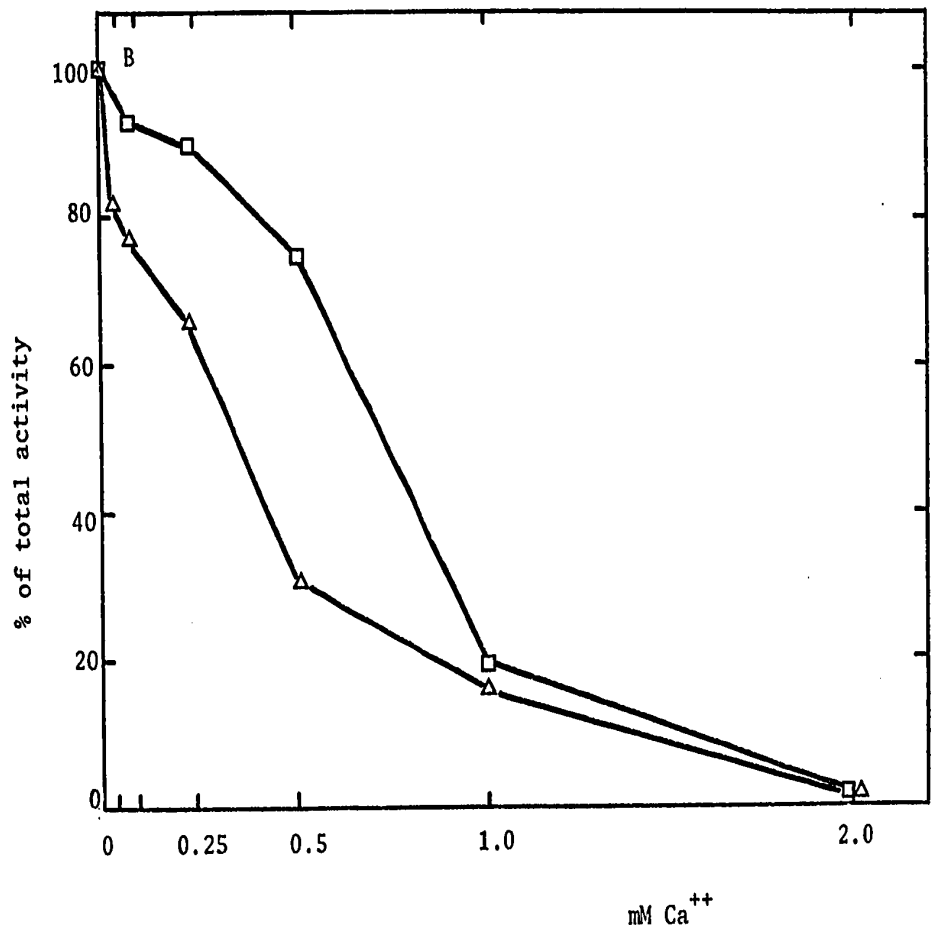
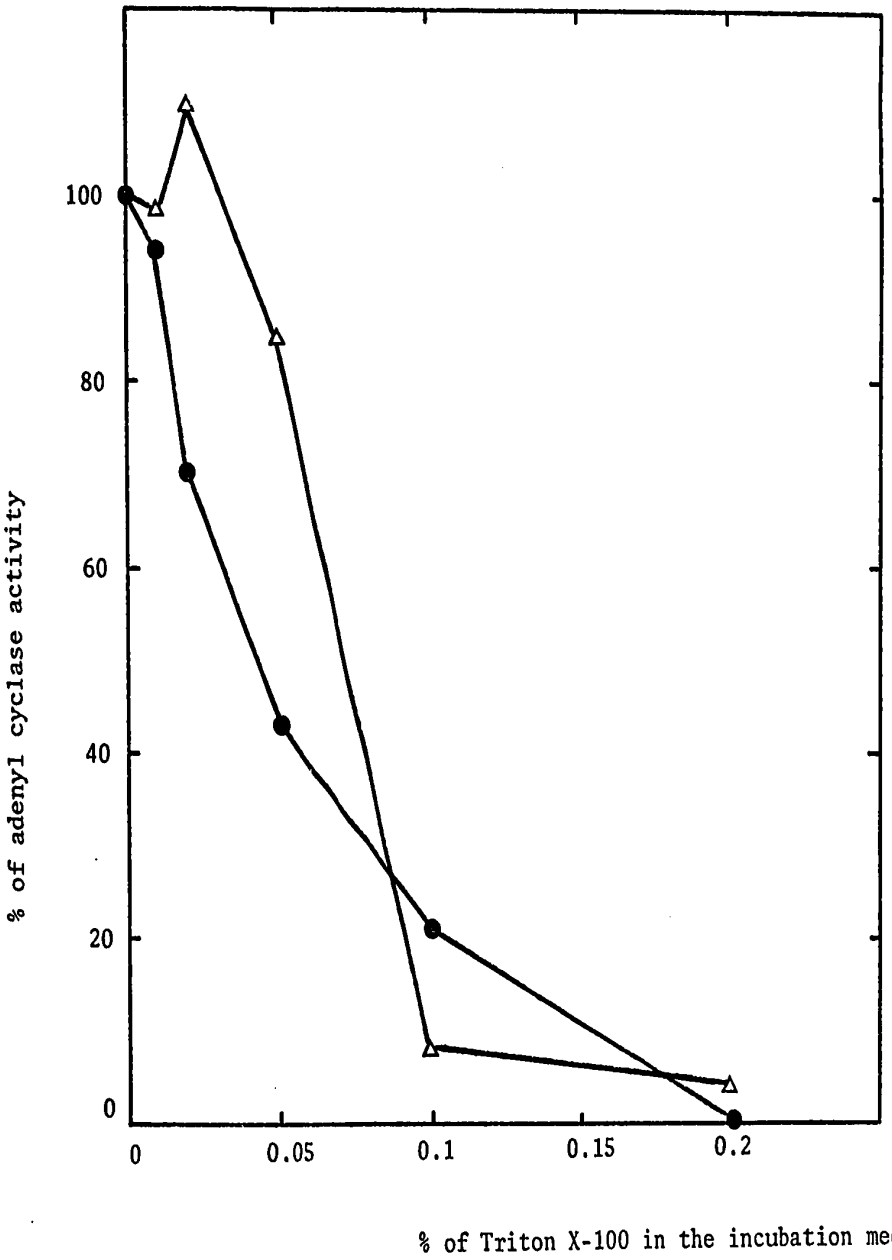


Figure 15: Inhibition by Triton X-100 of adenyl cyclase of brown adipose tissue from rats exposed to cold for 2 days.

• represents the basal activity

Δ represents the fluoride-stimulated activity

Activities are expressed as % of the activity obtained in the absence of Triton X-100.



addition of Triton X-100 (0.01% to 0.2% in the incubation medium) did not increase the basal or the NaF-stimulated adenyl cyclase activities but inhibited both (figure 15). A 0.2% concentration inhibited completely the basal and the fluoride-stimulated adenyl cyclase activities (figure 15). In rat cerebral cortex homogenate, the non specific interactions of Triton X-100 with the cell membrane structure activated adenyl cyclase but in the BAT homogenate, these non specific interactions with the cell membrane structure were detrimental to adenyl cyclase and it makes our hypothesis of the NaF-stimulated activity representing the total adenyl cyclase activity present still valid.

F) Discussion

An enhancement of the calorigenic response to noradrenaline as observed in cold acclimated rats could be reflected by an increase in the noradrenaline-responsive receptors in the cell membrane of the tissues which are important in the process of nonshivering thermogenesis. For these reasons the noradrenaline stimulated adenyl cyclase activity of interscapular brown adipose tissue was measured during acclimation to cold. The anticipated growth of the interscapular brown adipose tissue occurred in response to cold but the formation of the noradrenaline-stimulated adenyl cyclase did not keep pace with the growth of the tissue; the specific activity of this enzyme was reduced in the tissue of the cold acclimated animals. Thus no evidence was obtained for any increase in the noradrenaline-

sensitive system in the cold acclimated animals. The enhancement of the calorigenic response to noradrenaline in cold acclimated rats is not accompanied either by an increase in the sensitivity of adenyl cyclase from brown adipose tissue to noradrenaline. The dose-response curve for the stimulation of adenyl cyclase by noradrenaline is the same either in cold acclimated or in warm acclimated rats or in rats that have been exposed to cold for two days.

Unlike the noradrenaline-stimulated adenyl cyclase, the fluoride-stimulated enzyme does undergo rapid changes in activity during exposure to cold. A marked increase in specific activity (almost two-fold) occurred during the first two days of exposure to cold at a time when the noradrenaline-stimulated enzyme decreased in activity and when the growth of the tissue had not yet started. This extra activity persisted throughout the eight weeks of cold acclimation but practically disappeared within one day when the rats were returned to room temperature. This is clearly a rather rapid and reversible change occurring in response to a change in environmental temperature. The changes observed are unlikely to be due simply to the growth of the tissue. Another rapidly growing tissue, regenerating rat liver, maintains a constant specific activity of both adrenaline-stimulated and glucagon-stimulated adenyl cyclase throughout the growth period (Becker and Bitensky, 1969).

This extra adenyl cyclase activity, not stimulated by noradrenaline, does not seem to be due to increased protein synthesis in the brown adipose tissue since doses of cycloheximide

and actinomycin D which inhibited growth and protein synthesis in the brown adipose tissue did not block the appearance of the extra adenyl cyclase activity. Careful interpretation of results obtained with such potent drugs as cycloheximide and actinomycin D which can be toxic to the animals, must be done. If, for example, a particular physiological function or a synthetic process is impaired by a high dose of actinomycin D, it cannot be concluded that this function or synthetic process is immediately dependent upon transcription of DNA. Actinomycin D can cause dissolution of cells and secondary effects not directly related to the blockade of RNA synthesis (Harris, 1968). In the same way if a particular reaction is impaired by protein synthesis inhibitors (one of the immediate consequences of the use of actinomycin D is the inhibition of protein synthesis and in that sense it is considered as a protein synthesis inhibitor), it cannot be concluded that the impairment is due to a direct inhibition of protein synthesis. But if the function studied persists in the presence of proven inhibitory concentrations of the drug, it can be said with more certitude that the given function does not require protein synthesis (Harris, 1968). In the present study, growth and protein synthesis of the BAT were inhibited by both actinomycin D and cycloheximide while the extra adenyl cyclase activity still appeared upon exposure to cold. It can then be concluded with some certitude that the increase in the specific activity of the NaF-stimulated adenyl cyclase observed during cold exposure is not due to increased protein synthesis or to synthesis of a new protein.

The extra adenyl cyclase activity does not appear to be due to stimulation of brown adipose tissue adenyl cyclase during cold exposure by hormones which do not stimulate the enzyme in warm acclimated rats, since ACTH, glucagon, dexamethasone, growth hormone, corticosterone, L-triiodothyronine, and serotonin did not have any stimulatory effect on the enzyme in rats that had been exposed to cold for two days (figure 13) or in warm acclimated rats (Skala et al., 1970a). But, as was mentioned before, ACTH, glucagon, TSH and serotonin, as well as adrenaline and noradrenaline can stimulate oxygen consumption in brown adipose tissue slices (Joel, 1965; Beviz et al., 1971), and it was shown that the stimulatory effect of noradrenaline was mediated by cyclic AMP (Beviz et al., 1971). The fact that no stimulation of adenyl cyclase was observed in the brown adipose tissue homogenate could mean that the receptors for these different hormones able to stimulate respiration in the tissue slices, had been damaged by homogenization. The stimulation of brown adipose tissue respiration and lipolysis by hormones other than catecholamines has only been observed in tissue slices and not in isolated fat cells or in homogenates (Girardier and Seydoux, 1971). So in order to see any stimulation of adenyl cyclase by hormones other than catecholamines, it may be necessary to use tissue slices. For that purpose, the method of determination of adenyl cyclase activity used in the present study is inadequate (Krishna et al., 1968a). Incubating tissue slices with adenosine-C^{14} would be a proper way of measuring adenyl cyclase activity in brown adipose tissue slices (Kuo and

Dill, 1968) (see section 1c) Even if adenyl cyclase from tissue slices was stimulated by some other hormones than catecholamines, it would not necessarily mean that there was a direct stimulation of adenyl cyclase by these hormones. It has been postulated that the fact that stimulation of respiration in isolated fat cells from brown adipose tissue by hormones other than catecholamines does not occur, while it occurs in slices of the same tissue, means that these hormones have an indirect effect on the cells via the release of catecholamines from nerve endings (Girardier and Seydoux, 1971). If adenyl cyclase from tissue slices was more active in the cold exposed rats in the presence of some hormone other than catecholamines, it could mean that in vivo adenyl cyclase was more stimulated, even if indirectly, by an increased release of catecholamines from nerve endings caused by an increased secretion of some other hormone. The extra adenyl cyclase activity being observed in homogenates, it would be expected that the factor responsible for its existence could stimulate the enzyme in the same conditions, i.e. in the tissue homogenates. This extra adenyl cyclase activity could still be due to stimulation of adenyl cyclase by hormones other than catecholamines, even if this was not observed in the experiments performed. Some hormones could require the presence of additional cofactors for stimulation of respiration and lipolysis as well as for stimulation of adenyl cyclase, factors which could have been removed or damaged by homogenization of the tissue or isolation of the cells. Stimulation of lipolysis by ACTH in white adipose tissue, requires Ca^{++} (Bär

and Hechter, 1969). Adding calcium to brown adipose tissue homogenate did not cause any stimulating effect of ACTH. No effect of serotonin on lipolysis in white adipose tissue is observed unless the monoamine oxidase activity of the tissue preparation is inhibited by pargyline. Serotonin is rapidly inactivated by oxidative deamination by monoamine oxidase, during incubation (Bieck et al., 1966). In the brown adipose tissue, no effect of serotonin on lipolysis could be observed even when safrazine (an inhibitor of monoamine oxidase) was present (Yoshimura et al., 1969a), but when theophylline was added with serotonin (theophylline alone had a stimulatory effect on lipolysis) a lipolytic action of serotonin on tissue slices could be observed. The effect of serotonin on brown adipose tissue slices respiration was also potentiated by theophylline (Yoshimura et al., 1969a). In the present study, neither pargyline nor theophylline had any effect on the action of serotonin on brown adipose tissue adenyl cyclase activity. The effects of serotonin on the lipolysis and the respiration of slices of brown adipose tissue were completely abolished by reserpinization of the animals or significantly diminished by denervation of the brown adipose tissue (Yoshimura et al., 1969b). The effects of theophylline or adrenaline on the same parameters were not influenced by reserpinization or denervation (Yoshimura et al., 1969b). Stimulation of adenyl cyclase of the brown adipose tissue by noradrenaline or fluoride was not influenced by denervation of the brown adipose tissue (Forn et al., 1970a). The effects of noradrenaline on the respiration and the lipolysis of brown adipose tissue slices could be potentiated by serotonin

and it was concluded that serotonin could only exert an effect on brown adipose tissue slices in the presence of noradrenaline or theophylline (Yoshimura et al., 1969b). Theophylline could cause the release of catecholamines from nerve endings (Strubelt and Siegers, 1969) and that it is probably via those catecholamines that serotonin had an effect on respiration and lipolysis while in the presence of theophylline.

White adipose tissue ghost adenyl cyclase from adrenalectomized or hypophysectomized rats exhibits a striking reduction in response to ACTH but not in the response to adrenaline, glucagon and fluoride (Braun and Hechter, 1970). Cold exposure can elicit hypersecretion of ACTH. After twenty hours of cold exposure, the ACTH content of the pituitary gland is only 35% of its normal value. After two hours of cold exposure, there is a 300% increase in the level of plasma corticosterone, as a consequence of increased secretion of ACTH from the pituitary gland, but after twenty hours in the cold, the plasma level of corticosterone is back to normal (Maickel et al., 1961). If hyposecretion of glucocorticoids can decrease the ACTH stimulation of adenyl cyclase in the white adipose tissue, it is a pleasing hypothesis that the increased adenyl cyclase activity in brown adipose tissue could be due to an increased stimulation of the enzyme by ACTH occurring in response to a hypersecretion of glucocorticoids at the beginning of cold exposure. Unfortunately, brown adipose tissue adenyl cyclase is not stimulated by ACTH in rats that have been exposed to cold for two days, although there was an increased secretion of glucocorticoids in the animals, in the first twenty hours of cold exposure.

The extra adenyl cyclase activity observed in brown adipose tissue during exposure to cold is not due to synthesis of a new protein and is not due, as far as can be told, to stimulation of the enzyme from the cold exposed rats by a hormone which did not stimulate the enzyme in the warm acclimated rats. It is possible that the extra adenyl cyclase be due to variation in the concentration of some intracellular or extracellular metabolites induced by cold exposure. Calcium can either stimulate or inhibit adenyl cyclase activity (see section I, B 2c) and it is also an important regulator of cell function (Rasmussen and Tenehouse, 1968). In rats exposed to cold for two days, calcium inhibited basal, noradrenaline, adrenaline and NaF stimulated adenyl cyclase activities in the brown adipose tissue and certainly could not account for the increased stimulation of adenyl cyclase activity observed under those conditions.

It would be desirable to know if the changes in adenyl cyclase activity observed during cold acclimation were actually occurring in brown fat cells themselves. But although it is possible to prepare brown fat cells from warm acclimated rats, (Fain et al., 1967) it is at the moment impossible to isolate brown fat cells from cold acclimated rats (Behrens and Himms-Hagen, unpublished observations). Studies of the ultrastructure of the interscapular brown adipose tissue during adaptation of rats to cold (Suter, 1969; Thomson et al., 1969) have stressed the heterogeneous appearance of the brown fat cells, which may vary from unilocular through multilocular with a few large lipid droplets to multilocular with many or few very small lipid drop-

lets; this heterogeneous appearance of the cells persists during the first week of exposure to cold yielding at later stages of adaptation to a more homogeneous appearance of the cells (Thomson et al., 1969). The low proportion of brown fat cells in total interscapular brown adipose tissue reported in the original method for the isolation of these cells (Fain et al., 1967) together with the more recent information from the ultrastructure studies (Suter, 1969; Thomson et al., 1969) suggest that many of the brown fat cells are not isolated by this technique. The variable lipid content is the most probable explanation for the difficulty encountered in preparing a representative population of isolated brown fat cells from rats during the course of adaptation to cold. Thus although it would be desirable to use a relatively pure preparation of brown adipose tissue cells to prove that the changes in adenyl cyclase activity observed are actually occurring in brown fat cells themselves, this is not feasible with currently available techniques. The preponderance of brown adipose tissue cells of various kinds in the interscapular brown adipose tissue (Suter, 1969; Thomson et al., 1969) suggests that the change in enzyme activity is most probably occurring in these cells.

No function can at present be attributed to the extra fluoride-stimulated adenyl cyclase activity that appears so rapidly after exposure to cold, persists throughout acclimation and disappears almost completely when the rats are returned to room temperature for one day. All that can be concluded here is that adenyl cyclase of the brown adipose tissue is modified by

exposure to cold in a way which decreases its ability to be stimulated in vitro by noradrenaline, and that the way by which the adenyl cyclase is modified is unknown at the present moment.

Section III: Experimental: adenyl cyclase in skeletal muscle

A) Material and methods

1) *animals*

see section II A, 1

2) preparation of skeletal muscle homogenates

Leg muscles were removed immediately after the rat was killed and placed in cold KCl 0.15 M. The muscle was then cleaned from fat and connective tissue, weighed and immersed in Chappell-Perry medium without ATP (Chappell and Perry, 1954). (The composition of the Chappell-Perry medium is: 0.1 M KCl, 0.05 M Tris-HCl buffer pH 7.4, 0.001 M Na-ATP, 0.005 M $MgSO_4$ and 0.001 M EDTA). The tissue was then minced finely with scissors and homogenized twice, first in a glass homogenizer and then in a Teflon homogenizer. The homogenate was then filtered through glass wool and diluted with Chappell-Perry medium (without ATP) to a final concentration of 100mg of tissue per ml of homogenate.

3) assay of adenyl cyclase

Adenyl cyclase was assayed essentially as described in section II A, 4. The ATP-regenerating consisted of 13mM phosphoenolpyruvic acid (tricyclohexylamine salt) and in most assays (unless otherwise specified) of one unit of pyruvate kinase type II (obtained from Sigma and prepared from rabbit skeletal muscle,

substantially free of lactic dehydrogenase, creatine phosphokinase and phosphoglucomutase).

4) protein estimation

Protein was estimated by the Lowry method (Lowry et al., 1951).

5) chemicals

See section II, A, 6.

B) Properties

The following optimal concentrations of the different components required for the adenyl cyclase reaction have been found for skeletal muscle homogenates

1) *enzyme concentration*

The velocity of the reaction is linear at 37°C for 30 minutes with concentrations of enzyme corresponding to amounts of tissue up to ten mg (total amount of skeletal muscle present in the incubation medium). With fifteen mg of skeletal muscle, the reaction is linear only for 20 minutes (Figure 16A). Ten mg of skeletal muscle was the amount used in most assays and the incubation time was usually 20 minutes. The homogenate was diluted in Chappell-Perry medium (without ATP) to a concentration of 100mg/ml and 0.1ml of that solution was added to the incubation mixture to initiate the reaction.

Figure 16: Adenyl cyclase activity in skeletal muscle homogenates; effects of enzyme concentration and temperature.

A) Velocity versus enzyme concentration

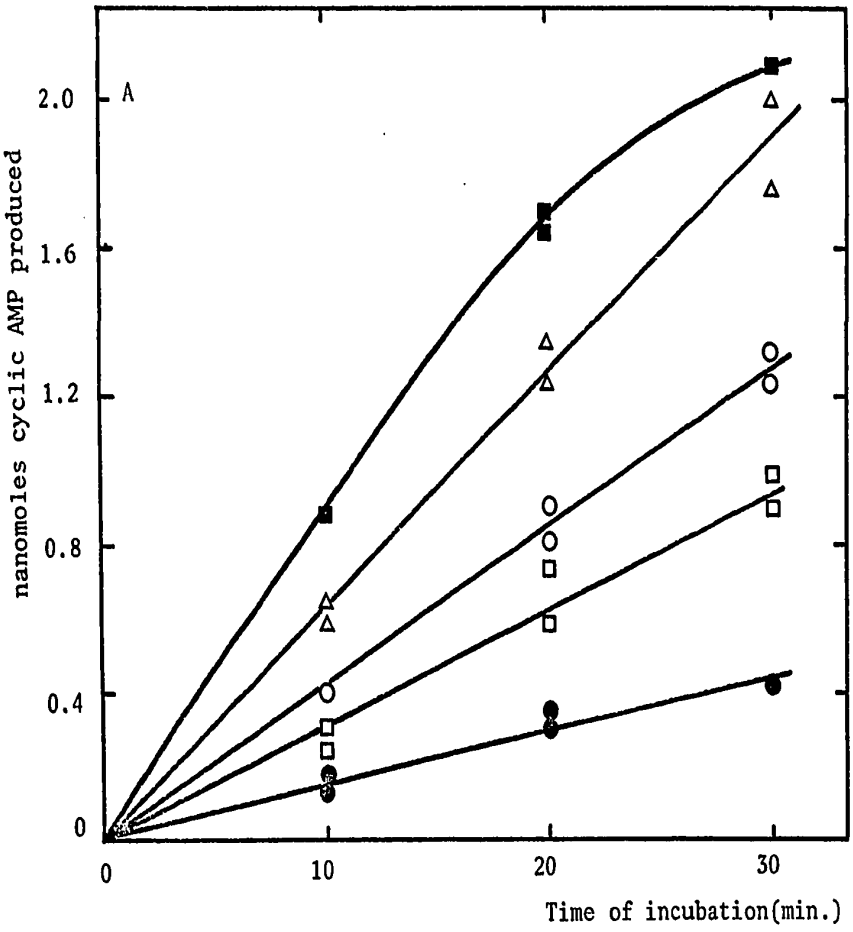
The incubation was carried out at 37°C and contained different amounts of skeletal muscle added in 0.1 ml of homogenate to the other 0.3 ml of the incubation medium

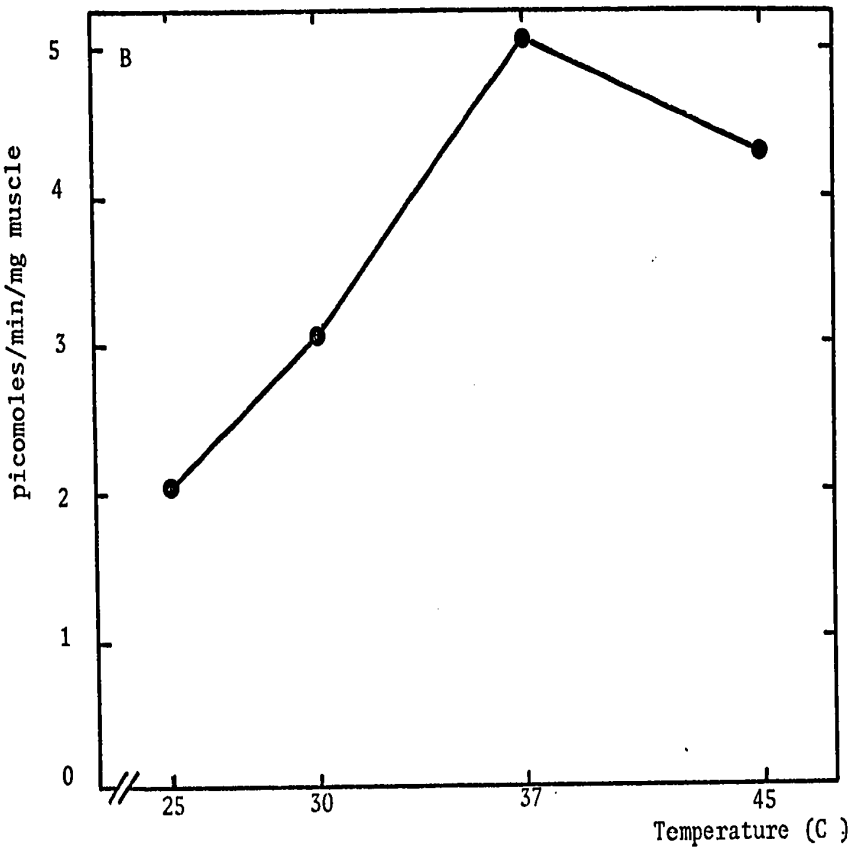
- ● 2 mg;
- □ 4 mg;
- o 6 mg;
- Δ 10 mg;
- ■ 15 mg.

The incubation mixture contained 10mM NaF, 3mM ATP, 16mM $MgSO_4$, 13mM phosphoenolpyruvate, 1 unit of pyruvate kinase type I, 10mM theophylline, and 0.25mM EDTA in 40mM Tris-HCl buffer, pH 7.4.

B) Velocity versus temperature

Ten mg of skeletal muscle were incubated for 20 minutes with 2.5mM ATP, 8.75mM $MgSO_4$, 10mM NaF, 13mM phosphoenolpyruvate, 1 unit of pyruvate kinase type I, 10mM theophylline and 0.25mM EDTA in 40mM Tris-HCl, pH 7.4.





2) temperature of the reaction

Adenyl cyclase is much less active at 30°C than at 37°C (figure 16B). The latter temperature was adopted for most assays. Adenyl cyclase specific activity is approximatively ten times less in skeletal muscle than in brown adipose tissue. The basal activity of adenyl cyclase of skeletal muscle is hardly measurable at 30°C and that is why 37°C was adopted for most assays of adenyl cyclase in skeletal muscle.

3) fluoride concentration

Adenyl cyclase activity is maximal with 7.5mM NaF (figure 18B); higher concentrations of NaF give stimulation of adenyl cyclase less than that observed with optimal concentration (figure 18B).

4) concentrations of adrenaline and noradrenaline

Figure 22 illustrates the dose-response curve of skeletal muscle adenyl cyclase to noradrenaline. The optimal noradrenaline concentration is between 0.25 and 0.75mM. Higher concentrations inhibit the stimulation of the enzyme. A concentration of 0.75mM was used in most assays.

The dose-response curve to adrenaline is shown in figure 24. Adenyl cyclase is maximally stimulated at adrenaline concentrations between 0.1 and 0.5mM. Concentrations higher than optimal give less stimulation of the enzyme. A concentration of 0.1mM was used in most assays.

The sensitivity of adenyl cyclase to adrenaline is greater than its sensitivity to noradrenaline which suggests that

the catecholamine receptor is of the β-type in skeletal muscle (Bowman and Raper, 1967).

5) concentrations of ATP and Mg^{++}

Fluoride-stimulated activity is maximal with a substrate concentration between 2mM and 3mM, and is inhibited by higher substrate concentrations (figure 17A). With a substrate concentration of 2.5mM, the optimal Mg^{++} concentration is 8.25mM (final concentration in the incubation medium) (figure 17B). The enzyme activity is completely dependent on Mg^{++}.

6) concentration of calcium

Calcium plays an important role in muscular contraction but it has no stimulatory effect on the adenyl cyclase from skeletal muscle. Concentrations higher than 1mM cause inhibition which reaches 100% at 4mM Ca^{++} (figure 18B). The fact that concentrations smaller than 1mM do not cause inhibition may be due to the presence of EDTA in the medium. EDTA is a chelating agent which can bind Ca^{++} to a certain extent. The fact that Ca^{++} inhibits adenyl cyclase activity could be of physiological significance since concentrations of that order of magnitude can occur in vivo in the tissue. Watchorn and McCance (1937) have estimated the amount of calcium in rat skeletal muscle to 13 mg/100g wet weight which would roughly correspond to a 3mM concentration. Himms-Hagen (unpublished observations) has estimated it to 4mg/100g wet weight which would roughly correspond to a 1mM concentration.

Figure 17: Adenyl cyclase in skeletal muscle; velocity versus ATP and Mg^{++} concentrations.

Ten mg of skeletal muscle were incubated for 20 minutes at 37°C. The incubation mixture contained in Tris buffer, pH 7.4 (40mM), 10mM NaF, 10mM theophylline, 0.25mM EDTA, 13mM phosphoenolpyruvate, 1 unit of pyruvate kinase type I and different amounts of ATP or Mg^{++}.

A) Velocity versus ATP concentration

The Mg^{++} concentrations were as follows:

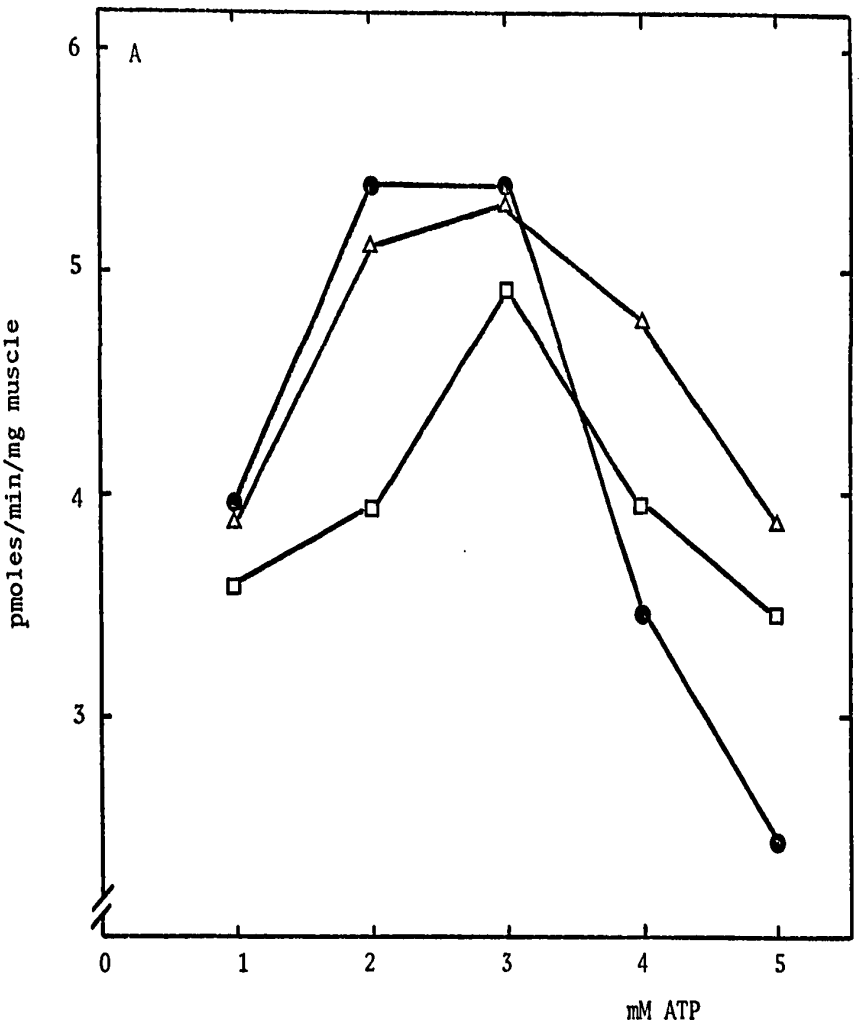
- ● 6.25mM;
- Δ 11.25mM;
- □ 16.25mM.

Notice the inhibition at substrate concentration greater than 3mM for the three different Mg^{++} concentrations used.

B) Velocity versus magnesium concentration

The optimal substrate concentration was used (2.5mM ATP) and the velocity was measured with different amounts of $MgSO_4$ (0 to 20mM) in the incubation medium.

Notice that there is inhibition with concentrations higher than optimal.



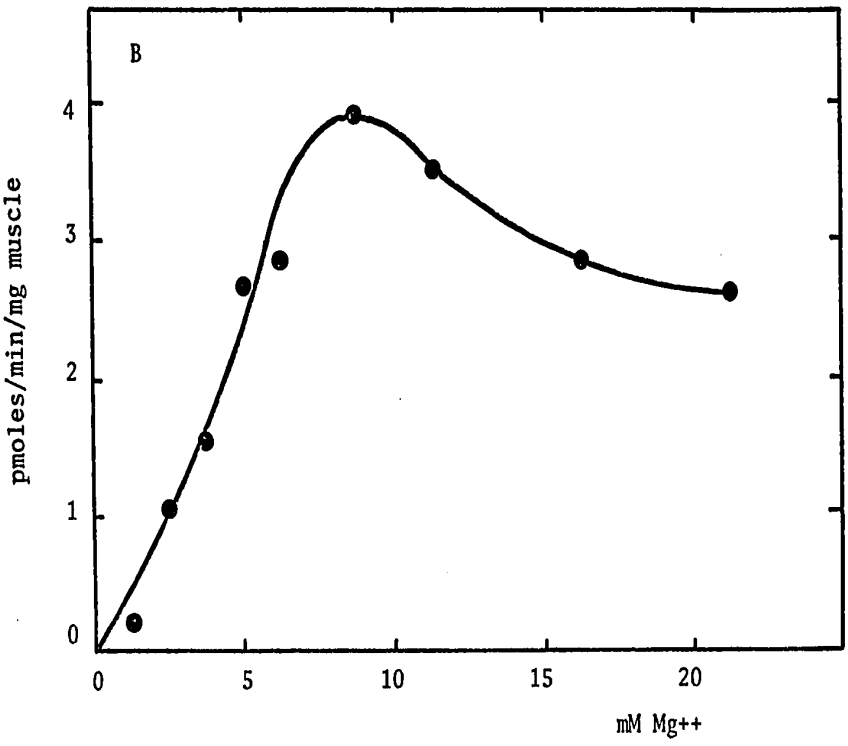


Figure 18: Adenyl cyclase in skeletal muscle; velocity versus fluoride concentration; velocity versus calcium concentration.

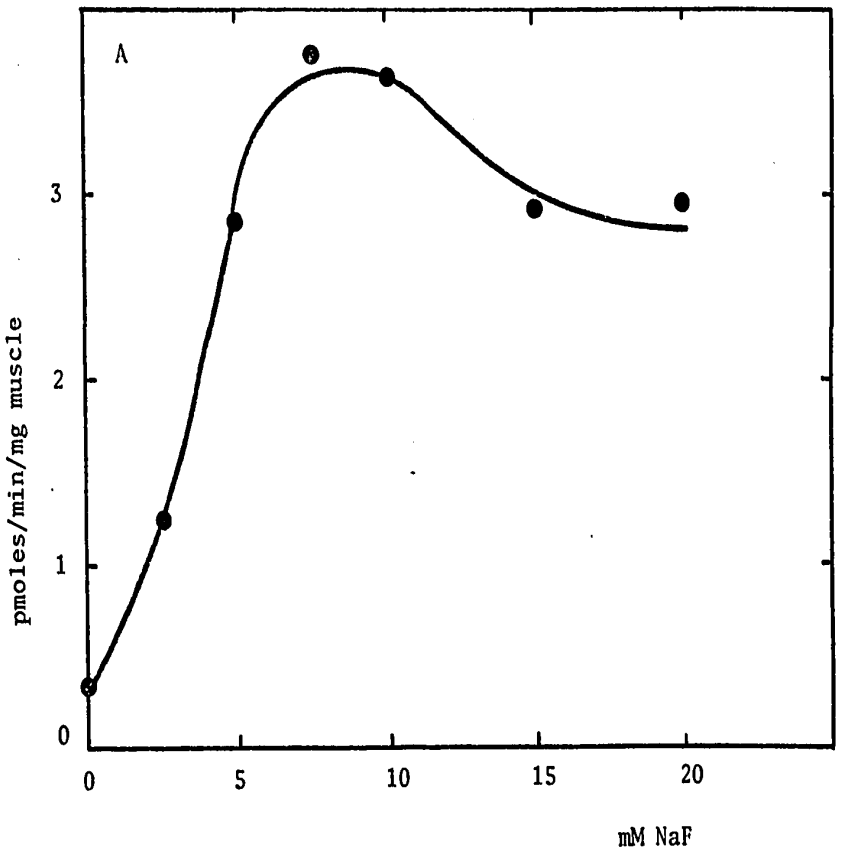
The incubation was carried on for 20 minutes at 37°C. The incubation mixture contained 2.5mM ATP, 8.25mM $MgSO_4$, 10mM theophylline 0.25mM EDTA, 13mM phosphoenolpyruvate and 1 unit of pyruvate kinase type I, in 40mM Tris buffer, pH 7.4 and 10 mg of muscle homogenate.

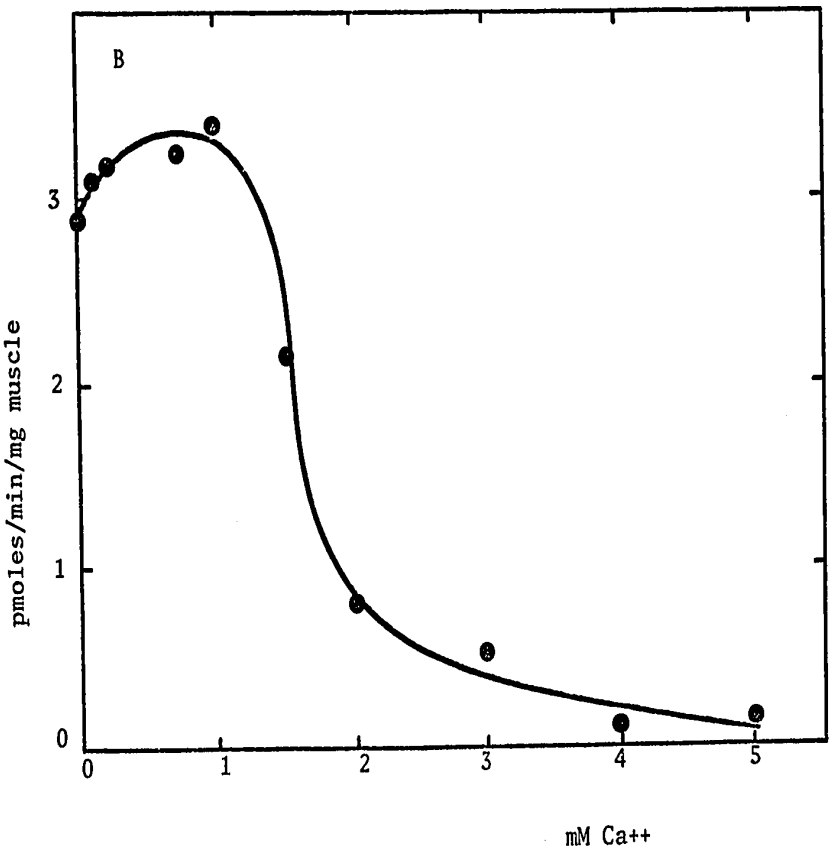
A) Velocity versus NaF concentration

Maximal stimulation was obtained with 7.5mM NaF and inhibition was observed with higher concentrations. Concentrations tested were between 0 and 20mM.

B) Velocity versus calcium concentration

No stimulation by calcium was observed but inhibition was observed at concentrations greater than 1mM. Calcium concentrations tested were between 0 and 5mM.


A
pmoles/min/mg muscle
0
1
2
3
0
5
10
15
20
mM NaF


B
pmoles/min/mg muscle
0
1
2
3
0
1
2
3
4
5
mM Ca++

C) Effects of cold exposure and cold acclimation on skeletal muscle adenyl cyclase

1) *cold acclimation*

There is no difference between cold and warm acclimated rats, for the basal adenyl cyclase activity (figure 19, bars 35 and W35 of the basal activity), the noradrenaline-stimulated activity (figure 19, bars 35 and W35 of the noradrenaline-stimulated activity, 0.75mM), the adrenaline-stimulated activity (figure 20, bars 35 and W35 of the adrenaline-stimulated activity, 0.1mM) and the fluoride-stimulated activity (figure 21) of skeletal muscle adenyl cyclase.

2) cold exposure

In rats exposed to cold for three to seven days, there is no significant increase in the basal adenyl cyclase activity (figure 19, comparing bars 3, 4 and 7 to bar 1 of the basal activity). There is significantly more stimulation of adenyl cyclase by noradrenaline (with concentrations 10μM, 100μM and 750μM) in the rats that have been exposed to cold for three and four days than in the warm acclimated rats (figure 19). By day seven of cold exposure, the level of the noradrenaline-stimulated adenyl cyclase activity has returned to the value obtained for warm acclimated rats.

There is also significantly more stimulation of adenyl cyclase by adrenaline (5μM, 10μM, and 100μM) at days three, four and seven of cold exposure than in the warm acclimated rats (day zero and day 35) (figure 20).

There is a 40% increase in the fluoride-stimulated adenyl cyclase after seven days of cold exposure (figure 21). The ac-

Figure 19: Changes in specific activity of basal and noradrenaline-stimulated adenyl cyclase in the skeletal muscle of rats during acclimation to cold.

Values shown are means ± standard errors. The number of animals in each group is as follows: day 0, 3, 4 and 35, four rats each; day 7, three rats; day W35, 5 rats. These are the same animals as those illustrated in figures 20 and 21. The group W35 refers to the warm acclimated rats that have been at room temperature for thirty-five days and the group zero refers to the rats that were killed on the day the other rats were placed in the cold. All the other numbers refer to the number of days of exposure to cold.

Noradrenaline was tested at three concentrations, 10μM, 100μM and 750μM (final concentration in the incubation mixture) in the rats that have been exposed to cold for 3, 4 and 7 days and at a concentration of 750μM in the rats of the other groups.

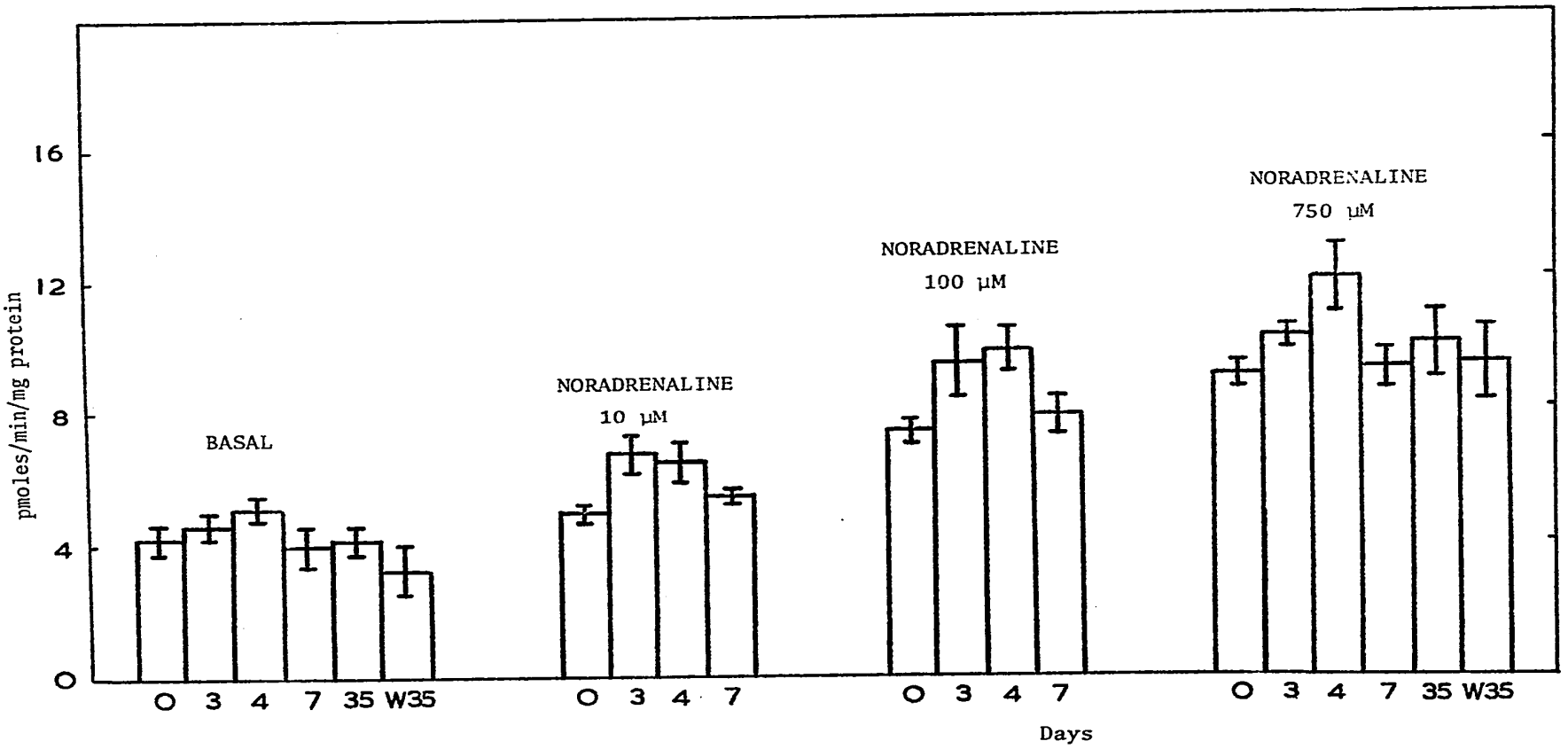


Figure 20: Changes in the specific activity of basal and adrenaline-stimulated adenyl cyclase in the skeletal muscle of rats during acclimation to cold.

Values shown are means ± standard errors. The numbers of animals are the same as in figure 19.

Adrenaline was tested at three different concentrations in the rats exposed to cold for 3, 4 and 7 days (5μM, 10μM and 100μM) and at a concentration of 100μM in the rats of the other groups.

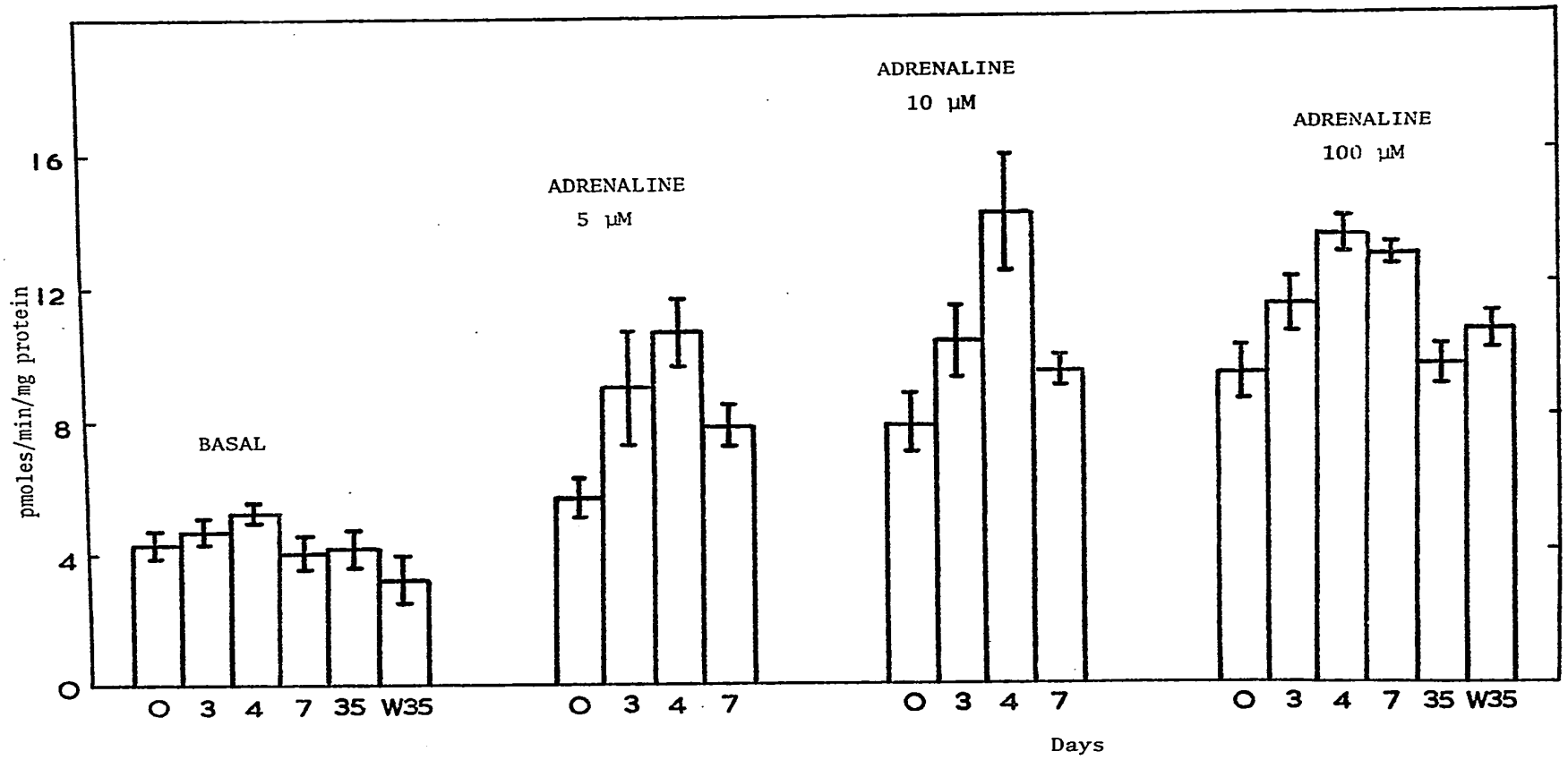


Figure 21: Changes in the specific activity of the fluoride-stimulated adenyl cyclase in the skeletal muscle of rats during cold acclimation.

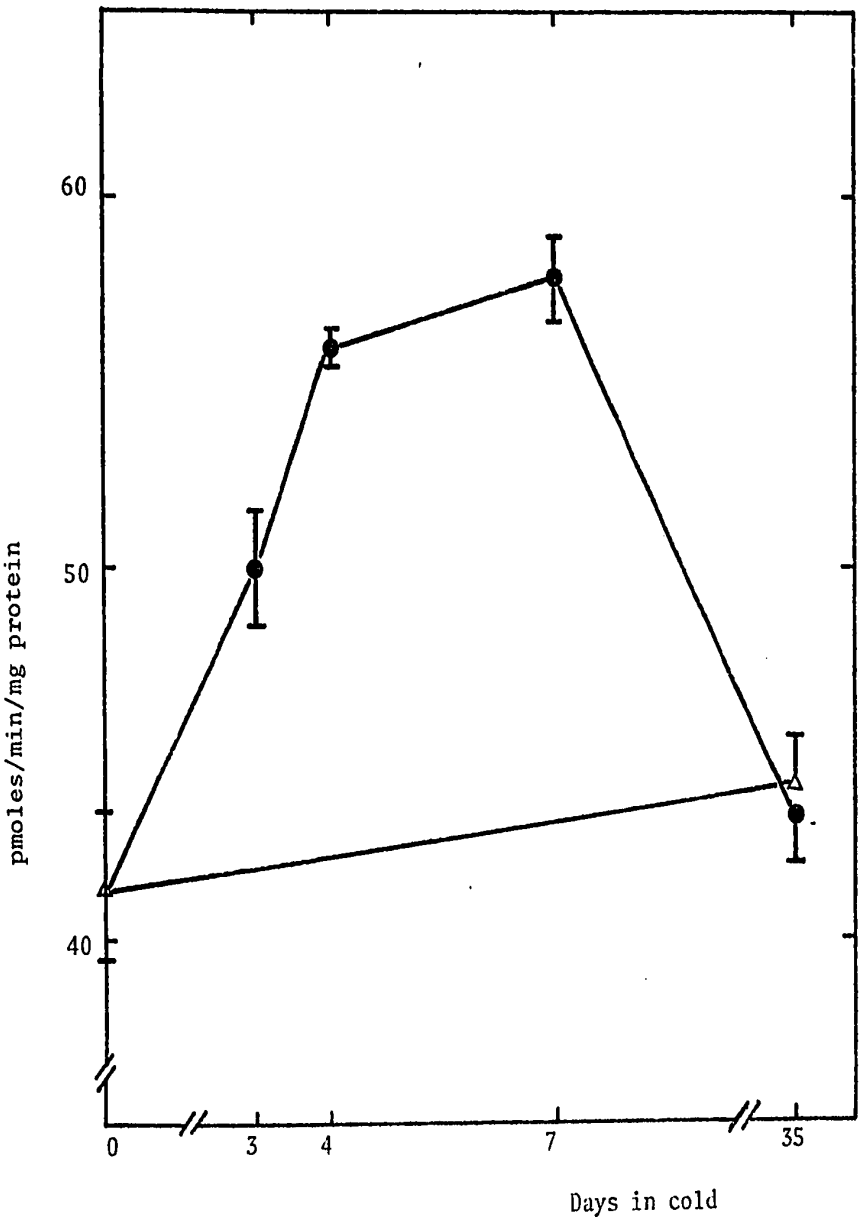
Values shown are means ± standard errors. The numbers of animals are the same as in figure 19 and 20.

Δ : warm acclimated rats, day zero and day 35.

• : cold exposed rats, day 3, 4, 7 and 35.

Notice that the scale for the adenyl cyclase activity is different from those of figures 19 and 20.

The fluoride concentration is 7.5mM in all assays.



tivity is also higher than in the warm acclimated rats, at days three and four of cold exposure (Figure 21).

3) sensitivity of adenyl cyclase to noradrenaline

The dose-response curves to noradrenaline of adenyl cyclase from cold and warm acclimated rats are not very different (figure 22). Maximum stimulation is obtained with concentrations between 0.25mM and 0.75mM. So there is no difference in the sensitivity to noradrenaline of skeletal muscle adenyl cyclase between cold acclimated and warm acclimated rats. Figure 23 illustrates the dose-response curves for the stimulation of adenyl cyclase by noradrenaline in rats that were exposed to cold for four days and in warm acclimated rats (day zero). Although there is more stimulation of adenyl cyclase by the three noradrenaline concentrations tested (10, 100 and 750µM) in the cold exposed rats, there is no shift in the dose-response curve and consequently no increase in the sensitivity of adenyl cyclase to noradrenaline with exposure to cold.

4) sensitivity of adenyl cyclase to adrenaline

The dose-response curves of adenyl cyclase to adrenaline are not significantly different in warm and cold acclimated rats (figure 24). Maximum stimulation is obtained between 0.1mM and 0.5mM adrenaline. There is then no increase in the sensitivity of adenyl cyclase to adrenaline in cold acclimated rats.

In rats that have been exposed to cold for four days, there is more stimulation by adrenaline of adenyl cyclase at the three concentrations tested (5, 10 and 100µM) (figure 25).

Figure 22: Dose-response curve for the stimulation of adenyl cyclase of rat skeletal muscle by noradrenaline, in cold and warm acclimated animals.

Each graph represents values for one rat (mean of duplicate determinations). The upper graph shows the dose-response curve for a cold acclimated rat (CC) and the lower graph shows the dose-response curve for a warm acclimated rat (WW). The period of acclimation was five weeks in both cases.

Activities are expressed in terms of the maximum activity (activity obtained with optimal concentration of noradrenaline), shown here as 100%.

Notice inhibition with noradrenaline concentrations higher than 0.75mM.

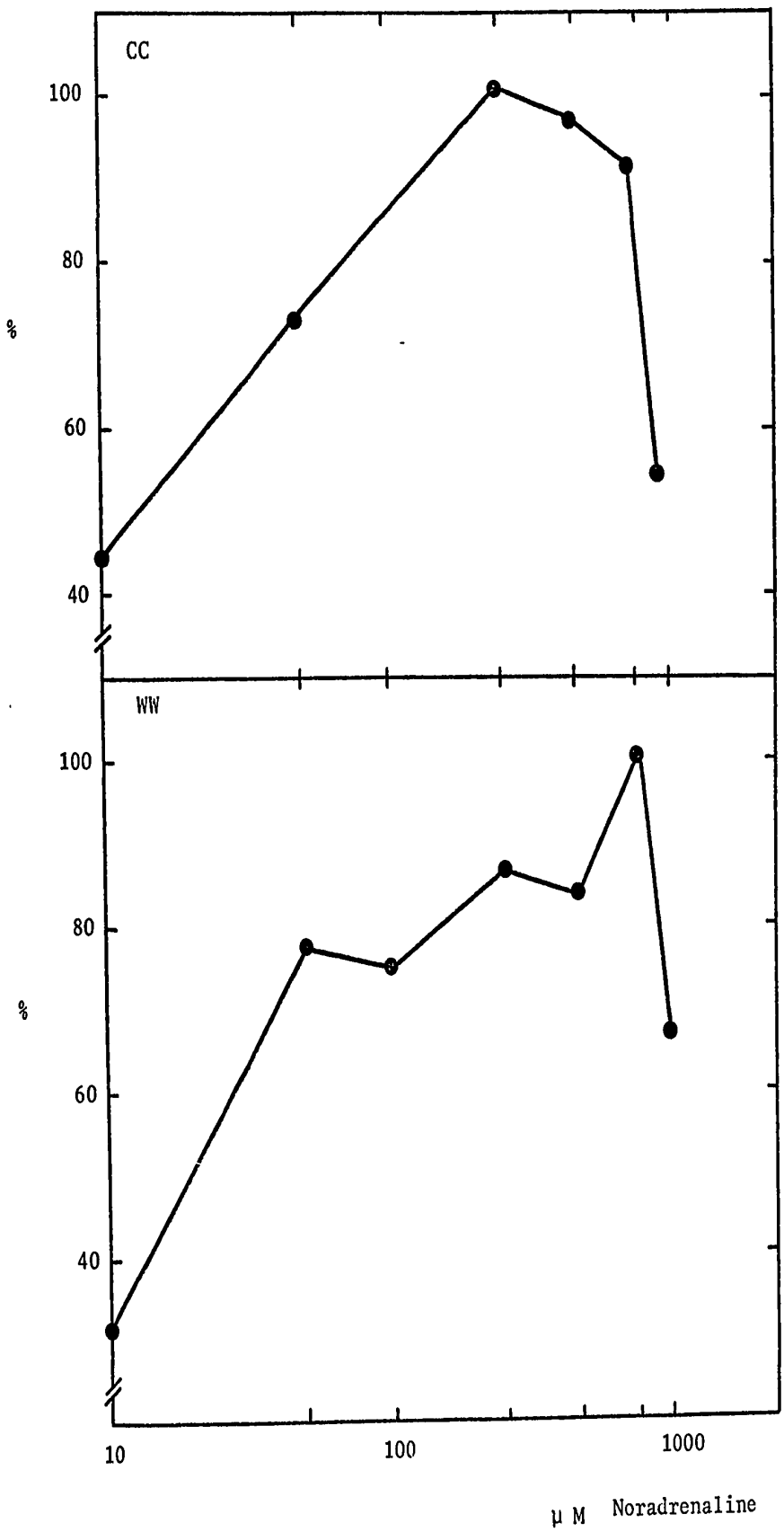


Figure 23: Dose-response curve for the stimulation by noradrenaline of adenyl cyclase from skeletal muscle in rats exposed to cold.

● : dose-response curve to noradrenaline of skeletal muscle adenyl cyclase in warm acclimated rats (day zero)

▲ : dose-response curve to noradrenaline of skeletal muscle adenyl cyclase in rats exposed to cold for four days.

Values shown are means ± standard errors. The rats four per group, were the same as the one represented in figures 19, 20 and 21 in the groups day zero and day four.

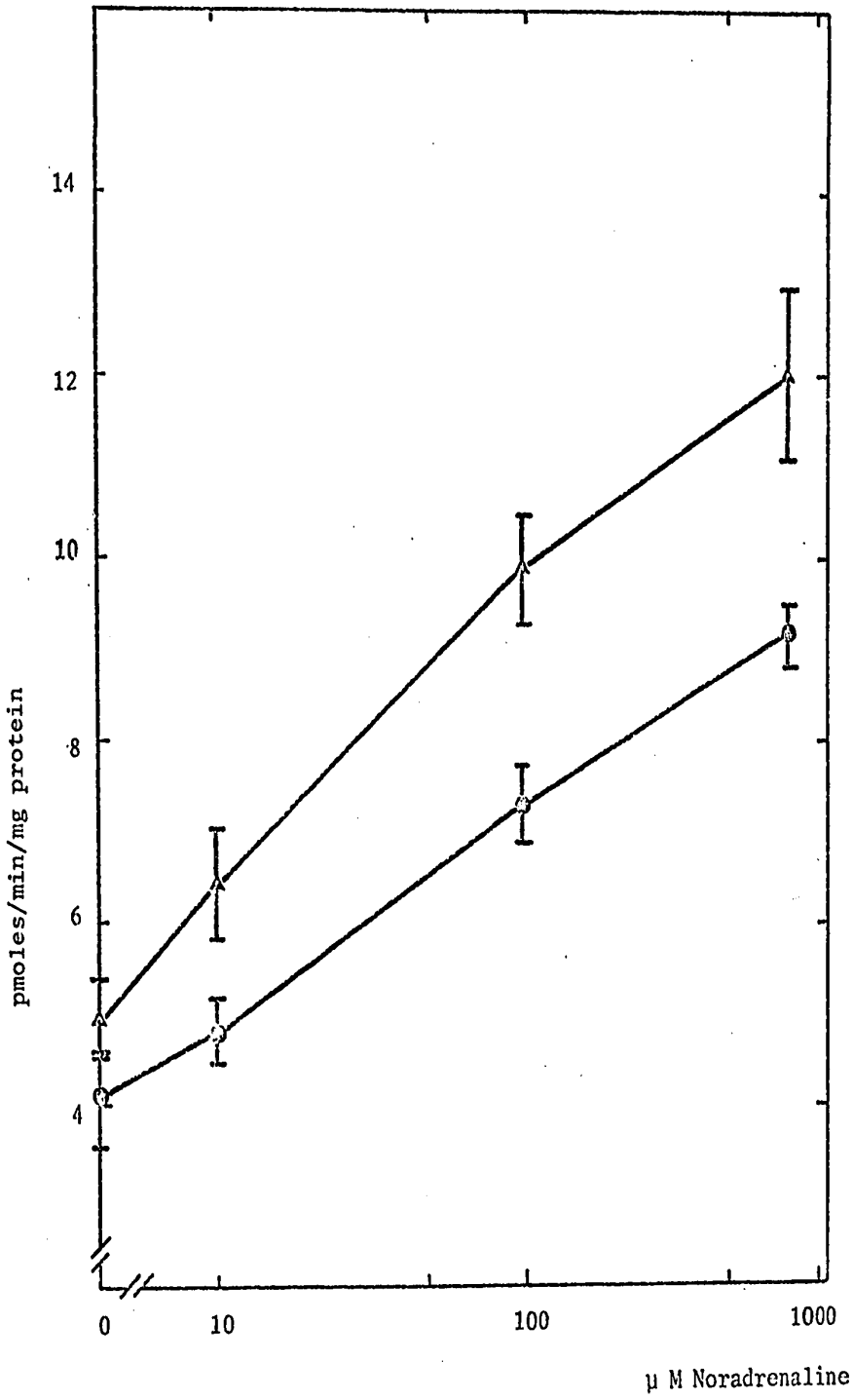

14
12
10
8
6
4
pmoles/min/mg protein
0
10
100
1000
μ M Noradrenaline

Figure 24: Dose-response curve for the stimulation of adenyl cyclase from rat skeletal muscle by adrenaline, in cold and warm acclimated animals.

Each graph represents values for one rat (mean of duplicate determinations). The upper graph illustrates the dose-response curve for a cold acclimated rat, and the lower graph illustrates the dose-response curve for a warm acclimated rat. The period of acclimation was five weeks in both cases. Activities are expressed in terms of the maximum activity (observed with optimal concentration of adrenaline), shown here as 100%. Notice inhibition at concentrations higher than optimal.

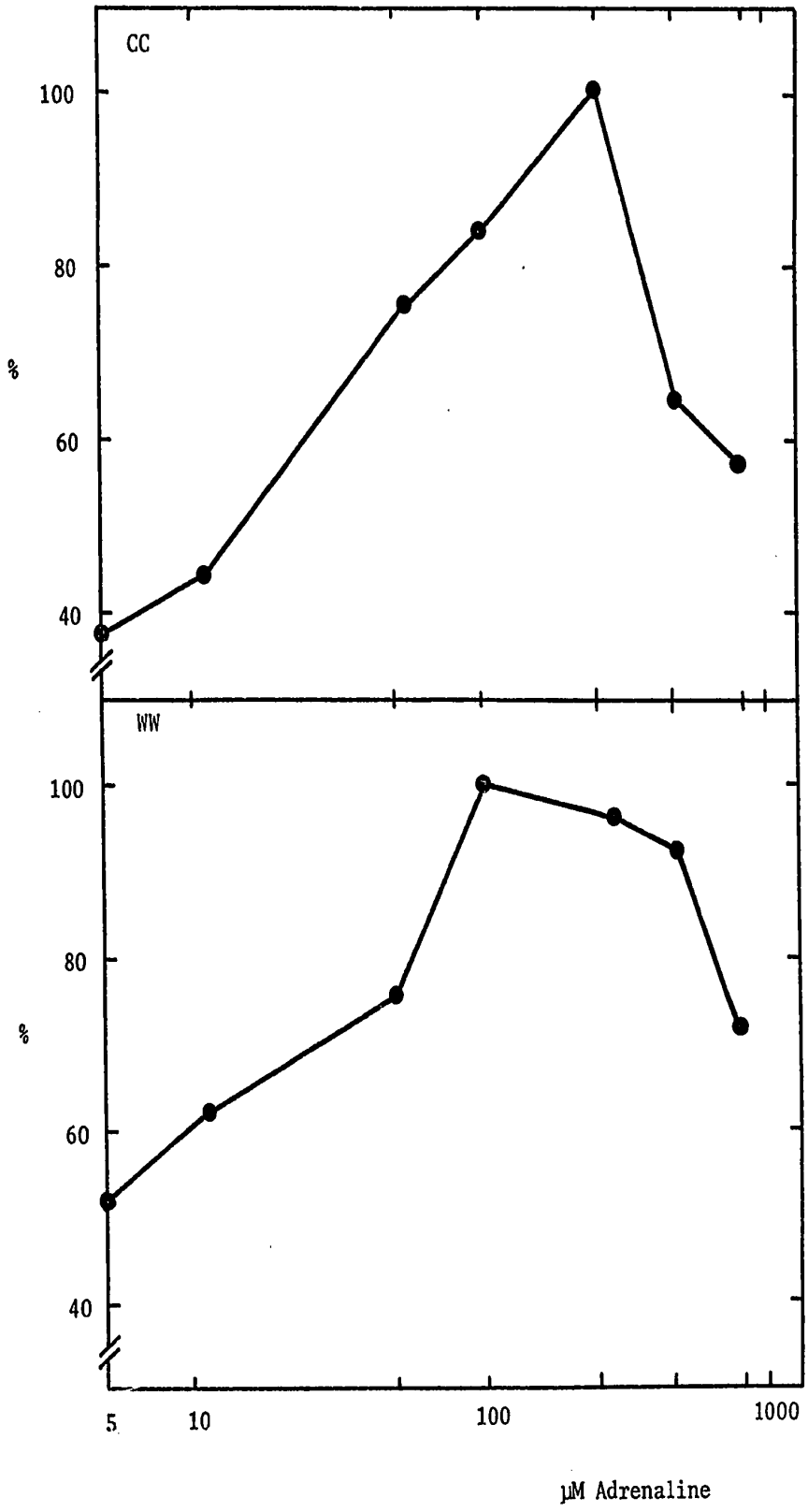


Figure 25: Dose-response curve for the stimulation by adrenaline of adenyl cyclase of skeletal muscle of rats exposed to cold.

o : dose-response curve to adrenaline of skeletal muscle adenyl cyclase in -arm acclimated rats (day zero).

Δ : dose-response curve to adrenaline of skeletal muscle adenyl cyclase in rats exposed to cold for four days.

Values shown are means ± standard errors. The rats (four per group) were the same as the one represented in figures 19, 20 and 21 in the groups day zero and day four.

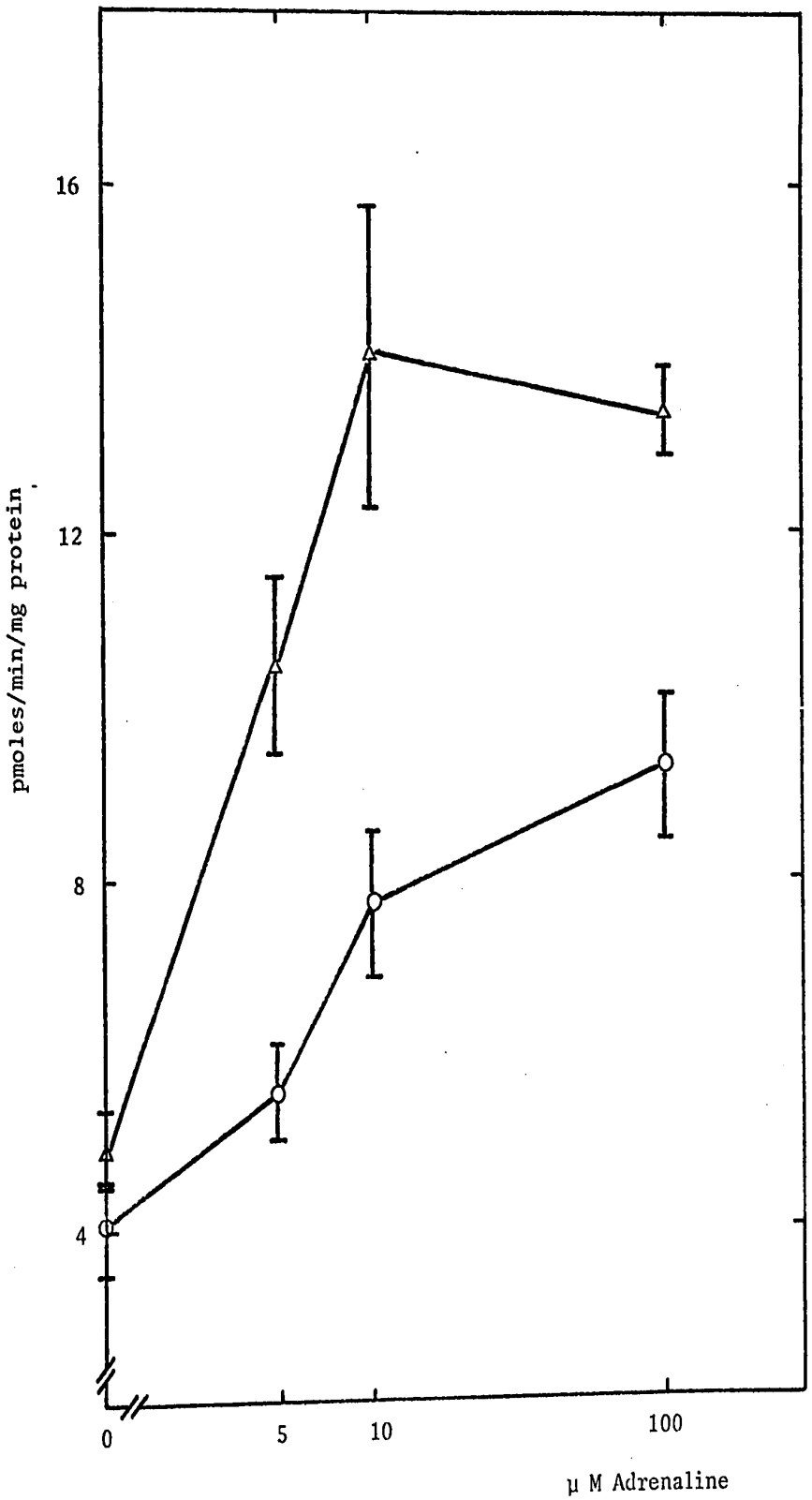

16
12
8
4
pmoles/min/mg protein
0
5
10
100
μ M Adrenaline

Table 3

Percentage of stimulation of adenyl cyclase by adrenaline during cold exposure

Adrenaline concentration μM	Duration of exposure to cold (days)			
	zero	three	four	seven
5	29%	65%	60%	39%
10	71%	84%	100%	61%
100	100%	100%	92%	100%

There is also a shift in the dose-response curve of adenyl cyclase to adrenaline in the cold exposed rats, when compared to that of the warm acclimated rats (day zero). Maximum stimulation is obtained with 10μM in the cold exposed rats (four days) while it is obtained at 100μM in the warm acclimated rats (day zero). Table 3 illustrates this increase in sensitivity to adrenaline observed during cold exposure. After the rats have been exposed to cold for three days, the skeletal muscle adenyl cyclase is stimulated more by 5μM and by 10μM than it is in the warm acclimated rats. The increase in sensitivity is maximum after four days of cold exposure and is no longer visible after seven days of cold exposure. The percentage of stimulation by adrenaline is expressed as:

$$\frac{\text{activity with given adrenaline concentration - basal activity}}{\text{activity with optimal adrenaline concentration - basal activity}}$$

It may be concluded that the sensitivity of adenyl cyclase to adrenaline is increased in the first few days of cold exposure (three and four days) as well as the stimulation of the enzyme by adrenaline, at any of the concentrations tested (figure 20).

D) Discussion

There is no difference between basal, noradrenaline-stimulated, adrenaline-stimulated and fluoride-stimulated adenyl cyclase activities of cold and warm acclimated rats skeletal muscle. The adenyl cyclase in skeletal muscle then is not altered by cold acclimation.

During the first few days of exposure to cold, there is an increase in the specific activities of the noradrenaline-stimulated, the adrenaline-stimulated and the fluoride-stimulated adenyl cyclase. The increase in specific activity of the adrenaline component is accompanied by a shift in the dose-response curve for the stimulation of adenyl cyclase by adrenaline, indicating an increased sensitivity of the enzyme to stimulation by adrenaline. This increase in sensitivity is only seen at days three and four of cold exposure; by day seven, the dose-response curve for the stimulation of adenyl cyclase by adrenaline, has returned to normal although the enzyme is still stimulated more by adrenaline than the enzyme of the warm acclimated rats.

The increase in the calorigenic response of rats to catecholamines observed during cold acclimation is not accompanied by an increase in the specific activity of adenyl cyclase in the skeletal muscle or by an increase in the sensitivity of the enzyme to adrenaline or noradrenaline in cold acclimated rats.

The increase in adenyl cyclase activity observed during the first week of exposure to cold as well as the increase in the sensitivity of the enzyme to adrenaline after three and four days of exposure to cold are quite puzzling. The time course of the changes observed for the stimulation of adenyl cyclase by adrenaline corresponds to the changes in the secretion of adrenaline by the adrenal medulla (Leduc, 1961).

Adrenaline has two different effects on the contraction of skeletal muscle. First it exerts a direct effect on the muscle fibers themselves. It was shown as early as 1895 (Oliver and

Schafer, 1895) that extracts of adrenal medulla increased the tension and prolonged the duration of maximal twitches of the non-fatigued gastrocnemius muscle of the dog. This action of adrenaline was confirmed by many workers and it was shown that adrenaline exerted this effect directly on the muscle fibers themselves, since the effect was still observed in fully curarized or chronically denervated muscles (see review by Bowman and Raper, 1967). Adrenaline exerts another effect on skeletal muscle: it influences neuromuscular transmission. This effect of adrenaline is potentiated by theophylline (Goldberg and Singer, 1969; Breckenridge et al., 1967) and is mimicked by dibutyryl cyclic AMP (Goldberg and Singer, 1969). The fact that adrenaline and dibutyryl cyclic AMP increase the frequency of single cell and plate potentials and the height of the compound end plate potential has led to the conclusion that their effects are due to an acceleration of the release of packets of acetylcholine from the motor neurone (Goldberg and Singer, 1969).

The mechanism by which adrenaline has a direct effect on the muscle fibers is not known but it could involve the participation of the adenyl cyclase system.

Although both actions of adrenaline occur simultaneously, the one action which is important at any one time depends upon the condition of the muscle. In the normal non-fatigued, non-curarized muscle, the effect of adrenaline on contractions are mostly the result of a direct action on muscle fibers; in a partially curarized muscle, the action on neuromuscular transmission plays the more important part whereas the defatiguing effect of

adrenaline probably involves both sites of action (Bowman and Raper, 1967).

Fatigued muscles lose the response to further stimulus as well as the ability to maintain phosphorylase in the a form (Danforth and Helmreich, 1964; Piras and Staneloni, 1969); but if the muscles are pretreated with adrenaline the resistance to fatique is increased and there is a slower fall in the percentage of phosphorylase in the a form (Danforth and Helmreich, 1964).

If adrenaline can increase the resistance of a muscle to fatigue by maintaining muscle phosphorylase in the a form, it is possible that it does so by the intermediary of the adenyl cyclase system which is well known to mediate the effects of adrenaline on muscle phosphorylase conversion from the b to the a form.

Muscles continuously shivering during cold exposure (Héroux et al., 1956; Hart et al., 1956) can be considered as potentially fatigued muscles. These muscles show an increase in adenyl cyclase specific activity (noradrenaline-stimulated, adrenaline-stimulated and fluoride-stimulated) and an increase in the sensitivity of the enzyme to adrenaline. It is possible that these changes in the adenyl cyclase system be associated with the defatiguing effect of adrenaline directly on muscle fibers. Adrenaline would then possibly exerts its defatiguing effect on muscle fibers by increasing adenyl cyclase activity. The increased stimulation of adenyl cyclase by adrenaline as well as the increase sensitivity of the enzyme to adrenaline,

could amplify quite a bit the effects of adrenaline on muscle phosphorylase. Thus, in the cold exposed rat, it is possible that the increase in adenyl cyclase observed is associated with the maintenance of shivering in the fatigued muscles. The changes in adenyl cyclase seem to be in favor of an increased response of the enzyme to adrenaline since both stimulation and sensitivity of the enzyme to adrenaline are increased in the rats exposed to cold for three and four days. This would then possibly be a very good example of the mediation of the defatiguing effect of adrenaline on muscle fibers themselves by the adenyl cyclase system.

As was mentioned earlier, the defatiguing effect of adrenaline probably involves both actions of adrenaline, i.e. the direct one on the muscle fibers themselves and the one on neuromuscular transmission. The results obtained with the adenyl cyclase system of the shivering muscles, only allows deductions or rather suppositions concerning the possible role of the adenyl cyclase system in the defatiguing effect of adrenaline directly on the muscle fibers. The adenyl cyclase measured is that present in the muscle cells and is not related to the possible adenyl cyclase from neurons involved in the neuromuscular transmission, probably at the presynaptic level.

So, stimulation of the adenyl cyclase system in early cold exposure may play a role in the defatiguing effect of adrenaline on shivering muscles. But the adenyl cyclase from rat skeletal muscle is not modified by the process of nonshivering thermogenesis since there is no difference between adenyl cyclase

of warm and cold acclimated rats for the basal, the adrenaline-stimulated, the noradrenaline-stimulated and the fluoride-stimulated activities respectively.

CHAPTER III: CYCLIC AMP PHOSPHODIESTERASE IN TISSUES

Section I: Survey of cyclic AMP phosphodiesterase

A) Distribution of cyclic AMP phosphodiesterase

1) *tissue distribution of cyclic AMP phosphodiesterase*

Cyclic AMP phosphodiesterase, an enzyme capable of destroying the biological activity of cyclic AMP, has been found in all mammalian tissues studied (Robison et al., 1971). It was first discovered by Sutherland and Rall (1958) in extracts of heart, brain and liver, and was identified later in many other mammalian tissues. In the dog, the enzyme was present in brain, adrenal gland, kidney, small intestine, liver pancreas, heart, lung, testis, sartorius muscle, aorta, femoral artery, diaphragm and femoral nerve (Butcher and Sutherland, 1962). The enzyme has also been studied in rat brain (Cheung, 1967), in beef heart (Butcher and Sutherland, 1962), in rabbit brain, kidney, heart, spleen, liver and skeletal muscle (Drummond and Perrott-Yee, 1961), and in rat white and brown adipose tissues (Hahn, 1970).

The enzyme is also present in species other than mammals. It is present in frog erythrocytes (Rosen, 1970), in the liver fluke Fasciola hepatica, in the earth worm Lumbricus terrestris, in fly larvae and in minnows (Butcher and Sutherland, 1962), in slime molds (Chang, 1968) and in bacteria (Brana and Chytil, 1966).

Cyclic AMP phosphodiesterase is then widely distributed, in mammalian species as well as in other species.

2) subcellular distribution of cyclic AMP phosphodiesterase

Cyclic AMP phosphodiesterase can be associated with the soluble or the particulate subcellular fractions or with both depending on the tissue or the species studied. Most of the phosphodiesterase activity from beef heart was found to be associated with the particulate material which sedimented at 2500*g* (Butcher and Sutherland, 1962). In dog heart, most of the phosphodiesterase activity was associated with the 4000*g* supernatant (Nair, 1966). In rabbit brain, the enzymatic activity was localized entirely in the 100,000*g* supernatant (Drummond and Perrott-Yee, 1961). In rat brain, the phosphodiesterase activity was 60% particulate but the highest specific activity of the enzyme was found in the soluble fraction; the particulate activity was found primarily in fractions rich in nerve endings. After osmotic shock about half of the activity associated with the 11,500*g* pellet became soluble (DeRobertis et al., 1967). Cheung and Salganicoff (1967) found that rat brain phosphodiesterase, when prepared in isotonic sucrose solution, was distributed as follows: 10% in the nuclear fraction, 40% in the mitochondrial fraction, 20% in the microsomal fraction and 30% in the 100,000*g* supernatant fraction. If the different subcellular fractions were exposed to Triton X-100 before being assayed, more than half of the total phosphodiesterase activity was then associated with the microsomal fraction indicating that the phosphodiesterase of the latter fraction was probably embedded in a lipoprotein matrix and inaccessible to the substrate. The remaining activity after treatment with Triton X-100 was found mostly in the mitochondrial and the soluble fractions (Cheung and Salganicoff, 1967).

It seems that phosphodiesterase can be either soluble or particulate in a tissue preparation or partially soluble and partially particulate depending on the tissue, the species and the methods of isolation used. Th e localization in the intact cell of cyclic AMP phosphodiesterase is therefore not completely clear.

B) Purification and properties of cyclic AMP phosphodiesterase

1) *purification of cyclic AMP phosphodiesterase*

Soluble phosphodiesterase has been purified with some success but particulate phosphodiesterase has not been purified yet. Soluble phosphodiesterase has been purified between 80- and 140-fold from beef heart 16,000*g* supernatant (Butcher and Sutherland, 1962) and 170-fold from dog heart 4,000*g* supernatant (Nair, 1966). A soluble phosphodiesterase has been purified more than 1000-fold from frog erythrocytes and the activity was always recovered in two different fractions and it was postulated that the enzyme existed in two different molecular forms (Rosen, 1970).

The existence of more than one form of cyclic AMP phosphodiesterase had been suggested by anomalous kinetic behavior of the rat brain soluble phosphodiesterase, behavior which was indicative of two apparent K_m (Brooker et al., 1968). Then by purification of rat brain phosphodiesterase it was possible to identify two active fractions, one with a low affinity for cyclic AMP (apparent K_m of 1×10^{-4}M) and the other one with a high affinity for cyclic AMP (apparent K_m of 5×10^{-6}M) (Thompson and

Appleman, 1971). Two molecular forms of phosphodiesterase have also been found in rat kidney and in frog bladder (Jard and Bernard, 1970). Direct evidence for the existence of more than one form of cyclic AMP phosphodiesterase in supernatants of rat and rabbit tissues was obtained using starch gel electrophoresis (Monn and Christiansen, 1971). All tissues studied contained more than one form of the enzyme but no tissue contained more than four components and various tissues had distinct patterns of components distribution; no complete separation of any component was achieved (Monn and Christiansen, 1971).

Soluble cyclic AMP phosphodiesterase has been purified to some extent in many preparations and it appears that the enzyme exists in more than one molecular form in many tissues. Identification of the different molecular forms of the enzyme and of their distribution in various tissues of different species will await further purification of the enzyme.

2) properties of cyclic AMP phosphodiesterase

a) substrate specificity

Cyclic 3',5'-nucleotide phosphodiesterase appears to be specific for purine 3'-5'-mononucleotides. It was initially reported that cyclic 3',5'-pyrimidines were also hydrolyzed by crude preparations of heart phosphodiesterase (Butcher and Sutherland, 1959; Nair, 1966) and brain phosphodiesterase (Drummond and Perrott-Yee, 1961). However a second phosphodiesterase was found in crude preparations of heart with a specificity for cyclic UMP (Hardman and Sutherland, 1965) and purification of heart cyclic AMP phos-

phodiesterase gave a preparation which hydrolyzed cyclic UMP at 12 to 15% of the rate observed with cyclic AMP (Nair, 1966).

The deoxy analog of 3',5'-cyclic AMP is also hydrolyzed by heart cyclic AMP phosphodiesterase at the same rate as cyclic AMP itself (Nair, 1966). Cyclic 2',3'-AMP is not hydrolyzed by cyclic AMP phosphodiesterase (Butcher and Sutherland, 1962; Nair, 1966). Polyadenylic acid, polyuridylic acid, DNA, RNA and 5'-AMP are not hydrolyzed by cyclic AMP phosphodiesterase (Sutherland and Rall, 1958; Butcher and Sutherland, 1962; Nair, 1966).

Cyclic GMP is hydrolyzed by phosphodiesterase. In a range of concentrations between 10^{-4}M and 10^{-3}M, it is hydrolyzed at about 70% of the rate of cyclic AMP by phosphodiesterase preparations from rat brain (Cheung, 1967) and beef heart (Beavo et al., 1970). At a concentration of 10^{-3}M the ratio of cyclic GMP hydrolysis to cyclic AMP hydrolysis is about 1 and varies little among homogenates of all tissues of the rat examined as well as among the subcellular fractions of several rat tissues (Beavo et al., 1970). But at lower substrate concentration (micromolar level) the rate of cyclic GMP hydrolysis can be several times faster, equal or slower than the rate of cyclic AMP hydrolysis depending on the tissue and/or the subcellular fraction studied (Beavo et al., 1970; Rosen, 1970; Thompson and Appleman, 1971) and it is possible that the different molecular forms of the enzyme (Monn and Christiansen, 1971) may have different relative affinities for cyclic AMP and cyclic GMP.

b) <u>stimulation of cyclic AMP phosphodiesterase</u>

In an attempt to elucidate the mechanisms regulating tissue

levels of cyclic AMP in an intact animal, factors affecting phosphodiesterase activity have been studied. Imidazole can stimulate phosphodiesterase activity in brain (Cheung, 1967) and heart (Butcher and Sutherland, 1962; Nair, 1966) preparations. The effect of imidazole on the level of cyclic AMP in intact cells has not been studied but it is unlikely that the stimulation of phosphodiesterase by imidazole is of physiological significance (see Robison et al., 1971).

The presence of an activator of phosphodiesterase in bovine brain was detected by Cheung (1970) who observed the partial loss of activity of the enzyme at the later stages of the purification procedure and the restoration of the enzymatic activity by the addition of a brain extract free of cyclic AMP phosphodiesterase activity. The activator appears to be a protein of molecular weight approximately equal to 40,000 which is gradually removed from the enzyme during the process of purification (Cheung, 1971b). The affinity of cyclic AMP for phosphodiesterase is increased in the presence of the activator but no evidence for the binding of cyclic AMP to the activator was obtained (Cheung, 1971b). The activator then does not stimulate phosphodiesterase activity through a direct binding of the substrate but probably by bringing about a conformational change in the enzyme which results in an increased affinity for the substrate (Cheung, 1971b). An activator and a relatively inactive purified phosphodiesterase were also isolated from human, porcine and rat brains and from bovine heart; an activator from one tissue could cross-activate effectively a purified phosphodiesterase from another tissue indicating the lack

of tissue specificity of the activator (Cheung, 1971b). The activator of phosphodiesterase may be very important in regulating tissue levels of cyclic AMP in the intact animal.

c) inhibition of cyclic AMP phosphodiesterase

The role of cyclic AMP phosphodiesterase is to inactivate cyclic AMP by hydrolyzing it to AMP; the inhibition of cyclic AMP phosphodiesterase would result, in the intact animal, in an acceleration of these metabolic processes able to be stimulated by cyclic AMP.

The best known inhibitors of phosphodiesterase are the methylxanthines which are capable of inhibiting the enzyme in every mammalian tissue that has been studied (Robison et al., 1971). Of the methylxanthines, theophylline is usually a more potent inhibitor of phosphodiesterase than either caffeine or theobromine. Theophylline was shown to act as a competitive inhibitor of beef heart phosphodiesterase (Butcher and Sutherland, 1962); caffeine acted noncompetitively on dog heart phosphodiesterase (Nair, 1966) but competitively on rat brain phosphodiesterase (Cheung, 1967).

The methylxanthines have been shown to potentiate the action of many hormones on different tissues (Robison et al., 1971); the potentiation of a hormonal action by the methylxanthines has been used as evidence in favor of the mediation of the given hormonal action by adenyl cyclase. Other agents such as puromycin (Appleman and Kemp, 1966), or triiodothyronine (Mandel and Kuehl, 1967; Rosen, 1970) can also inhibit phosphodiesterase activity in certain tissues. One of the problems

involved in the study of the inhibition of phosphodiesterase in different tissues by methylxanthines and other agents is that relatively high concentrations of the drugs are necessary to inhibit phosphodiesterase and that at these concentrations the drugs may have many other effects unrelated to their inhibition of phosphodiesterase.

Phosphodiesterase is inhibited by ATP and inorganic pyrophosphate (Cheung, 1966; Cheung, 1967; Gulyassy, 1971) and the inhibition can be virtually abolished by increasing the concentration of Mg^{++} in the incubation medium (Cheung, 1967; Gulyassy, 1971). Phosphodiesterase requires Mg^{++} for activity and it is probably by forming complexes with Mg^{++} that ATP and inorganic pyrophosphate inhibit phosphodiesterase activity (Cheung, 1967; Gulyassy, 1971). CTP, UTP, ITP, GTP, TTP, inorganic tripolyphosphate, inorganic tetrapolyphosphate and citrate, all of which are known to be metal-chelating agents, also inhibit phosphodiesterase activity probably by the same mechanism as ATP and inorganic pyrophosphate (Cheung, 1967).

Adenosine and adenine are also inhibitors of cyclic AMP phosphodiesterase but their inhibitory action is not affected by increasing the concentration of Mg^{++} in the medium (Gulyassy, 1971). The amino group of adenosine and adenine is involved in the inhibition of phosphodiesterase because inosine and purine derivatives have almost no inhibitory effects on phosphodiesterase (Gulyassy, 1971).

The physiological significance of the inhibition of phosphodiesterase by nucleotides depends on the relative intracellular

concentration of nucleotides and of Mg^{++} in their free and bound forms, and is rather difficult to estimate. The quantitative importance of the inhibition of cyclic AMP phosphodiesterase in the regulation of cyclic AMP levels in the intact animal cannot be assessed at the present moment and one can only postulate that the inhibition of phosphodiesterase may be of significance in the whole animal.

d) the cyclic AMP phosphodiesterase reaction

Cyclic AMP is inactivated by the phosphodiesterase reaction. Sutherland and Rall (1958) showed that cyclic AMP was quantitatively converted to 5'AMP by heart preparations. The product of the reaction, AMP, was identified by paper chromatography and by its enzymatic dephosphorylation by the 5'-nucleotidase activity of snake venom.

e) cofactor requirements

Mg^{++} is essential for the activity of phosphodiesterase (Drummond and Perrott-Yee, 1961; Cheung, 1967) and Mn^{++} can replace Mg^{++} completely with brain preparations (Cheung, 1967) but only partially with heart preparations (Drummond and Perrott-Yee, 1961). Partial stimulation of rat brain phosphodiesterase is obtained with Ca^{++}, Ni^{++}, and Ba^{++} but Ca^{++}, Cu^{++} and Zn^{++} are inhibitory (Cheung, 1967). In bovine brain, Cheung (1971a) showed that Ca^{++}, Mn^{++}, Co^{++}, and Zn^{++} were stimulatory at low concentrations (smaller than 0.1 mM) but inhibitory at high concentrations (larger than 1 mM). Lead is a potent inhibitor of brain phosphodiesterase even when Mg^{++} is present (Breckenridge and Johnston, 1969). The presence of a Ca^{++}-dependent phosphodiesterase activity

has been reported in rat brain (Kakiuchi and Yamazaki, 1970). EDTA inhibits phosphodiesterase activity (Drummond and Perrott-Yee, 1961; Cheung, 1967; Cheung, 1971a; Gulyassy, 1971) and Mn^{++} can completely remove the inhibitory effects of EDTA (Cheung, 1971a) on the enzyme. The phosphodiesterase reaction requires Mg^{++} for activity in all preparations studied but other metals can stimulate the enzymatic activity in certain tissues of certain species and in a limited range of concentrations; no generalization concerning the requirement of the reaction for metals other than Mg^{++} can be made at the present moment.

f) pH optimum

In dog heart preparations, the pH optimum lies between 8.5 and 9.2 (Nair, 1966) while in beef heart preparations the pH optimum is 7.9 (Butcher and Sutherland, 1962); in rat brain, the pH optimum is 8.0 (Cheung, 1967). The pH optimum seems to vary with the tissue and the species studied but it is usually in the alkaline range of pH (Robison et al., 1971).

C) Methods of estimation of cyclic AMP phosphodiesterase

Phosphodiesterase hydrolyzes cyclic AMP into AMP. The AMP produced can be treated with 5'-nucleotidase obtained from snake venom which hydrolyzes AMP into adenosine and inorganic phosphate and the latter can be measured colorimetrically by the method of Fiske and Subbarow (1925). The AMP formed during the phosphodiesterase reaction can be transformed into IMP by an excess of AMP deaminase, a reaction which can be followed spectropho-

tometrically (Drummond and Perrott-Yee, 1961); AMP can also be assayed by an enzymatic recycling method using excesses of myokinase, pyruvate kinase and lactate dehydrogenase (Cheung, 1966). A titrimetric method based on the fact that the phosphate group of cyclic AMP has one titratable species while that of AMP has two has been designed by Cheung (1969). The amount of cyclic AMP present before and after the phosphodiesterase reaction can be estimated and the difference represents the amount of cyclic AMP hydrolyzed during the reaction (Butcher and Sutherland, 1962).

More sensitive methods using labelled cyclic AMP for substrate have been reported recently and they have the advantage of measuring the reaction at much smaller substrate concentrations. The formation of tritiated adenosine in the presence of an excess of 5'-nucleotidase or the disappearance of tritiated cyclic AMP during the reaction can be estimated since adenosine and cyclic AMP can be separated from each other and from other nucleotides by ion-exchange chromatography (Beavo et al., 1970; Somerville et al., 1970). The products of the phosphodiesterase reaction using C^{14}- or H^{3}-cyclic AMP as substrate, can also be isolated by thin layer chromatography (Therriault and Winters, 1970; Gulyassy and Oken, 1971). The liberation of P^{32}-inorganic phosphate from P^{32}-cyclic AMP can be used as a method of estimation of phosphodiesterase activity (Schönhöfer et al., 1970).

There are numerous methods of estimation of phosphodiesterase activity but the more sensitive ones involve labelled substrate. Much smaller concentrations of cyclic AMP can be used with these radioactive methods and they have permitted the detection of the existence of more than one molecular form of cyclic AMP phosphodiesterase.

Section II: Experimental: cyclic AMP phosphodiesterase in brown adipose tissue

A) Material and methods

1) *animals*

see chapter 2, section II, A, 1.

2) *preparation of the brown adipose tissue*

see chapter 2, section II, A, 3.

3) *assay of cyclic AMP phosphodiesterase*

The brown adipose tissue homogenate was centrifuged at 30,000*g* for 30 minutes and the supernatant was dialyzed overnight at 4°C against 0.25M sucrose. This fraction could then be stored at -20°C for a few months without loss of activity. Of the total cyclic AMP phosphodiesterase activity in the original homogenate 90% was found to be present in this fraction. Phosphodiesterase activity was measured essentially as described by Nair (1966). The 5'AMP produced as a result of the action of phosphodiesterase on cyclic AMP was treated with 5'-nucleotidase obtained from snake venom and the released inorganic phosphate was measured colorimetrically. The assay was performed in two stages. The first stage consisted of the incubation of cyclic AMP with the brown adipose tissue phosphodiesterase preparation for 30 minutes at 30°C. Optimal concentrations of cyclic AMP, $MgSO_4$, and enzyme preparation were present in a final volume of

0.8ml containing 37.5mM Tris buffer at pH 8.0. The reaction was terminated by boiling the tubes for three minutes. Snake venom (from Crotalus atrox; solution containing 5 mg/ml) with 5'-nucleotidase activity was added to the medium (0.05ml) and a second incubation was carried out at 37°C in order to convert the AMP formed by the action of phosphodiesterase during the first incubation into adenosine and inorganic phosphate. This second reaction was stopped by the addition of 0.2ml of ice-cold 25% trichloroacetic acid and the tubes were centrifuged.

Adaptations (Nair, 1966; Vernikos-Danellis and Harris, 1968) of the method of Fiske and Subbarow (1925) have been modified slightly for the determination of inorganic phosphate released by brown adipose tissue preparations. To 0.7ml of the supernatant of the second incubation medium (after the addition of trichloroacetic acid) were added 1.3ml of distilled water and 0.5ml of 1.25% ammonium molybdate in 2.5N H_2SO_4. The color was developed by the addition of 0.05ml of a solution of Fiske and Subbarow reducer (this mixture of 1-amino-2-naphthol-4-sulfonic acid, sodium sulfite and sodium bisulfite, was purchased from Sigma and according to the instructions, one gram was diluted in 6.3ml of distilled water; 0.05ml of that solution was added to develop the color). Ten minutes later, the optical density of the resulting solution was read at 660 nanometers on a Beckman DB-G spectrophotometer. Standard solutions of inorganic phosphate containing between zero and one micromole were made up and read with every set of experiments. The optical density at 660 nanometers is directly proportional to the amount of inorganic

phosphate present (Figure 26). The amount of cyclic AMP hydrolyzed is proportional to the amount of inorganic phosphate formed during the second incubation. Blanks containing a concentration of enzyme identical to that of the samples but containing no cyclic AMP were used and every sample had its own blank. The optical density of the blank was subtracted from that of the corresponding sample. The cyclic AMP phosphodiesterase activity is expressed as nanomoles of inorganic phosphate formed per minute.

4) protein estimation

Protein was estimated by the Lowry method (Lowry et al., 1951).

5) chemicals

Adenosine 3':5'-cyclic monophosphoric acid, sucrose, Trizma-HCl, Trizma-base, 1.25% ammonium molybdate in 2.5N H_2SO_4, Fiske and Subbarow reducer, snake venom from Crotalus atrox (Western Diamondback Rattlesnake) were all obtained from Sigma.

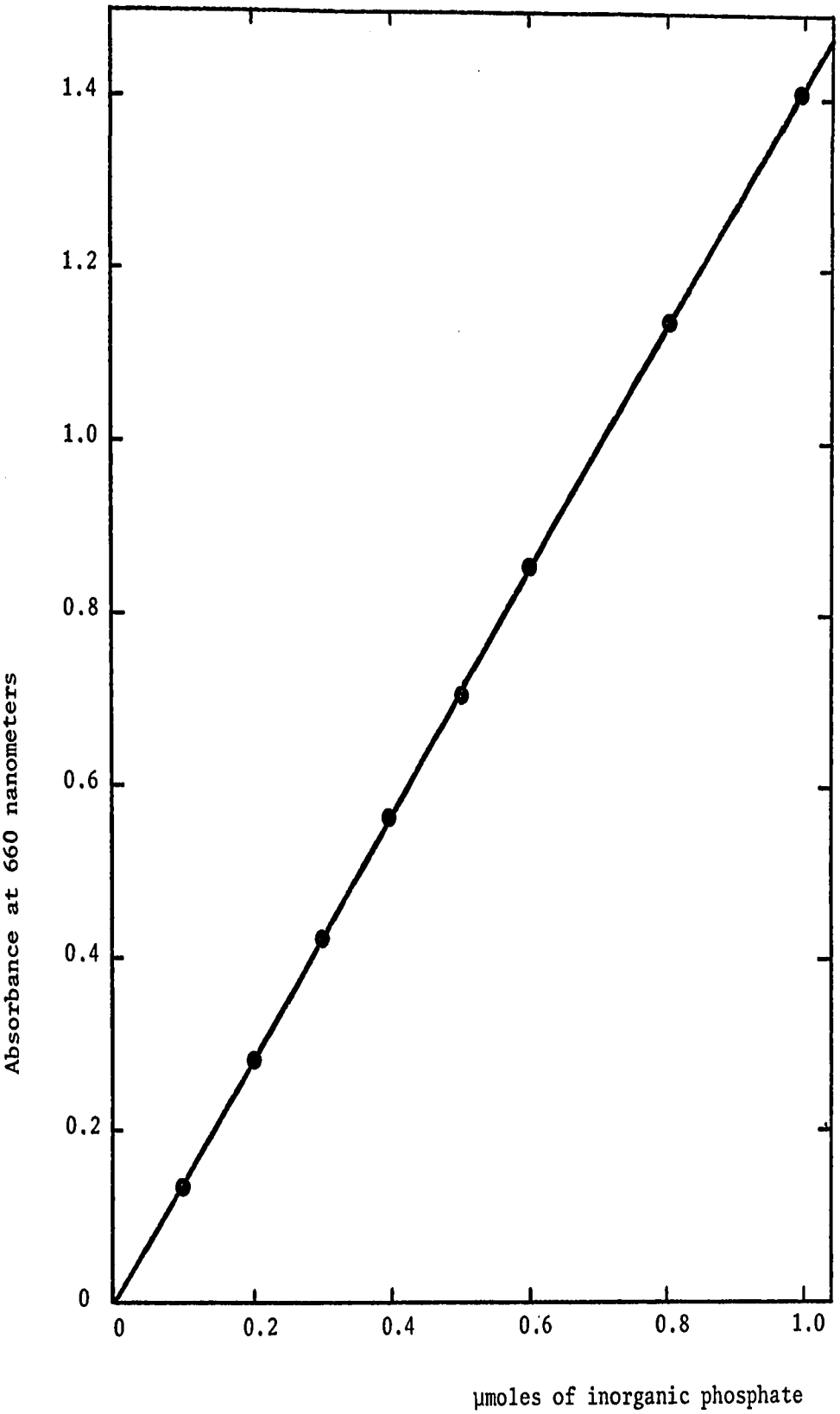
B) Properties

1) *time of incubation*

The hydrolysis of cyclic AMP by phosphodiesterase is linear for up to 40 minutes at 30°C with 0.5ml of the 30,000*g* supernatant of the brown adipose tissue homogenate after dialysis

Figure 26: Standard curve for the determination of inorganic phosphate; absorbance at 660 nanometers.

The standard solution of inorganic phosphate is made as follows: 1.36 gram of KH_2PO_4 is diluted in one liter of distilled water, a few drops of chloroform are added and the solution is stored in the refrigerator where it can be kept for several months. For use, it is diluted 1:10, so that 1ml corresponds to one micromole of inorganic phosphate. The final dilution is made in trichloroacetic acid 1.66% so that the acidity of the standards will be identical to that of the samples. The other steps of the procedure are explained in the text. Notice the linearity of the relationship between the absorbance at 660 nanometers and the concentration of inorganic phosphate in the range of concentrations studied.


Absorbance at 660 nanometers
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
µmoles of inorganic phosphate

(Figure 27). Higher concentrations of enzyme as well as longer periods of incubation were tested and found not to give rise to a linear relationship. In most assays, 0.5 ml of the 30,000*g* supernatant was used and the incubation was carried out at 30°C for 30 minutes.

2) concentration of cyclic AMP

The velocity of the reaction is maximum at a concentration of substrate of 0.5mM or more when determined at optimal Mg^{++} concentration (Figure 28A). A concentration of 1mM cyclic AMP was used in most assays.

3) concentration of Mg^{++}

The activity of cyclic AMP phosphodiesterase is stimulated by Mg^{++}. The stimulation seems maximal at all concentrations tested, i.e. between 1mM and 20mM (Figure 28B). A concentration of 3mM Mg^{++} was used in most assays.

4) pH optimum

Figure 29 shows that the pH optimum for phosphodiesterase of brown adipose tissue 30,000*g* supernatant lies between 7.6 and 8.5. Most assays were carried out at pH 8.0.

Figure 27: Velocity of the phosphodiesterase reaction versus the time of incubation of the brown adipose tissue preparation.

The incubation medium contained 2mM cyclic AMP, 2mM $MgSO_4$, 0.5ml of the dialyzed 30,000*g* supernatant of the homogenate containing 40mg/ml and 40mM Tris-HCl buffer, pH 7.8. The incubation was carried out at 30°C.

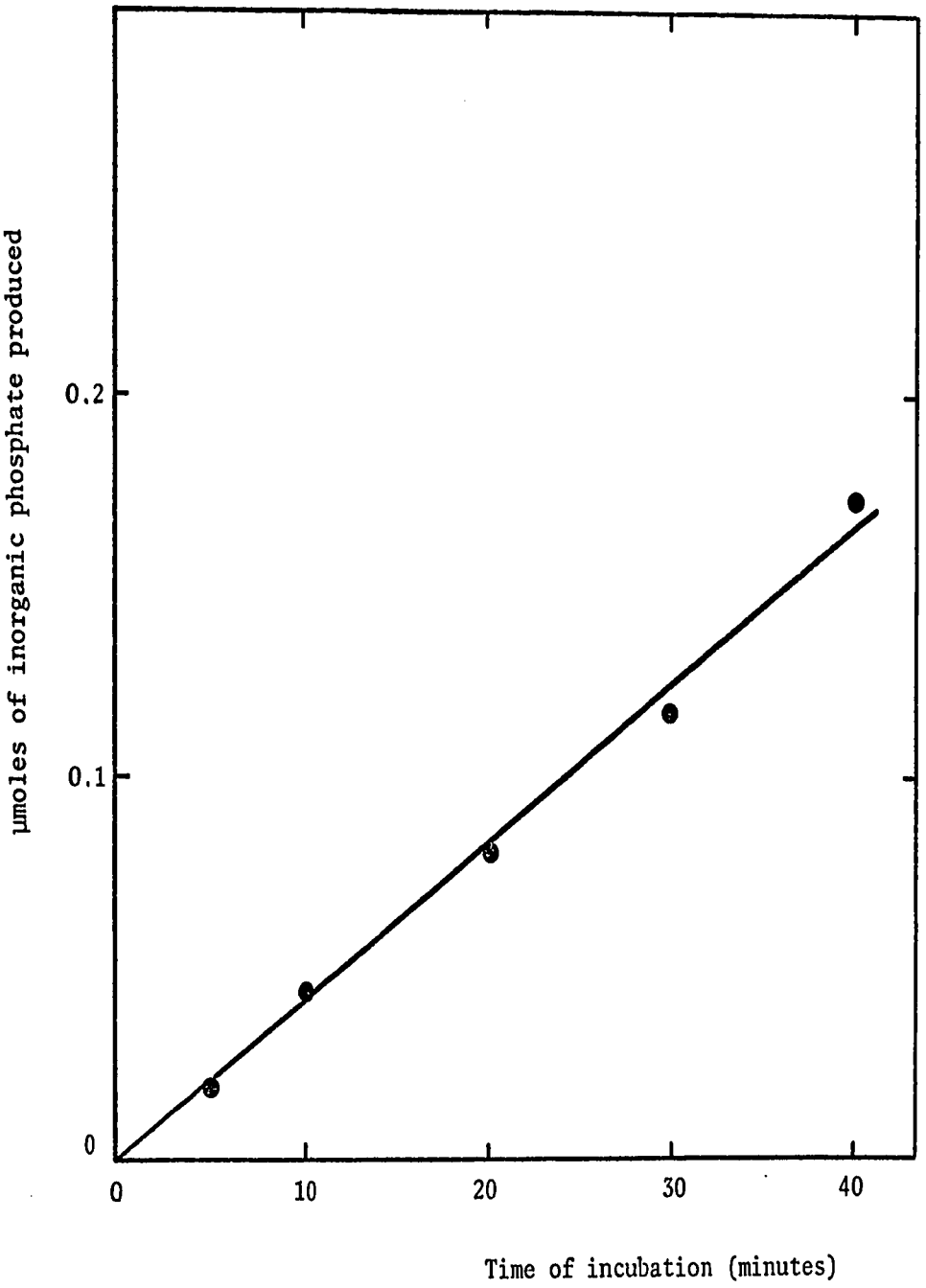

µmoles of inorganic phosphate produced
0.2
0.1
0
0
10
20
30
40
Time of incubation (minutes)

Figure 28A: Cyclic AMP phosphodiesterase from brown adipose tissue; velocity versus cyclic AMP concentration.

The incubation medium contained 3mM $MgSO_4$, 0.5ml of the dialyzed 30,000*g* supernatant, and 40mM Tris-HCl buffer, pH 8.0. The incubation was carried out at 30°C for 30 minutes.

Figure 28B: Cyclic AMP phosphodiesterase from brown adipose tissue; velocity versus Mg^{++} concentration.

The incubation medium contained 2mM cyclic AMP, 0.5ml of the dialyzed 30,000*g* supernatant, and 40mM Tris-HCl buffer, pH 8.0. The incubation was carried out at 30°C for 30 minutes.

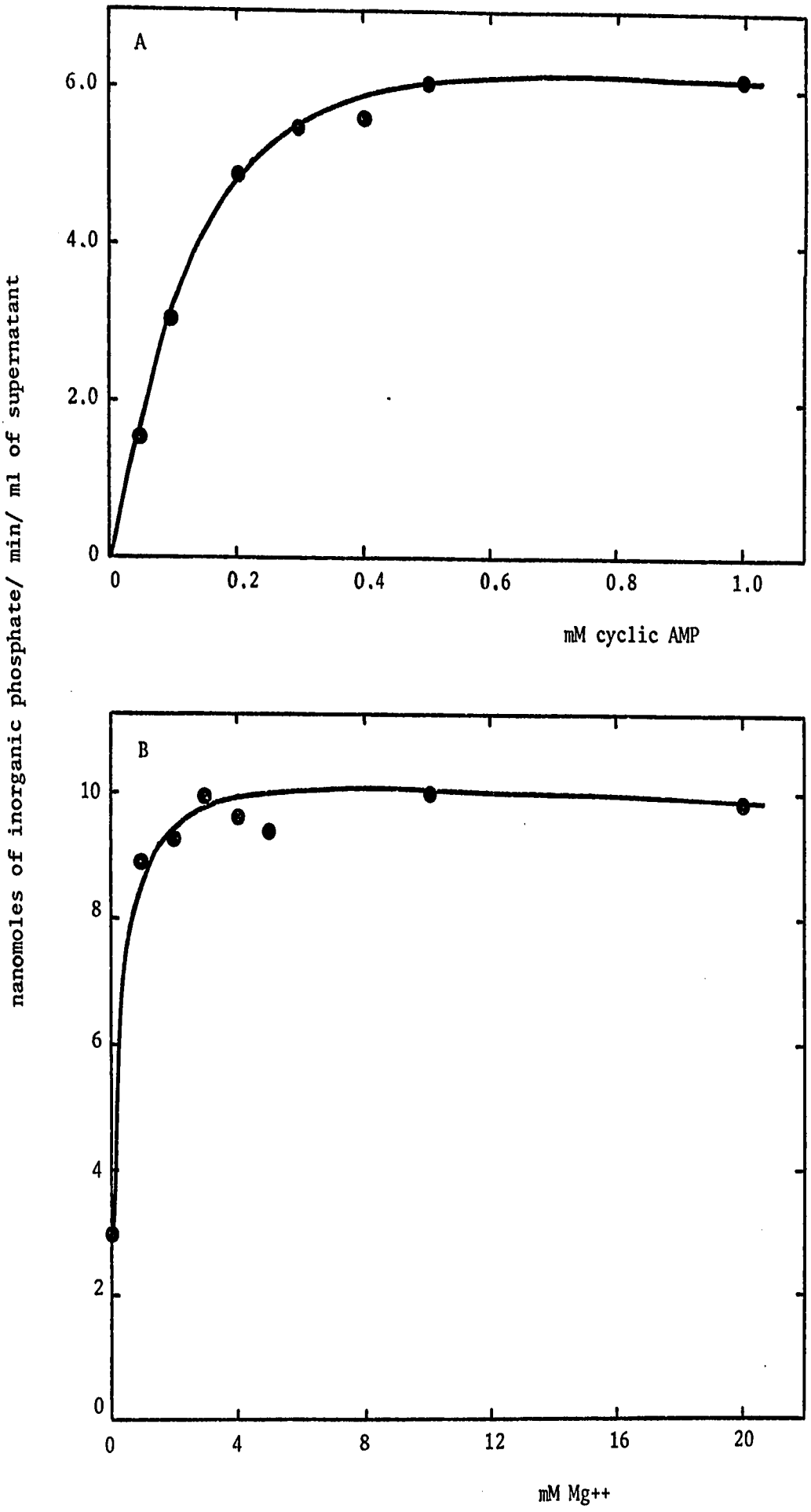
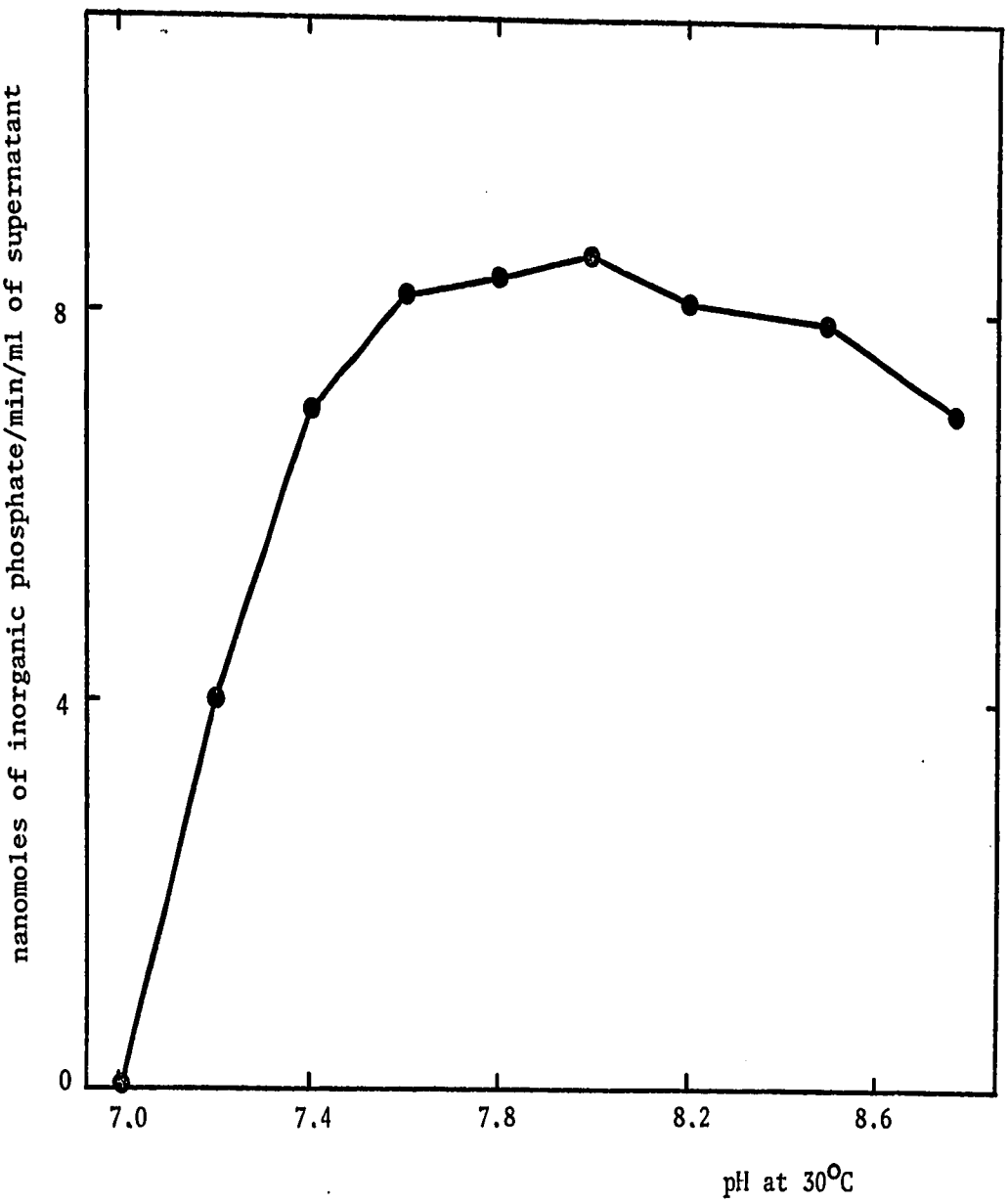

A
6.0
4.0
2.0
0
0
0.2
0.4
0.6
0.8
1.0
mM cyclic AMP
B
10
8
6
4
2
0
0
4
8
12
16
20
mM Mg++
nanomoles of inorganic phosphate/ min/ ml of supernatant

Figure 29: Cyclic AMP phosphodiesterase from brown adipose tissue; velocity versus pH.

The incubation medium contained 2mM cyclic AMP, 2mM $MgSO_4$, and 0.5ml of the dialyzed 30,000*g* supernatant. The incubation was carried out for 30 minutes at 30°C. The pH optimum lies over the range of pH from 7.6 to 8.5; at pH 7.0 there is no activity at all.


nanomoles of inorganic phosphate/min/ml of supernatant
0
4
8
7.0
7.4
7.8
8.2
8.6
pH at 30°C

The constituents of the incubation mixture had the following optimal concentrations: 1mM cyclic AMP, 3mM $MgSO_4$, 0.5ml of enzyme preparation (dialyzed 30,000*g* supernatnat of the homogenate containing 40 mg of brown adipose tissue per ml) and 37.5mM Tris buffer at pH 8.0.

C) Effects of cold exposure and cold acclimation on cyclic AMP phosphodiesterase activity in brown adipose tissue

There is no significant change in the specific activity of the cyclic AMP phosphodiesterase at any time during exposure to cold (Figure 30). There is an increase in the total activity of the enzyme in the tissue but this increase parallels the increase in protein content and in wet weight of the brown adipose tissue (Figures 30 and 6).

D) Discussion

The specific activity of cyclic AMP phosphodiesterase of the interscapular brown adipose tissue is not affected by exposure of the animals to cold. The total activity of the enzyme in the interscapular brown adipose tissue is increased by cold exposure but the increase in total activity of the enzyme parallels the increase in protein content of the tissue which explains why the specific activity of the enzyme is not modified. Hahn (1970) has reported that the activity of the

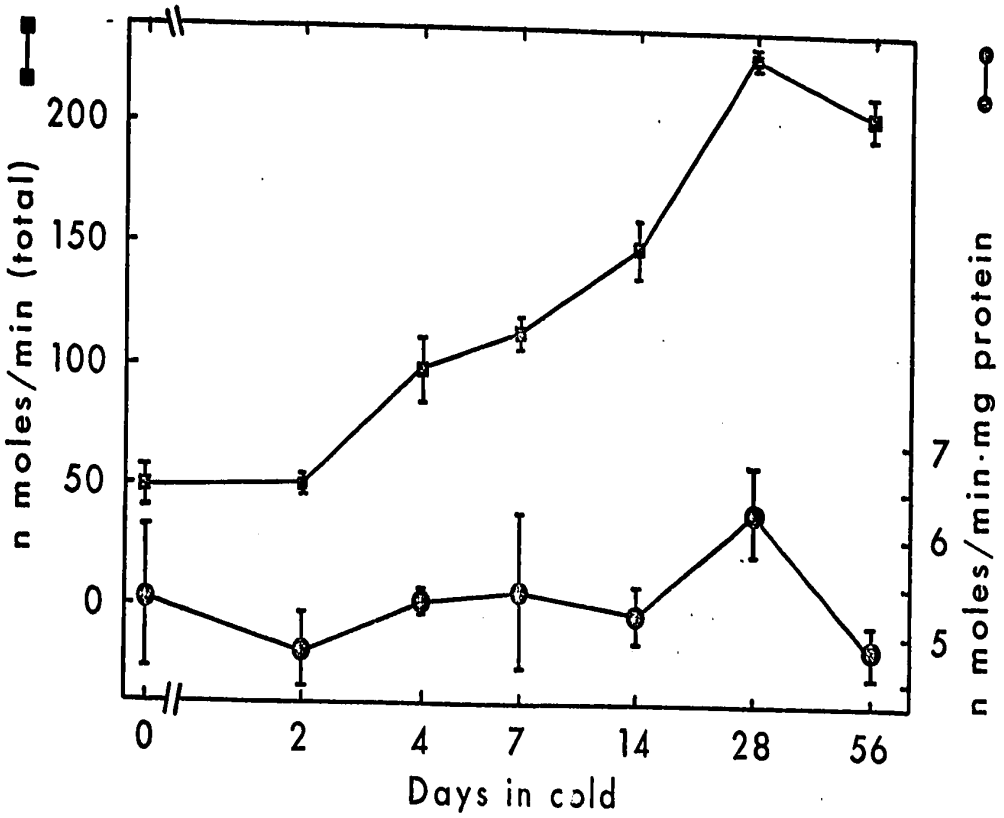
Figure 30: Cyclic AMP phosphodiesterase in brown adipose tissue; specific and total activities of the enzyme during acclimation of rats to cold.

The symbols used are:

■ : total activity;

● : specific activity..

The number of animals used is the same as in figure 6. The values are expressed as means ± standard errors.


n moles/min (total)
200
150
100
50
0
n moles/min·mg protein
7
6
5
0
2
4
7
14
28
56
Days in cold

cyclic AMP phosphodiesterase in the 10,000*g* supernatant of the interscapular brown adipose tissue was of the order of 5.5 nanomoles/min/mg protein. The values obtained in the present communication for the activity of phosphodiesterase in the 30,000*g* supernatant of the interscapular brown adipose tissue are also of the same order of magnitude.

The fact that no difference in the phosphodiesterase activity of the brown adipose tissue is observed during exposure to cold may be due to the lack of sensitivity of the method used. More sensitive methods using labelled cyclic AMP for substrate could reveal the presence of more than one molecular form of the enzyme in the brown adipose tissue. Studies of phosphodiesterase at micromolar substrate concentrations rather than at millimolar substrate concentrations have revealed the presence of more than one form of phosphodiesterase in many different tissues (Beavo et al., 1970; Thompson and Appleman, 1971). Multiple forms of phosphodiesterase have been partially separated by starch gel electrophoresis (Monn and Christiansen, 1971) and by gel filtration (Jard and Bernard, 1970; Rosen, 1970; Thompson and Appleman, 1971) in different tissues. The purification of phosphodiesterase from brown adipose tissue is beyond the scope of this presentation but it must be kept in mind that the results obtained at the millimolar level of substrate concentration may not be very representative of the situation present in the intact animal where the concentration of cyclic AMP in the different tissues is of the order of one picomole per mg of tissue. Although the results obtained show no difference for the phosphodiesterase activity

of the brown adipose tissue during cold exposure, it must be remembered that more sensitive methods of determination could possibly reveal differences in the enzyme activity during cold exposure of the animals. Unfortunately, such methods were not available when the experiments were performed and we can only conclude that in the conditions studied, with 1mM cyclic AMP, there is no difference observed for the phosphodiesterase activity of the brown adipose tissue during cold exposure.

Section III: Experimental: cyclic AMP phosphodiesterase activity in skeletal muscle

A) Material and Methods

1) *animals*

see chapter 2, section II, A, 1.

2) preparation of skeletal muscle

see chapter 2, section III, A, 2.

3) assay of cyclic AMP phosphodiesterase

The skeletal muscle homogenate was centrifuged at 4,000*g* for ten minutes (as described for dog heart by Nair, 1966) and the supernatant was dialyzed overnight, with two changes of buffer, against Chappell-Perry medium containing no ATP and no EDTA (EDTA is an inhibitor of phosphodiesterase and it was removed from the homogenate by dialysis against a medium containing no EDTA). This fraction can then be stored for months at -20°C without loss of activity.

Phosphodiesterase was measured essentially as described by Nair (1966), and as explained in the section II, A, 3, of this chapter except that the incubation was performed in one stage rather than in two stages. An excess of 5'-nucleotidase (0.05ml of a solution of snake venom containing 10mg/ml) was added to the other constituents of the incubation medium (optimal concentrations of cyclic AMP, $MgSO_4$, enzyme and Tris buffer) and the

reaction was carried on for 30 minutes at 37°C. The reaction was terminated by the addition of 0.2ml of 25% trichloroacetic acid. Inorganic phosphate produced during the reaction was measured as described in section II, A, 3 of this chapter. Determination of phosphodiesterase activity was performed at 37°C since the determination of adenyl cyclase in the same tissue was performed at 37°C because of the low specific activity of the enzyme in skeletal muscle.

4) protein estimation

Protein was estimated by the Lowry method (Lowry et al., 1951).

5) chemicals

as described in chapter 3, section II, A, 5.

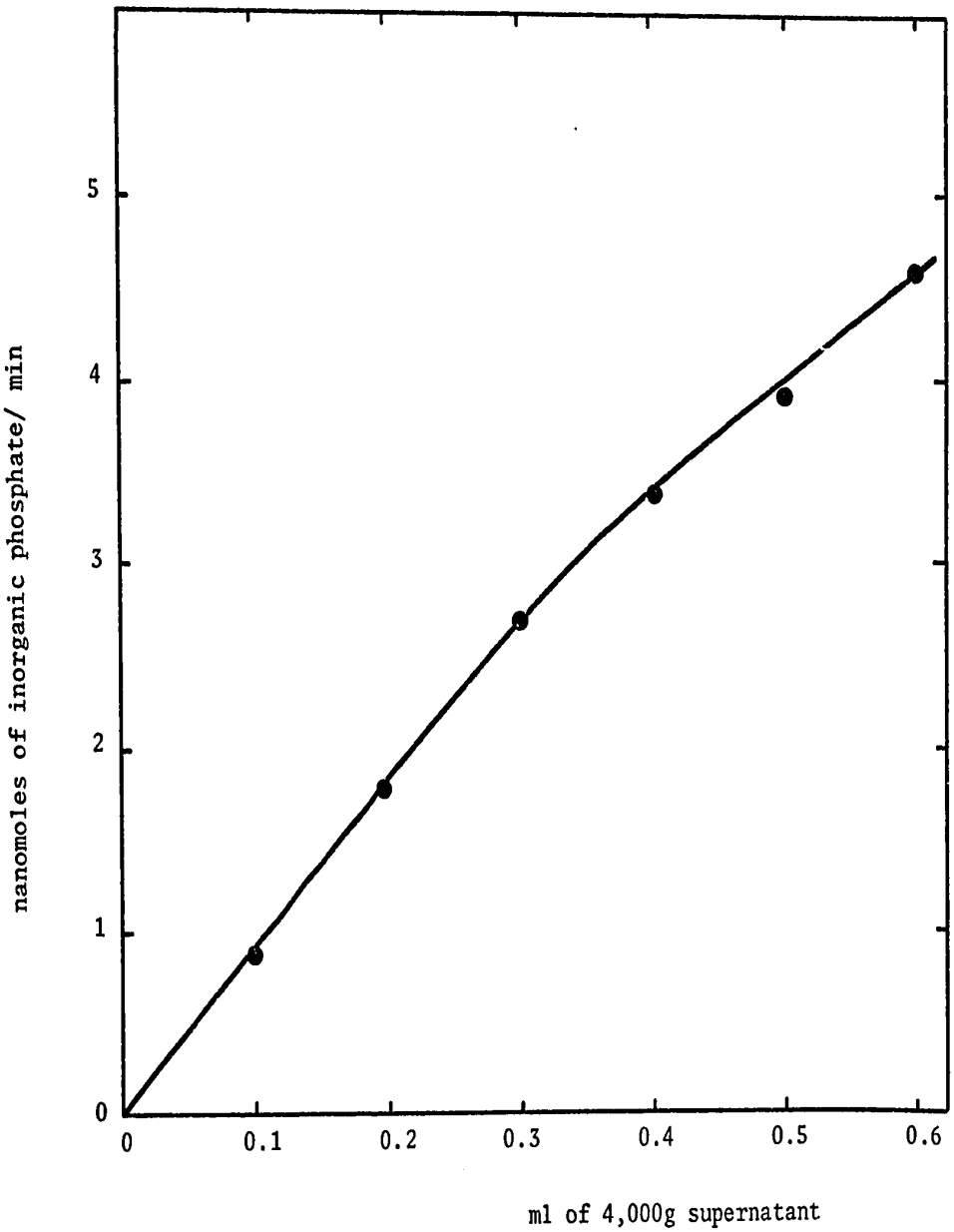
B) Properties

1) *enzyme concentration*

The rate of hydrolysis of cyclic AMP is linear for concentrations of enzyme up to 0.3ml of the 4,000*g* supernatant of skeletal muscle homogenate containing 100 mg of muscle per ml, during a 30 minute incubation period at 37°C (Figure 31). Higher concentrations of enzyme did not give rise to a linear rate of hydrolysis. In most assays, 0.3ml of the 4,000*g* supernatant was added to the incubation mixture to initiate the reaction which was carried out for 30 minutes at 37°C.

Figure 31: Cyclic AMP phosphodiesterase from skeletal muscle; velocity versus enzyme concentration.

The incubation medium contained 1mM cyclic AMP, 5mM $MgSO_4$, 80mM Tris buffer, pH 8.0 and different amounts of enzyme expressed in ml of the dialyzed 4,000*g* supernatant of the skeletal muscle homogenate containing 100 mg/ml. The reaction was carried out at 37°C for 30 minutes.


nanomoles of inorganic phosphate/ min
0
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5
0.6
ml of 4,000g supernatant

2) concentration of cyclic AMP

The velocity of the phosphodiesterase reaction is maximum at substrate concentrations higher than 0.2mM (Figure 32). A concentration of 0.5mM cyclic AMP was used in most assays, unless otherwise specified.

3) concentration of Mg^{++}

The velocity of the reaction at optimal substrate concentration is maximum at a Mg^{++} concentration (final concentration in the incubation medium) of 8.8mM (Figure 33 A). There is less stimulation of the enzyme with higher concentration of Mg^{++} (Figure 33 A). The activity of the enzyme in the absence of Mg^{++} could not be measured under these conditions because the enzyme preparation was diluted in Chappell-Perry medium containing 5mM Mg^{++}.

4) pH optimum

In expressing the final pH of the incubation medium, it must be remembered that the phosphodiesterase preparation was kept in Chappell-Perry medium containing 0.1M Tris-HCl buffer, pH 7.4. For that reason the pH of the reaction was measured at every concentration of Tris-HCl or glycine-NaOH buffer tested to insure that the pH illustrated in the figure 34 was the final pH of the incubation medium at 37°C. The pH optimum with Tris-HCl buffer was 8.45 and it seems that the enzyme was somewhat less active in glycine-NaOH buffer than in Tris-HCl buffer (Figure 34). Most assays, unless otherwise specified, were performed at the optimal pH of 8.45 in Tris-HCl buffer.

Figure 32: Cyclic AMP phosphodiesterase from skeletal muscle; velocity versus substrate concentration.

The incubation medium was composed of 5mM $MgSO_4$, 80mM Tris buffer, pH 8.45 and 0.3ml of the dialyzed 4,000*g* supernatant of the skeletal muscle homogenate containing 100 mg/ml. The reaction was carried out at 37°C for 30 minutes.

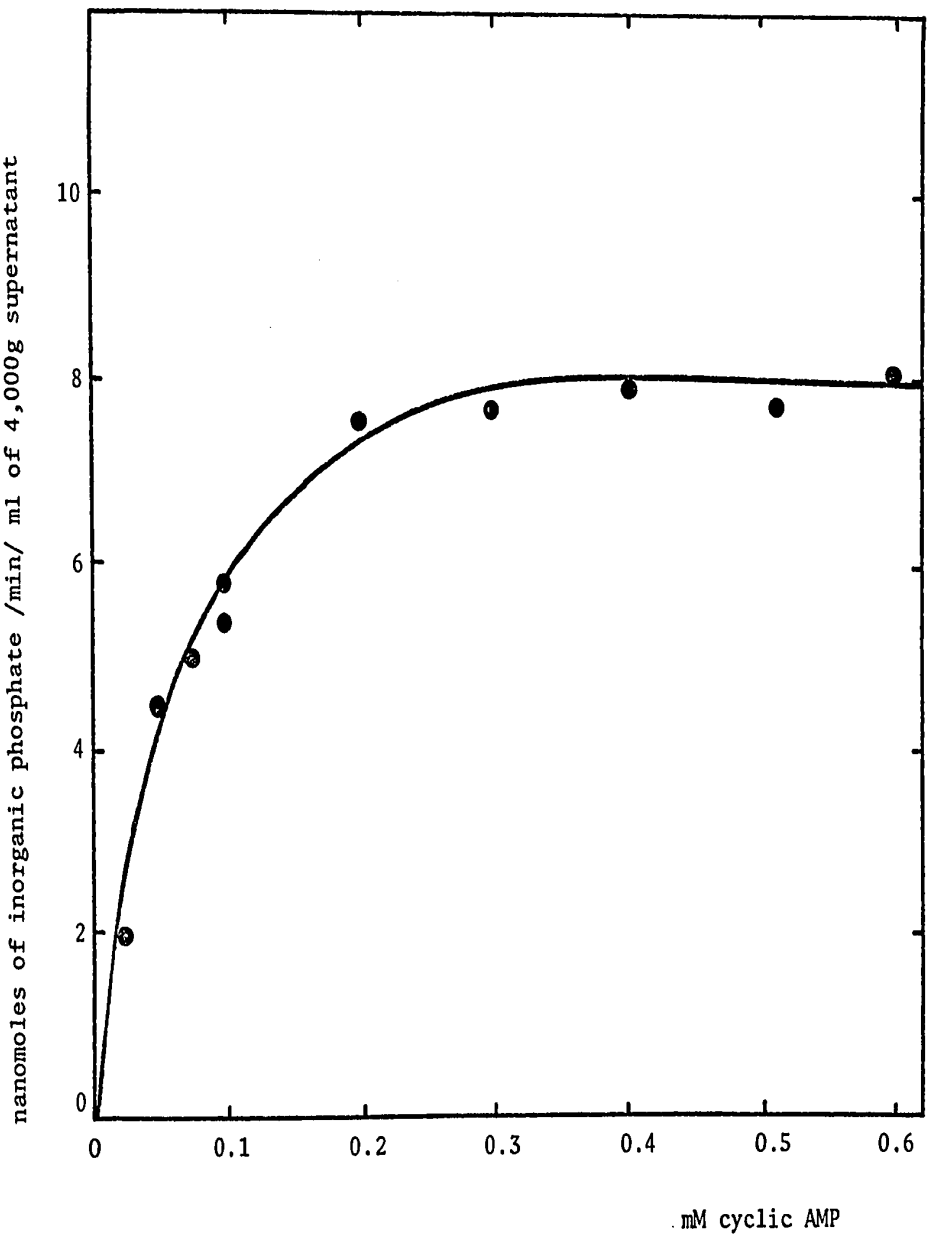
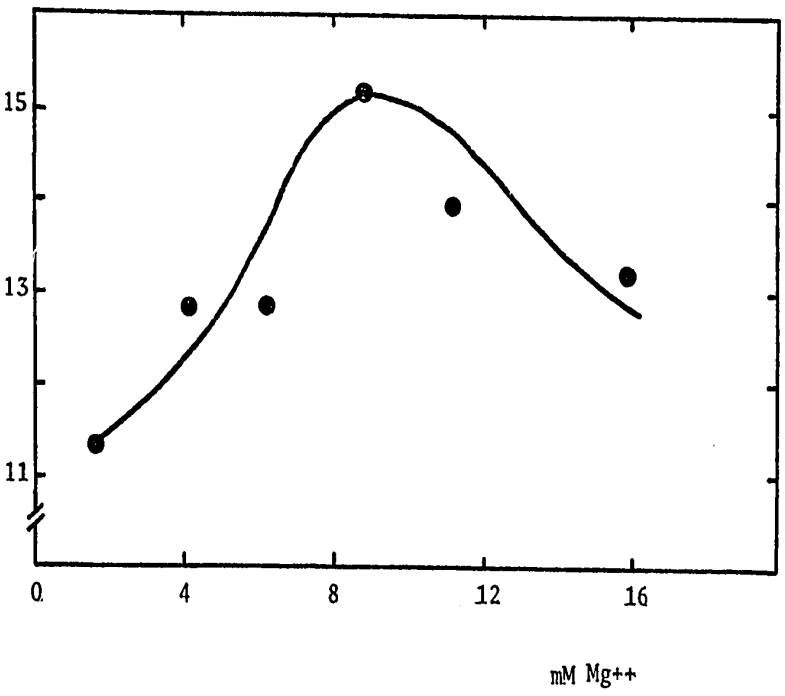

nanomoles of inorganic phosphate /min/ ml of 4,000g supernatant
0
2
4
6
8
10
0
0.1
0.2
0.3
0.4
0.5
0.6
mM cyclic AMP

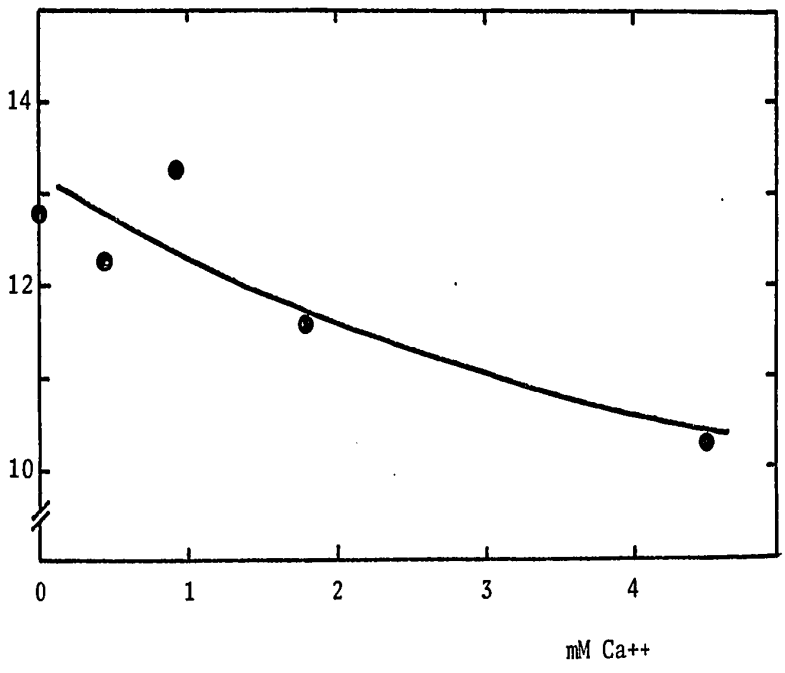
Figure 33 A: Cyclic AMP phosphodiesterase from skeletal muscle; velocity versus Mg^{++} concentration.

The incubation medium was composed of 0.5mM cyclic AMP, 80mM Tris buffer, pH 8.45 and 0.3ml of the dialyzed 4,000*g* supernatant of the skeletal muscle homogenate containing 100 mg/ml. The incubation was carried out at 37°C for 30 minutes. Notice that Mg^{++} concentrations higher than 8.8mM give less stimulation of the enzyme.

Figure 33 B: Cyclic AMP phosphodiesterase from skeletal muscle; velocity versus Ca^{++} concentration

The incubation medium was composed of 0.5mM cyclic AMP, 6.5mM $MgSO_4$, 80mM Tris buffer, pH 8.45 and 0.3 ml of the dialyzed 4,000*g* supernatant of the skeletal muscle homogenate containing 100 mg/ml. The incubation was carried out at 37°C for 30 minutes. Notice the inhibition of the enzymatic activity at calcium concentrations higher than 1mM.





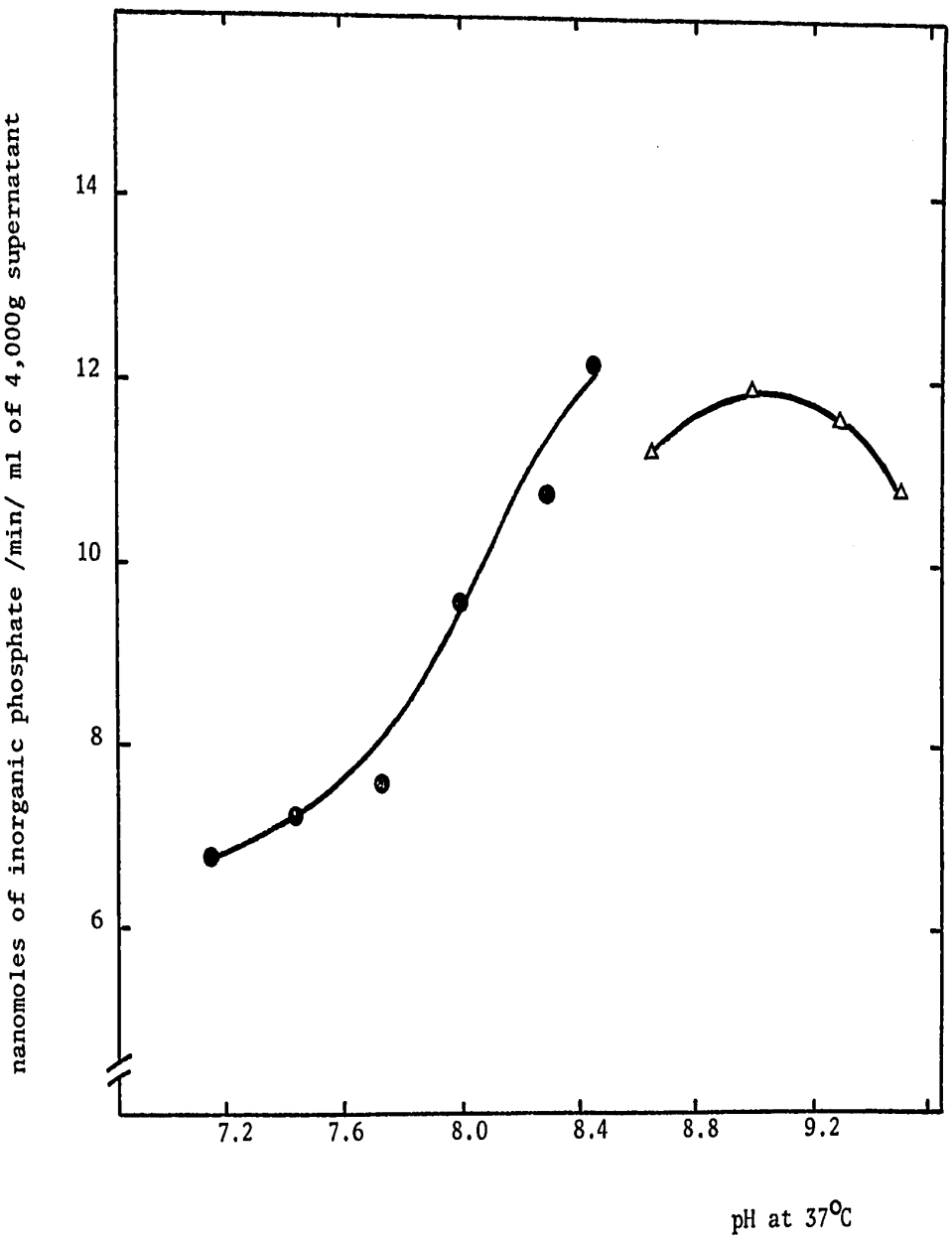
nanomoles of inorganic phosphate /min/ ml of 4,000g supernatant

Figure 34: Cyclic AMP phosphodiesterase from skeletal muscle; velocity versus pH.

The incubation medium was composed of 1mM cyclic AMP, 5mM $MgSO_4$, and 0.3ml of the dialyzed 4,000*g* supernatant of the skeletal muscle homogenate containing 100 mg/ml. The pH expressed is the final pH in the incubation medium at 37°C. In samples containing only Tris buffer, the concentration of Tris was 81.25mM; in samples containing glycine-NaOH buffer, the concentration of the latter was 62.5mM while the concentration of Tris due to the enzyme preparation was 18.75mM.

●: Tris-HCl buffer only;

Δ: glycine-NaOH buffer.


nanomoles of inorganic phosphate /min/ ml of 4,000g supernatant
14
12
10
8
6
7.2
7.6
8.0
8.4
8.8
9.2
pH at 37°C

5) concentration of Ca^{++}

Skeletal muscle phosphodiesterase is inhibited by calcium at concentrations higher than 1mM (Figure 33B). This may be of physiological significance since the concentration of calcium in skeletal muscle was estimated to be between 1mM (Himms-Hagen, unpublished observations) and 4mM (Watchorn and McCance, 1937).

C) Effects of cold exposure and cold acclimation on cyclic AMP phosphodiesterase activity in skeletal muscle

There is no significant change in the specific activity of cyclic AMP phosphodiesterase at any time during cold acclimation (Figure 35).

D) Discussion

Cyclic AMP phosphodiesterase activity is not modified in skeletal muscle during acclimation of rats to cold. It is possible that the lack of sensitivity of the method of determination of the enzyme activity may be responsible for the fact that no modification of the enzyme activity is observed. As was mentioned in section II, D, of this chapter, kinetic studies at the micromolar level, using radioactive methods of determination of the enzyme activity, have revealed the presence of more than one molecular form of phosphodiesterase in different tissues (Beavo et al., 1970;

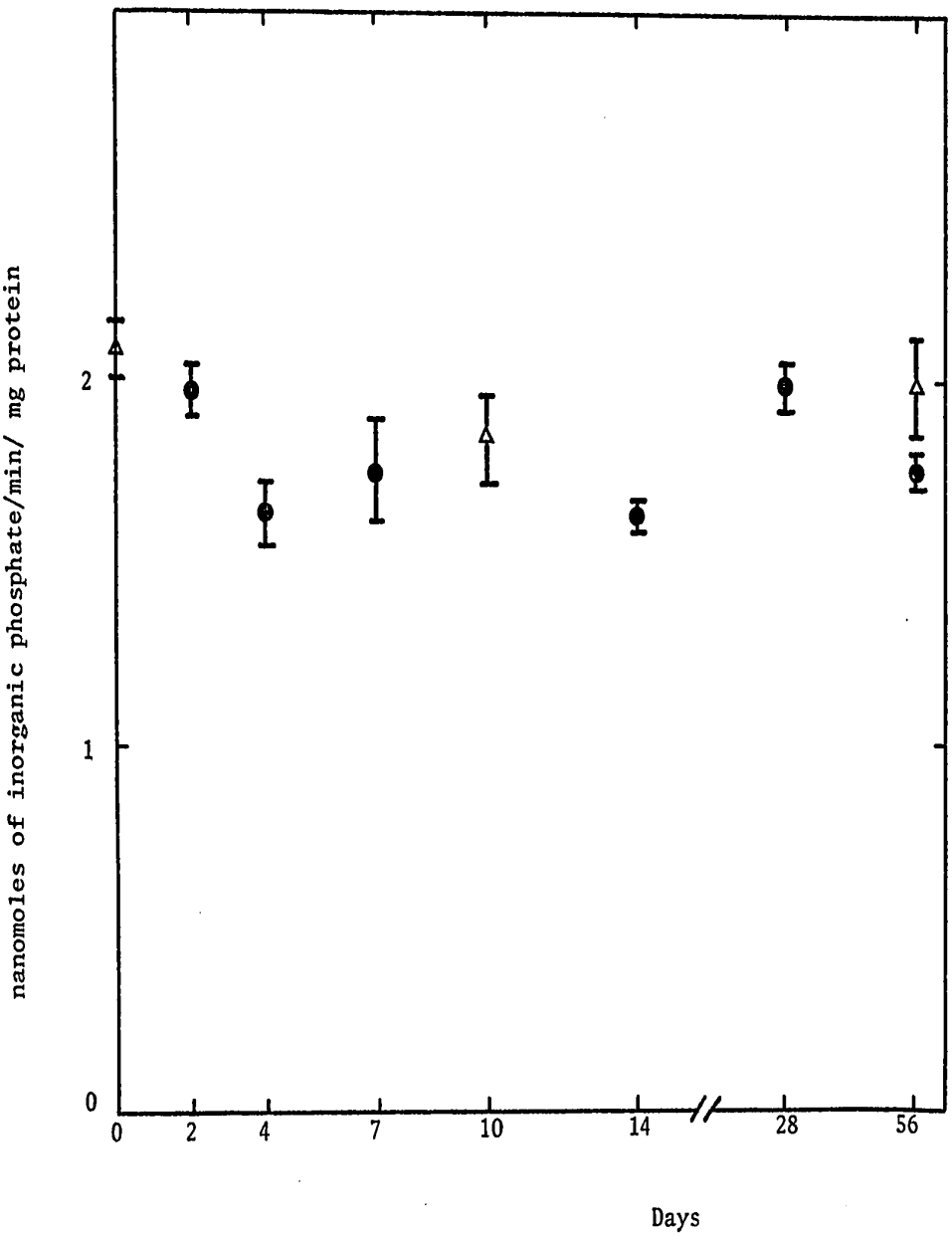
Figure 35: Cyclic AMP phosphodiesterase in skeletal muscle; specific activity of the enzyme during acclimation of rats to cold.

The symbols used are:

Δ : warm acclimated rats;

● : cold exposed or cold acclimated rats.

Five rats were used in each group; the values are expressed as means ± standard errors.


nanomoles of inorganic phosphate/min/ mg protein
2
1
0
0
2
4
7
10
14
28
56
Days

Thompson and Appleman, 1971) which was confirmed by partial separation of the different molecular forms from different tissues by starch gel electrophoresis (Monn and Christiansen, 1971) and by gel filtration (Jard and Bernard, 1970; Rosen, 1970; Thompson and Appleman, 1971). It is then possible that more than one molecular form of the enzyme may exist in skeletal muscle, a phenomenon which could only be revealed by determining the enzyme activity with more sensitive methods (using labelled cyclic AMP as the substrate of the reaction), and by purifying the phosphodiesterase of the tissue to resolve it in the different possible molecular forms. The results which would be obtained in these conditions could be different from the results obtained here at millimolar substrate concentrations and it can only be concluded that in the conditions studied, no modification of the phosphodiesterase of skeletal muscle was observed during cold acclimation of the rats.

CHAPTER 4: CYCLIC AMP LEVELS IN TISSUES

Section I: Survey of the levels of cyclic AMP in tissues

A) Levels of cyclic AMP in different tissues

The levels of cyclic AMP in different tissues of the rat have been reported and they are summarized in table 4 (taken from Ebadi et al., 1971). Tissue levels of cyclic AMP can be increased if a proper hormonal stimulus is applied to the given tissue. The increase in cyclic AMP levels will only be discussed for the four tissues that will be studied, namely skeletal muscle, liver, white adipose tissue and brown adipose tissue, and only the cases where the increase in the level of cyclic AMP is due to catecholamines will be reported.

Intracardial injection of adrenaline caused an increase in the level of cyclic AMP in the anterior tibialis and the soleus muscles of the cat (Butcher et al., 1965) and in the rat gastrocnemius (Posner et al., 1962). Intravenous injection of adrenaline provoked an increase in the level of cyclic AMP in the mouse gastrocnemius (Lyon and Mayer, 1969). Injection of isoproterenol into the artery of the rabbit gracilis muscle isolated in situ, greatly increased the level of cyclic AMP in the muscle (Mayer and Stull, 1971). The catecholamines are then able to increase the level of cyclic AMP in skeletal muscle; the main metabolic effect of the catecholamines in skeletal muscle is to increase the rate of glycogenolysis.

The accumulation of cyclic AMP in the intact liver has

Table 4

Levels of cyclic AMP in different tissues of the rat

Tissue	Cyclic AMP nanomoles/g wet weight of tissue
Stomach (pyloric region)	4.2 ± 0.4
Liver	3.3 ± 0.2
Adrenal (whole)	3.2 ± 0.6
Cerebral central cortex	3.0 ± 0.1
Kidney	3.0 ± 0.2
Thyroid (whole)	2.9
Pituitary (whole)	2.9 ± 0.3
Heart (left ventricle)	2.7 ± 0.4
Pancreas	2.3 ± 0.4
Muscle (gastrocnemius)	1.7 ± 0.3
Spleen	1.6 ± 0.2
Submaxillary gland	1.6 ± 0.2
Skin (abdominal)	1.5 ± 0.3
Small intestine (duodenum)	1.5 ± 0.2
Aorta (ascending)	1.5
Trachea (bifurcation)	1.5 ± 0.3
Testes (whole)	1.5 ± 0.2
Large intestine (descending colon)	1.3 ± 0.1
Thymus (adult)	1.2 ± 0.3
Lung (right superior lobe)	1.0 ± 0.2
White adipose tissue	0.35 ± 0.10
Brown adipose tissue*	3.0 ± 0.4

* All values obtained from a review by Ebadi et al., 1971 except for the value for the brown adipose tissue which was obtained from Beviz et al., 1971.

been studied in two different systems. Perfusion of the rat liver with adrenaline resulted in a three-fold increase in the level of cyclic AMP in the tissue (Robison et al., 1967b). Incubation of rabbit liver slices with adrenaline caused an increase in the level of cyclic AMP (Sutherland et al., 1965). The main metabolic effect of the catecholamines on the liver is also to increase the rate of glycogenolysis.

Incubation of whole fat pads in vitro with adrenaline caused an increase in the level of cyclic AMP and in the release of free fatty acids from the tissue (Butcher et al., 1965). The level of cyclic AMP in perfused fat pads was also increased by the introduction of adrenaline in the perfusate (Butcher et al., 1968); the addition of adrenaline to isolated fat cells caused a rapid increase in the level of cyclic AMP in the cells (Butcher et al., 1968; Weiss et al., 1966; Humes et al., 1969); the addition of adrenaline to white adipose tissue slices also caused a rapid increase in the level of cyclic AMP (Humes et al., 1969). The potentiation of the effect of adrenaline on the level of cyclic AMP in the white adipose tissue by methylxanthines has been observed in isolated fat cells (Weiss et al., 1966; Humes et al., 1969), in tissue slices (Humes et al., 1969) and in whole fat pads incubated in vitro (Butcher et al., 1965). The effects of adrenaline on white adipose tissue lipolysis are associated with an increase in the cyclic AMP level in the tissue.

Noradrenaline could increase the level of cyclic AMP in brown adipose tissue slices; it also increased the respiration of the tissue slices and lipolysis (Beviz et al., 1971).

B) Plasma and urinary levels of cyclic AMP and their modification by hormones

In addition to the rates of formation and degradation of cyclic AMP, the rate at which cells extrude the cyclic nucleotide may be a factor in the regulation of the internal concentration of the compound. The presence of cyclic AMP in extracellular fluids was first reported for human urine (Butcher and Sutherland, 1962). The concentration of cyclic AMP in the plasma and the urine of different mammalian species has now been reported (see review by Broadus et al., 1971). The average value for the cyclic AMP content of the rat plasma is 30 picomoles/ml and the rate of urinary excretion of cyclic AMP in the rat is between 12 and 115 nanomoles/100g/day.

Cyclic AMP is cleared from the plasma by glomerular filtration; the human kidney was found to add a variable quantity of endogenous cyclic AMP to the tubular urine, corresponding to approximately one third of the total level of cyclic AMP excreted (Broadus et al., 1970a). The sources of the normal plasma levels of cyclic AMP are unknown but, as will be discussed later, the liver and kidney seem capable of adding cyclic AMP to the plasma under the appropriate hormonal stimulation. It can be said that changes in the extracellular levels of cyclic AMP reflect alterations in the intracellular levels of the compound in response to hormonal stimulation (Hardman et al., 1971).

Adrenaline (Broadus et al., 1970b; Ball et al., 1970) and isopropylnoradrenaline (Ball et al., 1970) injected intravenously, elevated the human plasma level of cyclic AMP. The excretion of

cyclic AMP was also increased in man by intravenous injection of adrenaline (Broadus et al., 1970b) and isopropylnoradrenaline (Williams et al., 1972).

Hormones other than catecholamines can also increase the rate of production and/or the rate of excretion of cyclic AMP. Parathyroid hormone was shown to increase by several fold the excretion of cyclic AMP in many species (Chase and Aurbach, 1967; Chase et al., 1969; Kaminsky et al., 1970; Taylor et al., 1970) but did not increase very much the level of cyclic AMP in the plasma (Kaminsky et al., 1970). Urinary cyclic AMP was increased in hyperparathyroid patients (Taylor et al., 1970; Kaminsky et al., 1970) and depressed in hypoparathyroid patients (Taylor et al., 1970; Chase et al., 1969); parathyroidectomy of rats produced a 50% fall in the urinary excretion of cyclic AMP (Chase and Aurbach, 1967). Parathyroid hormone is known to increase adenyl cyclase activity in the kidney (Chase and Aurbach, 1968) and it is by increasing the level of cyclic AMP in the kidney that parathyroid hormone increases the urinary excretion of cyclic AMP (Kaminsky et al., 1970). The small increase in plasma level after the injection of parathyroid hormone is believed to be due to the increased release of cyclic AMP into the circulation by the kidney, since it was not observed in anephric patients (Kaminsky et al., 1970).

Glucagon, infused intravenously, into fasting normal man, induced up to 30-fold increases in both plasma and urine levels of cyclic AMP (Broadus et al., 1970b). The intravenous injection of glucagon to rats caused a seven-fold increase in the excretion

of cyclic AMP (Hardman et al., 1969). The increase in cyclic AMP levels in response to glucagon was shown not to be mediated by parathyroid hormone or adrenaline (Broadus et al., 1970b); the increment in the urinary excretion of cyclic AMP could be accounted for by glomerular filtration of higher levels of plasma cyclic AMP (Broadus et al., 1970b). The increased cyclic AMP concentration in the plasma following the injection of glucagon, probably results from an increased amount of cyclic AMP entering the plasma from the liver since the perfused rat liver can release cyclic AMP in response to glucagon (Broadus et al., 1970b).

Hypophysectomy can slightly decrease the excretion of cyclic AMP in rats and treatment of the rats with corticosteroids returns the excretion rate of cyclic AMP to normal values (Hardman et al., 1969). In man, growth hormone does not alter cyclic AMP excretion (Taylor et al., 1970); ACTH produces only small increases in plasma and urine levels of cyclic AMP (Broadus et al., 1971).

Cyclic AMP excretion in women is slightly increased during the mid-portion of the menstrual cycle and also following 30 weeks of gestation (Taylor et al., 1970). The excretion of cyclic AMP has been reported to be reduced in depressed patients and elevated in manic patients (Paul et al., 1970).

Many different hormonal stimuli can then induce changes in the plasma levels and/or the urinary excretion of cyclic AMP. Cold acclimation, which is accompanied by an increased calorigenic response to catecholamines, could possibly be associated with an increase in the production rate and/or the excretion rate of cyclic AMP.

C) Methods of determination of cyclic AMP levels in tissues

The first method of estimation of cyclic AMP was based on the activation of liver phosphorylase by cyclic AMP (Rall and Sutherland, 1958), and on the subsequent measurement of the inorganic phosphate formed during the reaction (Rall and Sutherland, 1958) or on the reduction of NADP in the presence of phosphoglucomutase and glucose-6-phosphate dehydrogenase (Brown et al., 1963; Scott and Falconer, 1965). Various materials were found to interfere with the assay and separation of cyclic AMP from other components of tissue extracts by ion exchange chromatography (Butcher et al., 1965; Kakuchi and Rall, 1968) improved the sensitivity of the method. The activation of muscle phosphorylase was also used as a method of determination of cyclic AMP (Posner et al., 1964; Namm and Mayer, 1968).

Another type of determination of cyclic AMP was based on the conversion of cyclic AMP to AMP by purified phosphodiesterase and on the conversion of the AMP formed to ATP by myokinase and pyruvate kinase (Breckenridge, 1964). The ATP produced during the reaction can be detected by several methods (Breckenridge, 1964; Goldberg et al., 1969; Aurbach and Houston, 1968; Kaneko and Field, 1969; Ebadi et al., 1971). All of those methods can detect cyclic AMP in a few milligrams of tissue but each requires a quantitative separation of cyclic AMP from other adenine nucleotides prior to the assay.

Methods of estimation of cyclic AMP based upon the competition between labelled and unlabelled cyclic AMP for the hydrolytic site of brain phosphodiesterase (Brooker et al., 1968) or

for the binding sites on a specific antibody (Steiner et al., 1969) have been designed. The stimulation of protein kinase activity has been used as the basis of a rapid determination of cyclic AMP levels; protein kinases transfer the labelled terminal phosphate of ATP to a variety of substrates (e.g. protamine, histone, casein and phosphorylase kinase) thus forming a labelled phosphoprotein which can be isolated on filter paper discs (Castagna, 1970; Kuo and Greengard, 1970; Wastila et al., 1970; Butcher, 1971).

It is now becoming evident that the protein kinases which are sensitive to stimulation by cyclic AMP are composed of a regulatory or receptor subunit and of a catalytic subunit (Reimann et al., 1971; Gill and Garren, 1970; Gill and Garren, 1971; Walton et al., 1971; Gill and Garren, 1969) which can be separated by chromatographic means (Gill and Garren, 1971; Reimann et al., 1971). In activating the kinase, cyclic AMP binds to the receptor subunit and causes it to dissociate from the catalytic subunit which is then no longer inhibited and is able to phosphorylate the protein substrate of the reaction. The binding of cyclic AMP to protein kinases has been used to measure the levels of tissue cyclic AMP (Gill and Garren, 1969; Gilman, 1970). Cyclic AMP in trichloroacetic extracts of tissues competes with tritiated cyclic AMP for binding to the partially purified protein kinases from skeletal muscle (Gilman, 1970) or adrenal cortex (Gill and Garren, 1969). The rapid separation of free cyclic AMP from that bound to the protein kinase is accomplished by passing the reaction mixture through a membrane filter with a

demonstrated affinity for the protein kinase-cyclic AMP complex (Kuwano and Schlessinger, 1970; Gilman, 1970; Gill and Garren, 1969). This method is simple, rapid and does not require purification of cyclic AMP prior to the assay.

All of these methods of determination of cyclic AMP levels plus others as well, have been discussed by Breckenridge (1971).

Section II: Experimental: Cyclic AMP levels in tissues of warm and cold acclimated rats

A) Material and methods

1) *preparation of tissues*

Rats were anesthetized with ether at the temperature of acclimation and were dissected rapidly. Pieces of brown adipose tissue, skeletal muscle, liver and white adipose tissue were frozen within five seconds of removal from the animal by clamping them with a reduced-size version of Wollenberger tongs precooled in liquid nitrogen*. Then the pieces of tissue were rapidly wrapped in aluminium foil and kept in liquid nitrogen until further used (not more than two hours). The Wollenberger tongs are made of aluminium plates between which tissues can be frozen very rapidly, the tongs being precooled in liquid nitrogen before use. This procedure increases the surface area of the tissue at the instant of freezing allowing faster cooling of the tissue (Wollenberger et al., 1960). It is important to freeze samples of animal tissues as quickly as possible because the tissue levels of many metabolic intermediates change rapidly either post-mortem or during the anoxic interval upon removal of the tissue from the animal (Williamson and Corkey, 1969); it was observed that the levels of cyclic AMP vary rapidly after the removal

---

* It was found that clamping the tissues within five seconds of their removal from the rats gave values for cyclic AMP levels identical to those obtained when the tissues were clamped in situ; clamping the tissues after their removal from the animals is much easier than clamping them in situ.

of the tissue from the animal (Ebadi et al., 1971).

2) extraction of cyclic AMP from tissues

The frozen tissues were powdered in a metallic mortar with a metallic pestle (obtained from Canlab), precooled in liquid nitrogen. Then the powdered tissues were weighed rapidly (amount used usually between 200 and 500 mg of tissue) and dropped in cold 0.5N perchloric acid. They were then homogenized in a glass homogenizer in 0.5N perchloric acid and brought to a final concentration of approximately 100 mg/ml. The samples were centrifuged at 8,000*g* for ten minutes at 0°C and the supernatants were neutralized with 0.5N KOH, added with a microsyringe using thymol blue as a pH indicator; this operation precipitated the perchloric acid as potassium perchlorate. After ten minutes on ice, the samples were centrifuged for ten minutes at 8,000*g* and aliquots of the supernatants were lyophilized and then dissolved in 0.05M Tris-HCl buffer, pH 7.4. The amount of cyclic AMP present in the sample could then be determined immediately or the samples could be frozen at -20°C. The amount was unchanged after two weeks of storage at -20°C.

3) preparation of plasma and urine samples

After the tissues were removed, the hepatic vein and the inferior vena cava were cut and the blood was collected with a heparinized Pasteur pipette and transferred to a heparinized centrifuge tube. The blood was mixed and centrifuged immediately at 8,000*g* for ten minutes at 0°C. One volume of 0.5N perchloric acid was added to one volume of plasma after centrifugation.

The samples were mixed and, after 30 minutes on ice, centrifuged at 8,000*g* for ten minutes at 0°C. The protein-free extracts could then be stored at 4°C for at least three months without loss of cyclic AMP (Broadus et al., 1970b). Before the determination of cyclic AMP, samples were neutralized with 0.5N KOH as described earlier on, lyophilized, dissolved in phosphate buffer and assayed immediately.

Urine was collected on ice for two consecutive days, the bottles being changed after a 24 hours period. The rats were kept without restraint, with food and water, in metabolic cages. These cages had a screening device which stopped the feces and allowed the urine to flow through a metallic funnel attached tightly to the bottle to avoid evaporation. The urine samples were treated with equal volumes of 0.5N perchloric acid, mixed, placed on ice for 30 minutes, centrifuged and the supernatants were stored at -20°C for several months without loss of cyclic AMP (Broadus et al., 1970a). Upon thawing they were treated in the same way as the plasma samples.

4) assay of cyclic AMP levels

Cyclic AMP levels are determined essentially as described by Walton and Garren (1970) with some modifications introduced to insure optimal experimental conditions. The assay is based upon the isotopic dilution of tritiated cyclic AMP by the cold cyclic AMP which is being measured and upon the subsequent binding of the cyclic nucleotide to the receptor protein. The free cyclic AMP is separated from the bound material by passing the mixture

through a membrane filter which has demonstrated an affinity for the protein-cyclic AMP complex (Kuwano and Schlessinger, 1970; Walton and Garren, 1970; Gilman, 1970). This assay is very sensitive and can detect as little as 0.1 picomole in the conditions used.

Some essential criteria must be met in setting up the method, in order to achieve accuracy. A review has been written on the theoretical aspects of saturation analysis (which is defined as a general analytical method relying on progressive saturation by the test compound of a specific reagent) (Ekins and Newman, 1970). The binding of the protein kinase by cyclic AMP is only one of the many examples of the so-called methods of saturation analysis. The most important point in the use of these methods is to saturate the binding material with the test compound. The total amount of cyclic AMP which binds to the protein kinase must be equal to the amount of labelled material present in the incubation medium, or in other words, the protein kinase must be saturated with tritiated cyclic AMP so that the addition of cold cyclic AMP will not cause further binding of the labelled material but a proportional dilution of the bound radioactivity. The protein kinase should bind less than 30% of the cyclic AMP present in order to achieve good saturation (Gilman, 1970).

The method involves purification of the binding protein kinase and determination of the optimal binding conditions. The protein kinase from beef adrenal is purified according to the method of Walton and Garren (1970). Fresh beef adrenal glands were collected at the local slaughter house and put immediately

in sucrose 0.25M. All the purification procedure was done on ice or in the cold room at 4°C. The adrenal glands were stripped of medulla and the cortex was homogenized in 2 volumes of medium containing 0.05M Tris-HCl pH 7.4, 0.25M sucrose, 0.05M KCl and 0.001M $MgCl_2$. The homogenate was first centrifuged at 500*g* for 15 minutes and the supernatant was recentrifuged at 12,000*g* for 10 minutes. The second supernatant was centrifuged at 105,000*g* for 90 minutes. The concentration of protein of the post-microsomal supernatant was determined by ultraviolet absorption and the fraction was diluted to reach a final concentration of 10 mg/ml. Then 0.32 mg/ml of ammonium sulfate was added slowly to the preparation, with constant mixing. The solution was neutralized with dilute NaOH to pH 7.4. After one hour on ice with constant mixing, the solution was centrifuged at 12,000*g* for 10 minutes and the precipitate was redissolved in 0.05M Tris-HCl pH 7.4 containing 6mM mercaptoethanol and dialyzed against 0.01M Tris-HCl, pH 7.4 containing also 6mM mercaptoethanol. The dialysed preparation which contained 8.6 mg/ml of protein was divided into 1 ml portions and frozen at -20°C. The activity of the protein kinase preparation was the same even after 6 months of storage. For use in the incubation medium, one portion of the protein mixture was thawed and diluted in phosphate buffer 0.225M used immediately, and never refrozen after use.

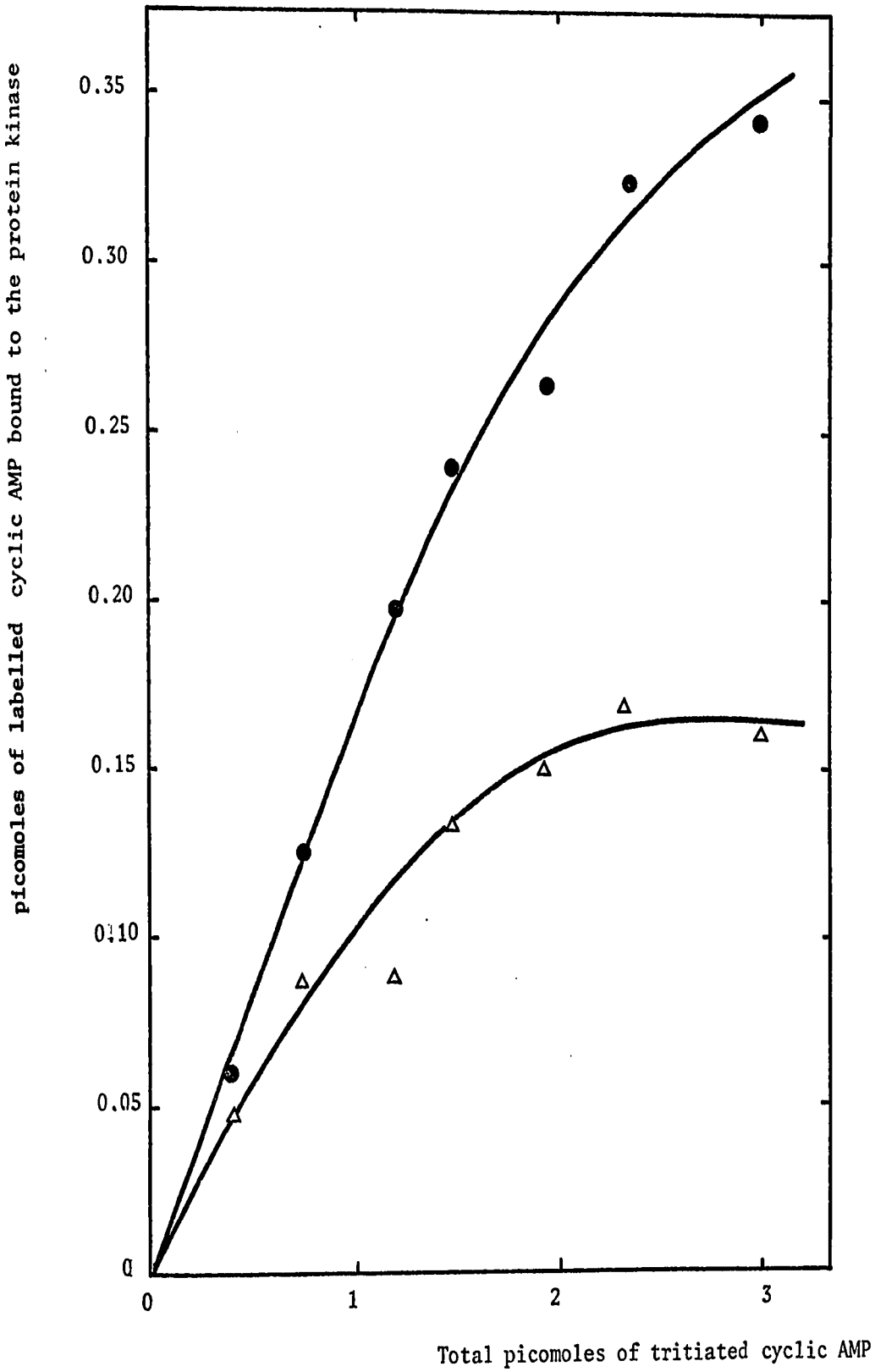
The binding capacity of the protein kinase is increased by decreasing the volume of the incubation medium. The latter usually contained in a final volume of 0.15 ml, 0.05 ml of tritiated cyclic AMP, 0.05 ml of cold (standard or unknown) cyclic

AMP diluted in Tris buffer pH 7.4 (0.05M) and 0.05 ml of the protein kinase preparation which was usually a 2.5-fold dilution in phosphate buffer, 0.225M of the frozen enzymatic preparation. The tritiated cyclic AMP was purchased at a specific activity of 24.1 Curies/mmole and was diluted with water to a final concentration of about 2.6 picomoles and 136,000 dpm (determined with accuracy for every new dilution of the radioactive material), in the final incubation medium. The reaction was initiated by the addition of the protein component and was carried on at $0^{o}C$ for 100-120 minutes. Then, 1.0 ml of Tris buffer, 0.025M pH 7.4, containing 10mM $MgCl_2$, was added to the incubation mixture and after five minutes, the mixture was transferred to the upper part of a Millipore filter apparatus containing 5 ml of the same buffer. A mild vacuum was applied and the liquid passed through the membrane filter which had been previously washed in the same buffer. Then, 10 ml of the buffer was passed through the system to rinse the filter. The membrane filter was then dried under an infra-red lamp for five minutes and then placed in 10 ml of modified Bray solution (5g of PPO and 100g of naphthalene brought to a volume of one liter with p-dioxane) and counted two hours or more later, in a Beckman LS-250 using channels ratio to correct for the quench due to the paper. The use of this mode of counting has been recommended for samples containing paper or gel to insure better accuracy.

Saturation of the protein kinase by labelled cyclic AMP is illustrated in Figure 36, A 7.5% binding was obtained with 85 µg of protein and 2.0 picomoles of cyclic AMP while a 15% binding was obtained with 170 µg of protein and 2.5 picomoles of

Figure 36: Binding of cyclic AMP by beef adrenal protein kinase; capacity of binding at two different substrate concentrations.

The incubation medium contained 0.05 ml of tritiated cyclic AMP (between 0.4 and 3.0 picomoles), 0.1 ml of the protein preparation (5-fold or 10-fold dilution in Tris buffer of the frozen enzymatic preparation) containing respectively 170 µg (●——●) and 85 µg (Δ——Δ) of protein, and 0.15 ml of Tris buffer, pH 7.4. The reaction was initiated by the addition of the protein mixture and was carried on at $0^{o}C$ for 100 minutes. There was no cold cyclic AMP in the incubation medium in order to determine the amount of labelled cyclic AMP necessary to saturate the protein kinase.


picomoles of labelled cyclic AMP bound to the protein kinase
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0
1
2
3
Total picomoles of tritiated cyclic AMP

cyclic AMP. Reducing the incubation volume from 0.3 ml to 0.15 ml increased the binding to 21% with 170 µg of protein and 2.5 picomoles of cyclic AMP; those conditions were used in most assays.

The binding of cyclic AMP to the protein kinase increases with the time of incubation at $0^{o}C$ and is maximum after 100 minutes (Figure 37A). Mg^{++} increases the binding of cyclic AMP to the protein kinase but the binding seems more stable in the absence of Mg^{++} (Figure 37 A, comparing o——o to •——•). Mg^{++} was then omitted from the incubation medium to insure a more stable binding, but it was included in the washing buffer because it increased the affinity of the bound complex for the membrane filter (Walton and Garren, 1970).

If the protein kinase preparation after thawing was diluted in Tris buffer, its binding capacity decreased by 28% over a period of two hours but if the protein preparation was diluted in phosphate buffer, its binding capacity was more stable and decreased only by 7% over a period of two hours (Figure 37 B). Consequently, the protein kinase preparation was thawed and diluted in phosphate buffer not more than one hour before use, a period during which the binding capacity of the protein kinase in phosphate buffer was almost constant (Figure 37 B).

The protein kinase is saturated with an amount of cyclic AMP equivalent to the amount of labelled material present in the medium so that addition of cold cyclic AMP dilutes the radioactive nucleotide and gives a proportional decrease in the radioactivity of the bound complex. Using standards containing up to 20 picomoles of cyclic AMP and plotting on a full logarithmic

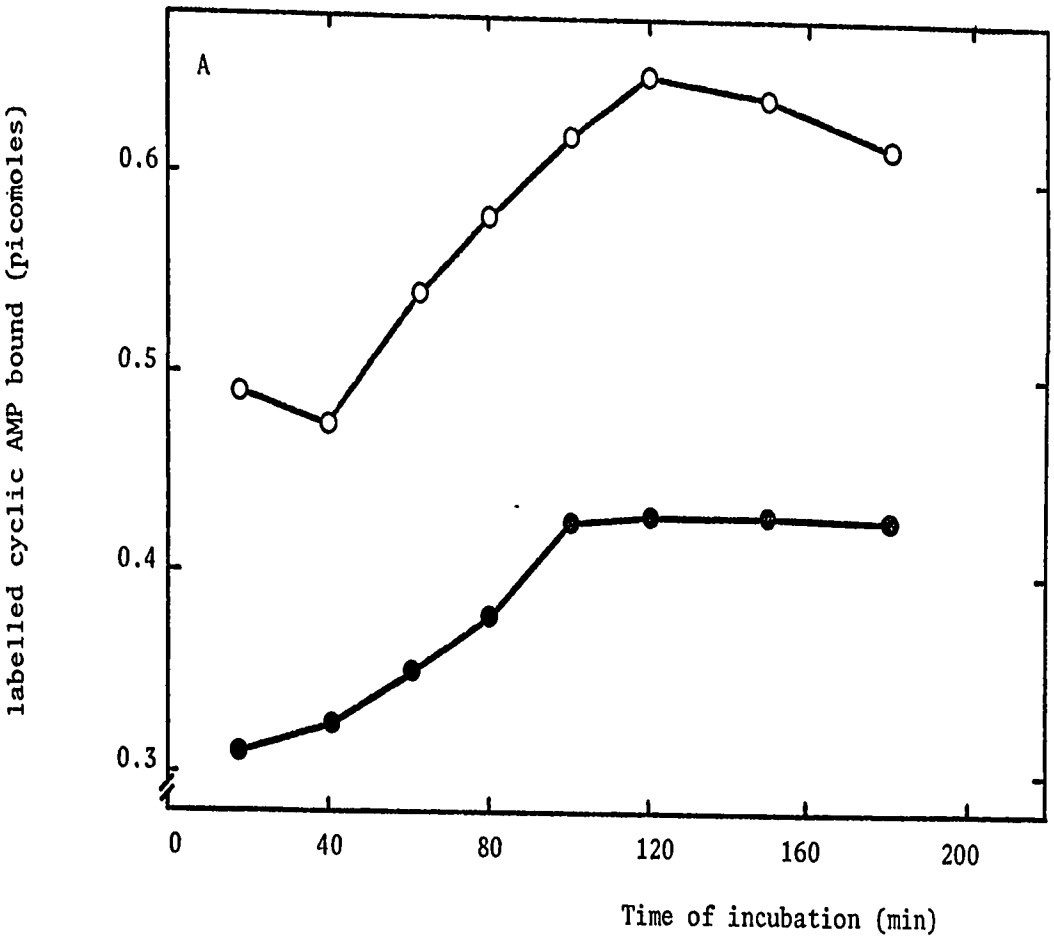
Figure 37 A: Binding of cyclic AMP to the protein kinase in function of the time of incubation.

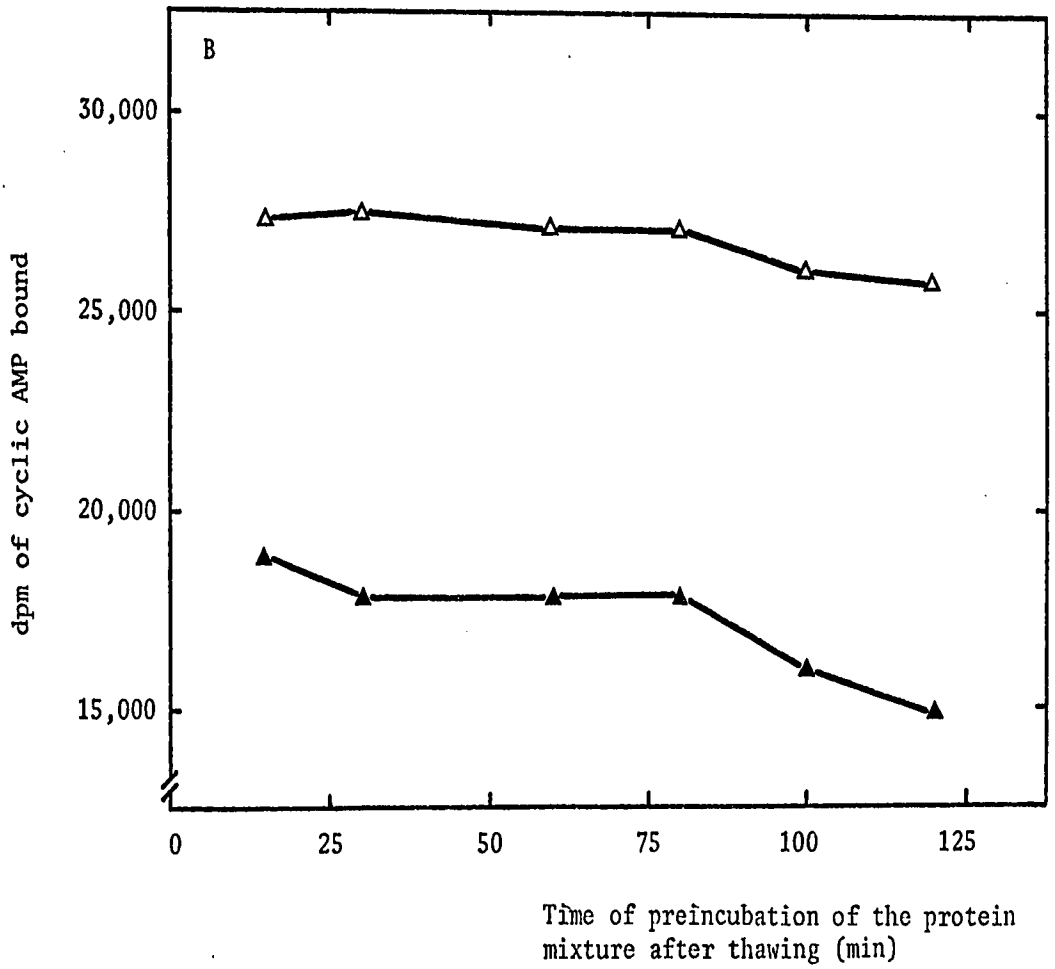
The incubation medium contained 0.05 ml of tritiated cyclic AMP (2.6 picomoles and 136,000 dpm), 0.05 ml of protein kinase (2.5 fold dilution of the frozen enzymatic preparation) containing 170 µg of protein and 0.05 ml of Tris buffer containing 8mM theophylline. The buffer contained either no Mg^{++} (•——•) or 11mM Mg^{++} (o——o). The medium contained no cold cyclic AMP in order to determine the maximum binding of the labelled material under different conditions.

Figure 37 B: Stability of the binding capacity of the protein kinase in phosphate buffer and in Tris-HCl buffer.

The incubation medium contained 0.05 ml of tritiated cyclic AMP (2.6 picomoles, 136,000 dpm), 0.05 ml of the protein kinase preparation (2.5 fold dilution of the frozen material) containing 170 µg of protein diluted in 0.05M Tris buffer containing 8mM theophylline (▲——▲) or in 0.225M phosphate buffer (Δ——Δ) both at pH 7.4, and 0.05 ml of Tris buffer, 0.05M.

The time of preincubation in buffer of the thawed protein kinase preparation prior to the assay, is that illustrated in the figure; the incubation time, during the assay was 100 minutes for all samples.


A
labelled cyclic AMP bound (picomoles)
0.6
0.5
0.4
0.3
0
40
80
120
160
200
Time of incubation (min)


B
dpm of cyclic AMP bound
30,000
25,000
20,000
15,000
0
25
50
75
100
125
Time of preincubation of the protein mixture after thawing (min)

scale the amount of radioactivity of the complex versus the total amount (labelled + cold) of cyclic AMP present, a linear relationship is obtained (Figure 38). There is no non-specific absorption of the free nucleotide to the membrane filter since in the absence of protein the radioactivity retained on the paper is not higher than the background. The protein kinase is quite specific for cyclic AMP; other nucleotides especially cyclic ones can interfere with the assay but at concentrations much higher than those found in mammalian tissues (Walton and Garren, 1970; Gilman, 1970).

5) chemicals

H^3-cyclic adenosine 3',5'-monophosphate was obtained from New England Nuclear. Cellulose ester membrane filters (HA 0.45 microns, 24 mm) were obtained from Millipore Corporation. Cyclic AMP, Trizma-HCl and Trizma-base were obtained from Sigma. Perchloric acid was obtained from Allied Chemical. Sodium pentobarbitone was obtained from British Drug House. Ether was a Baker Analyzed Reagent from Fisher Company.

B) <u>Effects of cold acclimation on cyclic AMP levels in tissues, plasma and urine of rats</u>

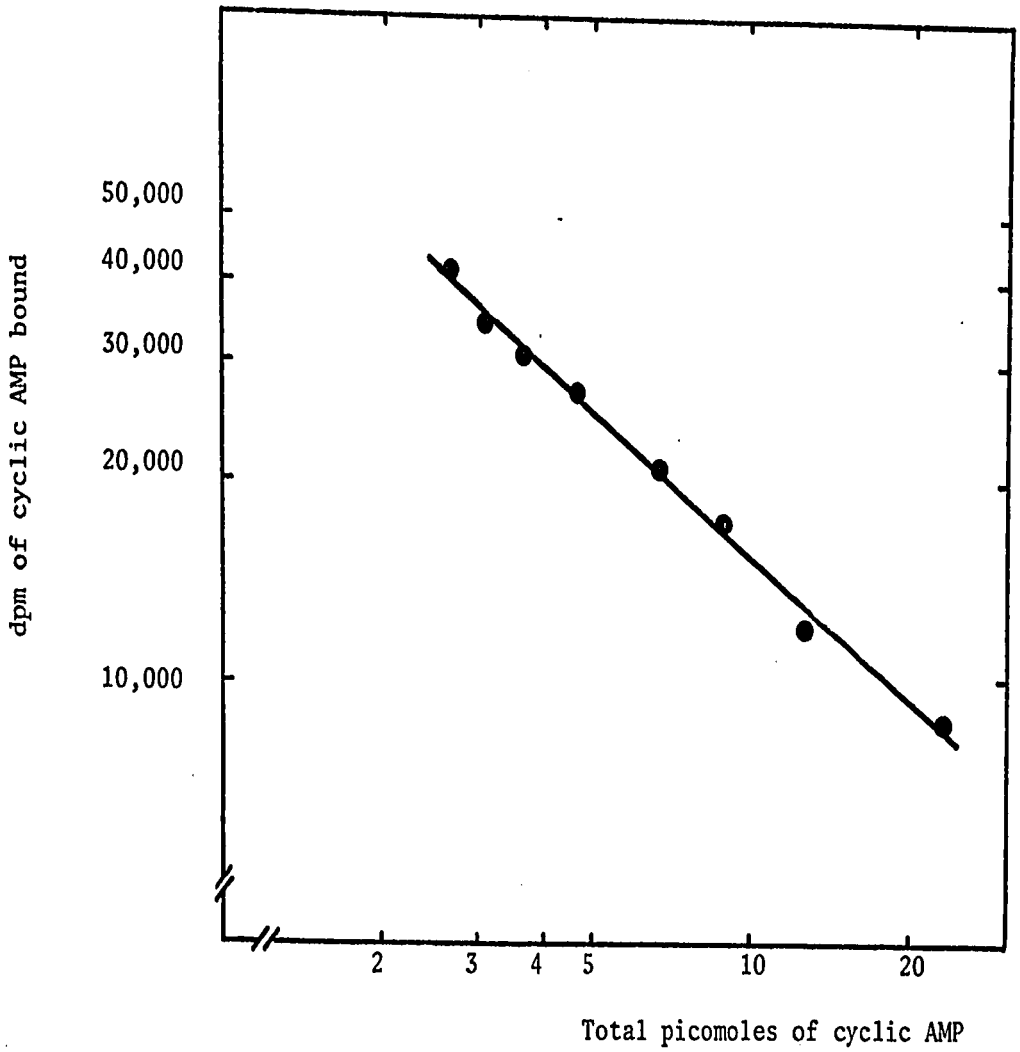
1) *tissue levels of cyclic AMP*

There is no significant difference in the levels of cyclic AMP in skeletal muscle, liver and white adipose tissue between warm acclimated rats, and cold acclimated rats living in the cold

Figure 38: Standard curve for the isotopic dilution of the labelled cyclic AMP by cold cyclic AMP.

The incubation medium contained 0.05 ml of tritiated cyclic AMP (2.66 picomoles and 136,000 dpm), 0.05 ml of Tris buffer 0.05M containing amounts of cold cyclic AMP between 0.5 and 20 picomoles, and 0.05 ml of protein kinase (2.5 fold dilution in 0.225M phosphate buffer, of the frozen material) containing 170 µg of protein.

Notice that both scales are logarithmic.


dpm of cyclic AMP bound
50,000
40,000
30,000
20,000
10,000
2
3
4
5
10
20
Total picomoles of cyclic AMP

or in the warm (Figure 39). The level of cyclic AMP in the brown adipose tissue of cold acclimated rats living in the cold or transferred to room temperature for one day, is significantly higher (55% increase) than in warm acclimated rats (Figure 39).

2) plasma levels of cyclic AMP

There is significantly more cyclic AMP in the plasma of cold acclimated rats (39% increase) than in the plasma of warm acclimated rats (Figure 40). The cold acclimated rats maintain this higher level of plasma cyclic AMP even when they are returned to room temperature for one day (Figure 40).

3) excretion of cyclic AMP

There is significantly more cyclic AMP excreted in cold acclimated rats than in warm acclimated rats (Table 5). The volume of urine excreted per day is the same in the cold and the warm acclimated rats so that the amount of cyclic AMP per ml of urine is also higher in cold than in warm acclimated rats (Table 5).

C) Discussion

The values obtained for the tissue levels of cyclic AMP correspond well to those reported in the literature (compare values of figure 39 with values of table 4). The concentration of cyclic AMP measured in the plasma of warm acclimated rats is 38 picomoles/ml while that reported by Broadus and coworkers (1971) is 30 picomoles/ml. The urinary excretion of cyclic AMP in warm

Figure 39: Effects of cold acclimation on the levels of cyclic AMP in brown adipose tissue, skeletal muscle, liver and white adipose tissue.

The white bars represent the warm acclimated rats, the hatched bars represent the cold acclimated rats in the cold and the black bars represent the cold acclimated rats after one day at room temperature.

The number of rats is as follows: four rats in the warm acclimated groups except for the white adipose tissue in which there are only three rats; five rats in the cold acclimated group in the cold; and three rats in the cold acclimated group at room temperature.

The values are expressed as means ± standard errors.

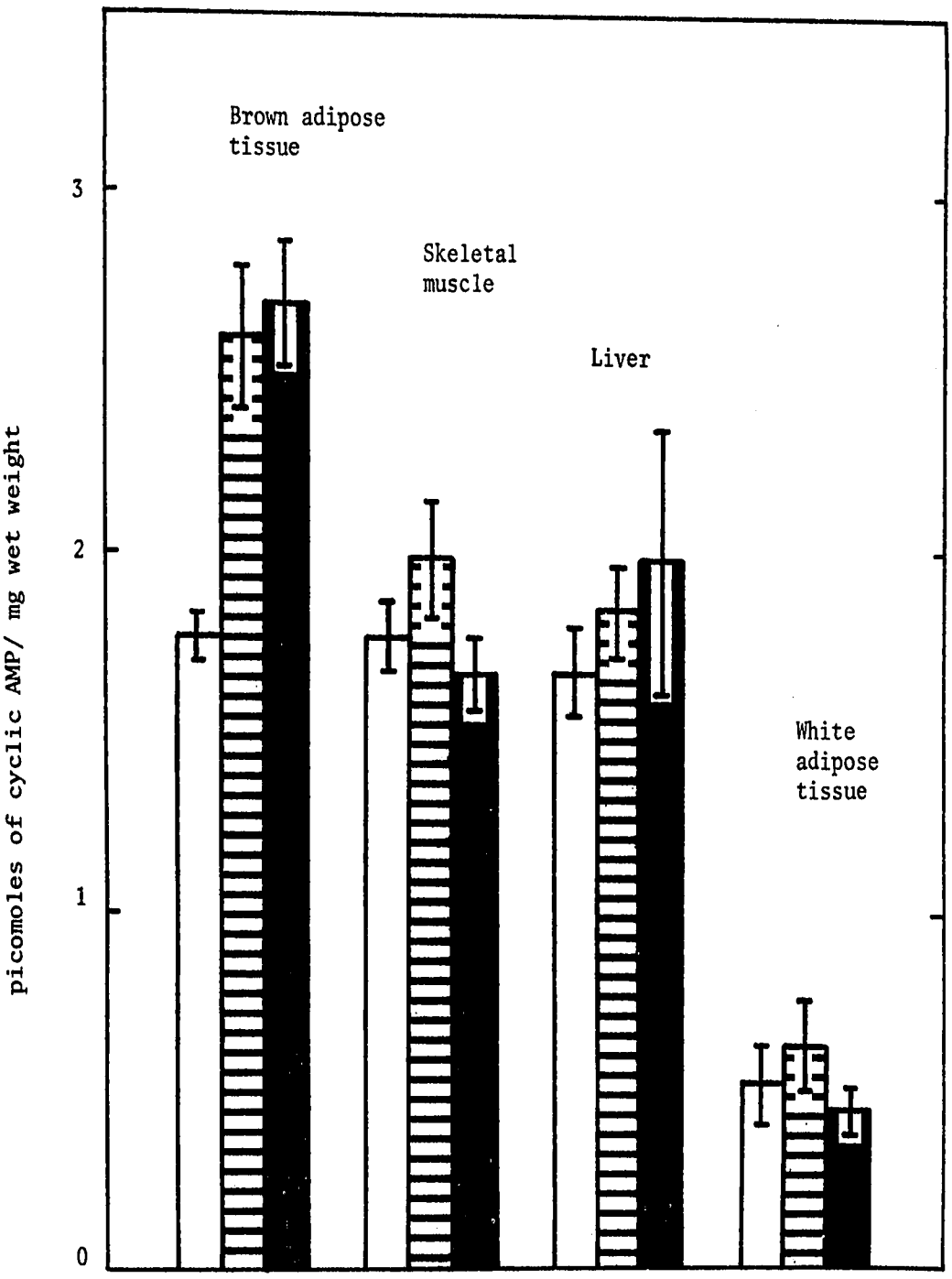

Brown adipose tissue
Skeletal muscle
Liver
White adipose tissue
picomoles of cyclic AMP/ mg wet weight
0
1
2
3

Figure 40: Effects of cold acclimation on plasma levels of cyclic AMP

The white bar represents the warm acclimated rats, the hatched bar represents the cold acclimated rats in the cold and the black bar represents the cold acclimated rats at room temperature. The number of rats is the following: six rats in the warm acclimated group, seven rats in the cold acclimated group in the cold and three rats in the cold acclimated group after one day at room temperature.

Values are expressed as means ± standard errors.

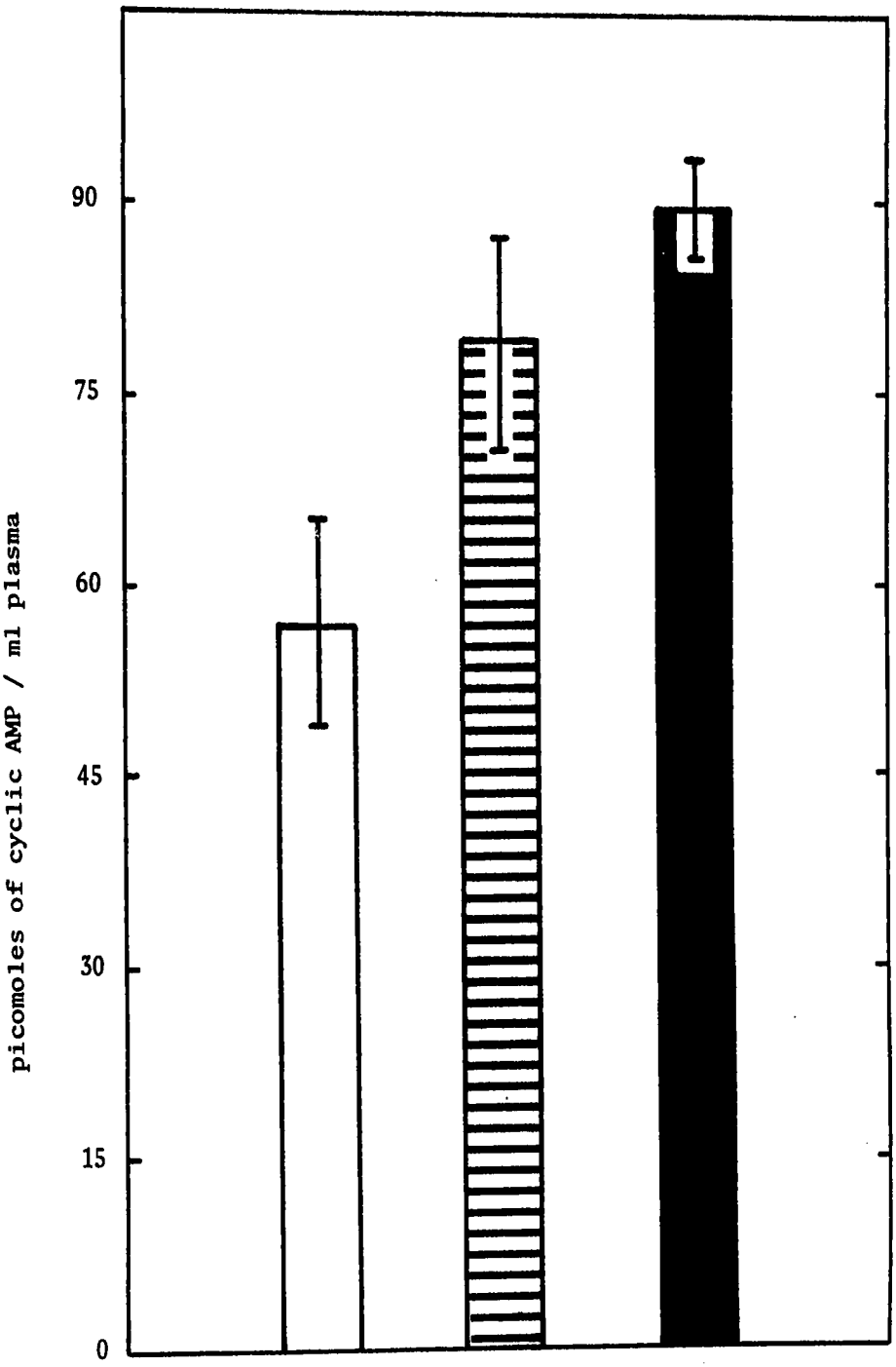

picomoles of cyclic AMP / ml plasma
90
75
60
45
30
15
0

Table 5

Excretion of cyclic AMP

	Warm acclimated rats at room temperature	Cold acclimated rats in the cold
Number of rats	11	12
Body weight (g)	376 ± 7	303 ± 3
Surface area (cm^2)	387 ± 5	343 ± 3
Volume of urine excreted per day (ml)	16.8 ± 1.4	17.1 ± 1.1
Nanomoles of cyclic AMP excreted per day	72.8 ± 5.1	88.9 ± 6.6 *
Nanomoles of cyclic AMP excreted/day/100cm^2	18.7 ± 1.2	25.8 ± 1.8 **
Nanomoles of cyclic AMP excreted/ml of urine	4.3 ± 0.4	5.2 ± 0.2

* significantly different at $p < 0.1$

** significantly different at $p < 0.01$

acclimated rats is 20 nanomoles/day/100g of body weight (calculated from Table 5) compared to reported values ranging from 12 to 115 nanomoles/day/100g (Broadus et al., 1971).

There is an increase in the level of cyclic AMP in brown adipose tissue with cold acclimation, from 1.7 picomoles/mg of tissue to 2.6 picomoles/mg of tissue. It is possible to estimate the increase in cyclic AMP in the total brown adipose tissue as follows. Taking values for the weight of the interscapular brown adipose tissue in warm and cold acclimated rats from table 1, the total amount of cyclic AMP in the interscapular brown adipose tissue is 738 picomoles in the warm acclimated rats and 2592 picomoles in cold acclimated rats. Assuming that the weight of the total brown adipose tissue in the rat is twice that of the interscapular brown adipose tissue, the increase in brown adipose tissue cyclic AMP with cold acclimation would be 3,708 picomoles which represents a 250% increase.

The increase in plasma cyclic AMP can be compared with the increase in urinary excretion of the cyclic nucleotide in order to see if the increase in the excretion of cyclic AMP is due entirely to the increase in production of the compound, i.e., the plasma level of the compound. The total plasma volume of a rat has been estimated to 40.4 ml/kg of body weight (Altman and Dittmer, 1964) and the glomerular filtration rate has been estimated to about 6ml/min/kg of body weight (Altman, 1961) so that it takes 6.73 minutes to filter the total plasma of the rat and the given plasma is filtered 214 times a day. The amount of cyclic AMP present in the total plasma of the warm acclimated

rats is equal to 563 picomoles and in the cold acclimated rats, is equal to 649 picomoles (Table 6). If all the cyclic AMP present in the plasma is eliminated in the urine, the total amount of cyclic AMP produced per day would be 120 nanomoles/day in the warm acclimated rats and 139 nanomoles/day in the cold acclimated rats (Table 6). These values, considering the assumptions made in the calculations, are of the same order of magnitude as the values measured for the excretion of cyclic AMP, 73 nanomoles/day in the warm acclimated rats and 89 nanomoles/day in the cold acclimated rats (Tables 5 and 6). The calculated increase in production rate of cyclic AMP with cold acclimation (19 nanomoles/day) corresponds well with the increase in urinary excretion observed experimentally (16 nanomoles/day).

The increased cyclic AMP levels in the brown adipose tissue and the plasma (and also the urinary excretion) of cold acclimated rats do not seem to be associated with nonshivering thermogenesis since the levels of cyclic AMP in brown adipose tissue and plasma do not return to the values obtained with warm acclimated rats when cold acclimated rats are returned to room temperature. Nonshivering thermogenesis is known to be switched off when cold acclimated rats are taken out of the cold. The increased cyclic AMP levels in the brown adipose tissue and the plasma appear to be associated with the state of adaptation rather than with the operation of the nonshivering thermogenesis process itself.

It would be possible for the increased concentration of cyclic AMP in the blood and the increased urinary excretion of cyclic AMP to be entirely due to the leakage of cyclic AMP from

Table 6

Cyclic AMP in cold acclimation

Parameters	Values for warm acclimated rats at room temperature	Values for cold acclimated rats in the cold	Increase
Total excretion of cyclic AMP nanomoles/day	73	89	16
Plasma clearance of cyclic AMP (calculated from plasma concn. and filtration rate) nanomoles/day	120	139	19
Concn. of cyclic AMP in plasma pmoles/ml	55	78	23
Total pmoles of cyclic AMP in plasma	563	649	86
Concn. of cyclic AMP in BAT pmoles/g	1700	2600	900
Total amount of cyclic AMP in total BAT picomoles	1476	5184	3708
Production rate needed for amount present in urine pmoles/min	50.6	61.8	11.2
Production rate calculated from plasma levels observed pmoles/min	83.3	96.5	13.2

the brown adipose tissue since the increase in the total brown adipose content of cyclic AMP with cold acclimation is 3708 picomoles while the increase in the excretion rate of the cyclic nucleotide is 11.2 picomoles/min and the increase in the production rate of the compound is 13.2 picomoles/min (Table 6). Other tissues cannot however be excluded as sources of the increase in the plasma and the urinary cyclic AMP, during cold acclimation.

Section III: Experimental: Effects of noradrenaline infusion on cyclic AMP levels in cold and warm acclimated rats..

A) Material and methods

1) *animals*

Warm or cold acclimated rats were brought to the laboratory less than two hours before the beginning of the experiment. They were anesthetized with sodium pentobarbitone (2.8 mg/100cm^2 body surface administered intraperitoneally) and a polyethylene cannula was placed in a tail vein. Then 0.9% NaCl or noradrenaline (l-arterenol monohydrate, 20 mg/ml in 0.01N HCl was stored at 0^{o}C for up to five days and was diluted in saline immediately before the experiment) was infused at a rate of 0.5 µg/100cm^2 of body surface/min for 30 minutes exactly. Then the rats were rapidly dissected under ether anaesthesia and the tissues were removed as described in section II, A, 1 of this chapter.

2) *extraction of cyclic AMP from tissues*

see chapter 4, section II, A, 2.

3) *preparation of plasma samples*

see chapter 4, section II, A, 3.

4) *determination of cyclic AMP levels*

see chapter 4, section II, A, 4.

5) *chemicals*

see chapter 4, section II, A, 5.

B) Effects of noradrenaline infusion on cyclic AMP levels in tissues of warm and cold acclimated rats

Noradrenaline infusion did not increase the levels of cyclic AMP in the brown adipose tissue and the white adipose tissue of either warm or cold acclimated rats (Figure 41). Noradrenaline infusion increased the levels of cyclic AMP in the leg muscle of the warm ($p < 0.02$) and the cold acclimated rats ($p < 0.2$) (Figure 42). Noradrenaline infusion increased the level of cyclic AMP in liver of warm acclimated rats ($p < 0.02$) but not in the liver of cold acclimated rats (Figure 42). Noradrenaline infusion increased the level of cyclic AMP in the plasma of warm acclimated rats ($p < 0.1$); the level of cyclic AMP in the plasma of cold acclimated rats was increased slightly by noradrenaline infusion ($p < 0.3$) but it must be remembered that the level of cyclic AMP in the plasma of cold acclimated rats was already higher than in warm acclimated rats (Figure 43).

C) Discussion

One question asked in the introduction was whether noradrenaline had any greater effect on cyclic AMP levels in cold acclimated rats than it did in warm acclimated rats. The answer is no: noradrenaline infusion increases the cyclic AMP levels in skeletal muscle of warm and cold acclimated rats to the same extent; it increases the level of cyclic AMP in the liver of warm acclimated rats but not in the liver of cold acclimated rats; it increases

Figure 41: Effects of noradrenaline infusion on the cyclic AMP levels of the brown and the white adipose tissues in cold and warm acclimated rats.

The white bars represent the rats receiving saline infusion and the black bars represent the rats receiving noradrenaline infusion. There are five rats in each group and the rats are the same as in figures 42 and 43.

Values are expressed as means ± standard errors.

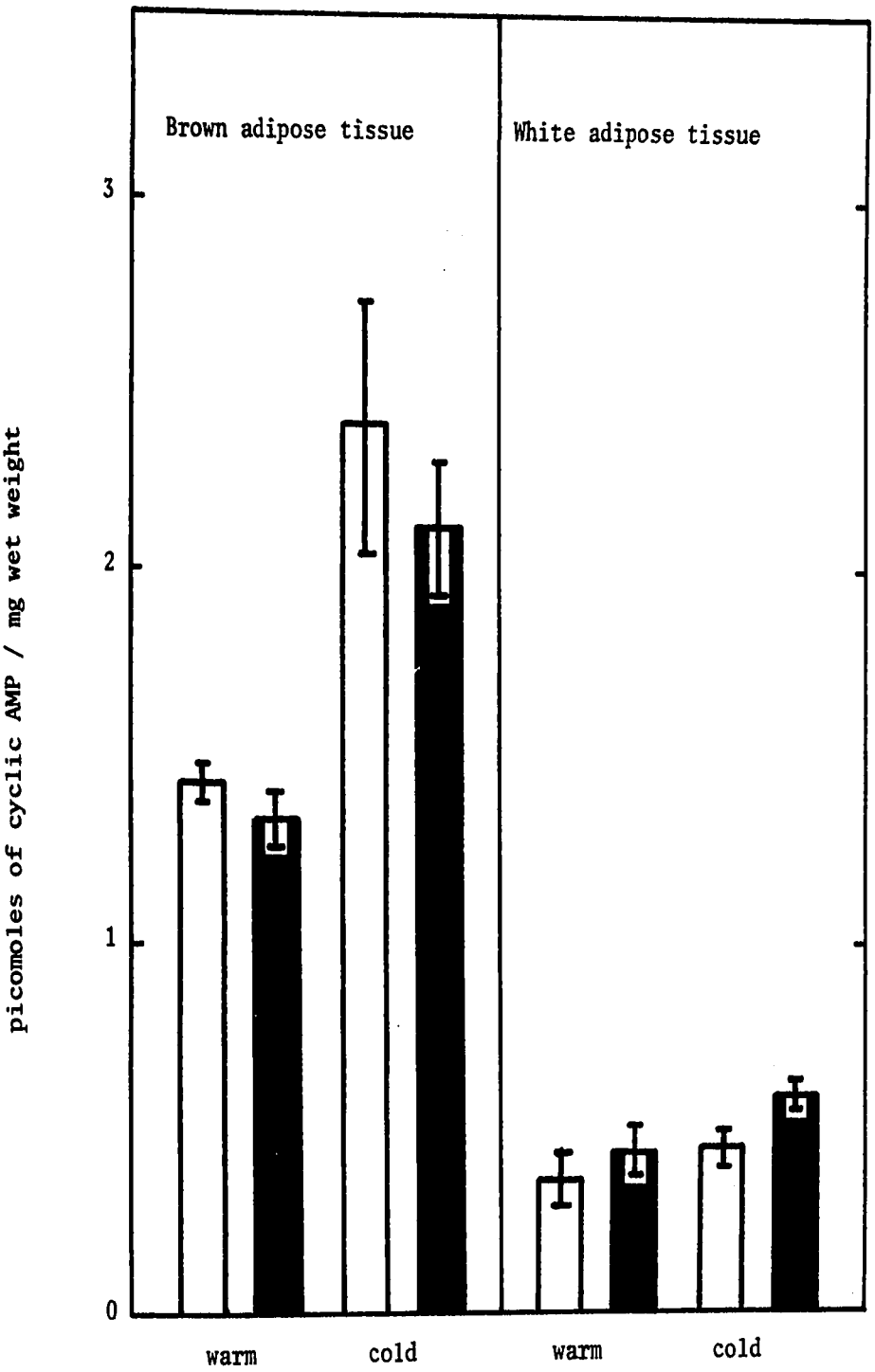

Brown adipose tissue
White adipose tissue
picomoles of cyclic AMP / mg wet weight
3
2
1
0
warm
cold
warm
cold

Figure 42: Effects of noradrenaline infusion of the cyclic AMP levels of the skeletal muscle and the liver in cold and warm acclimated rats.

The white bars represent the rats receiving saline infusion and the black bars represent the rats receiving the noradrenaline infusion. There are five rats in each group and they are the same as in figures41 and 43.

Values are expressed as means ± standard errors.

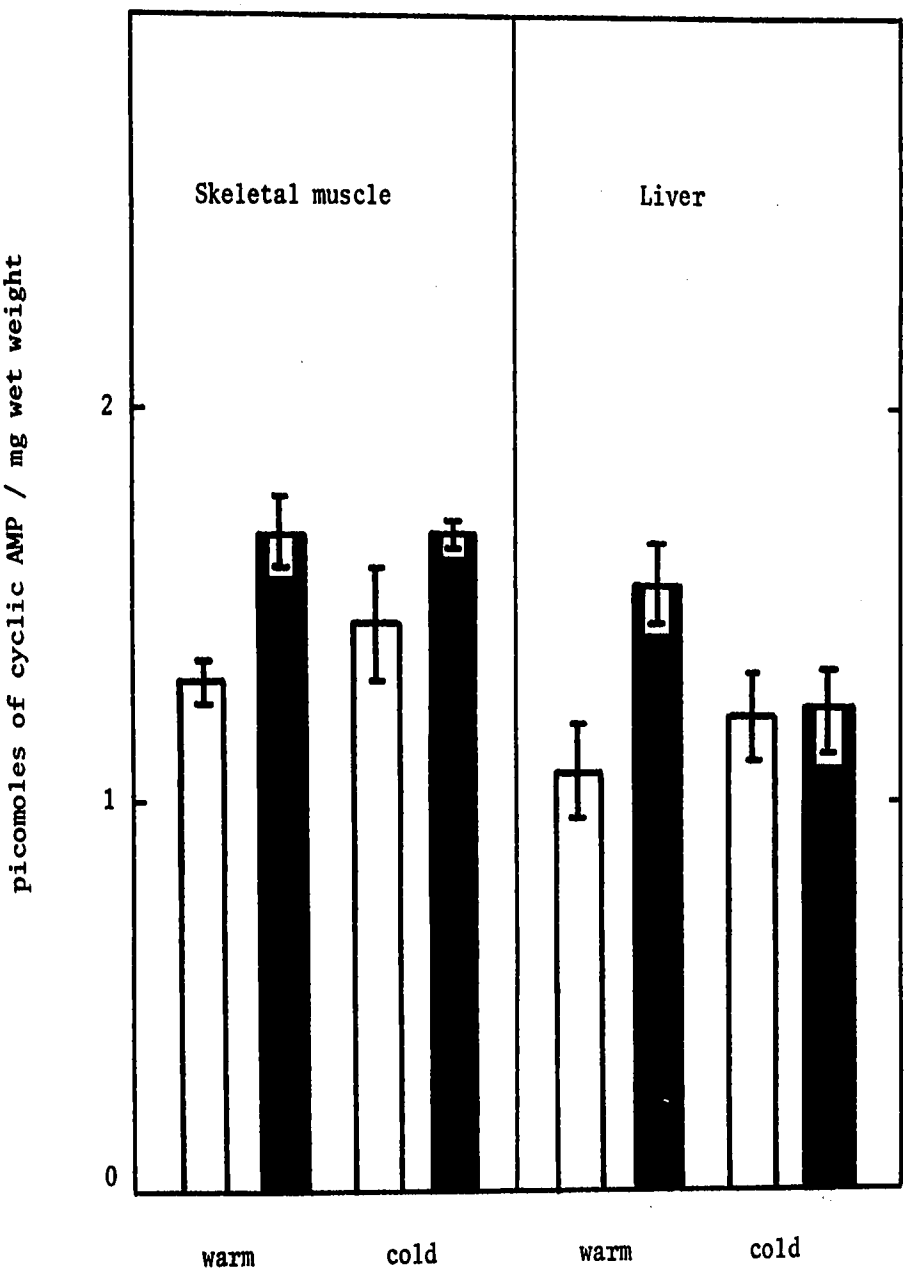
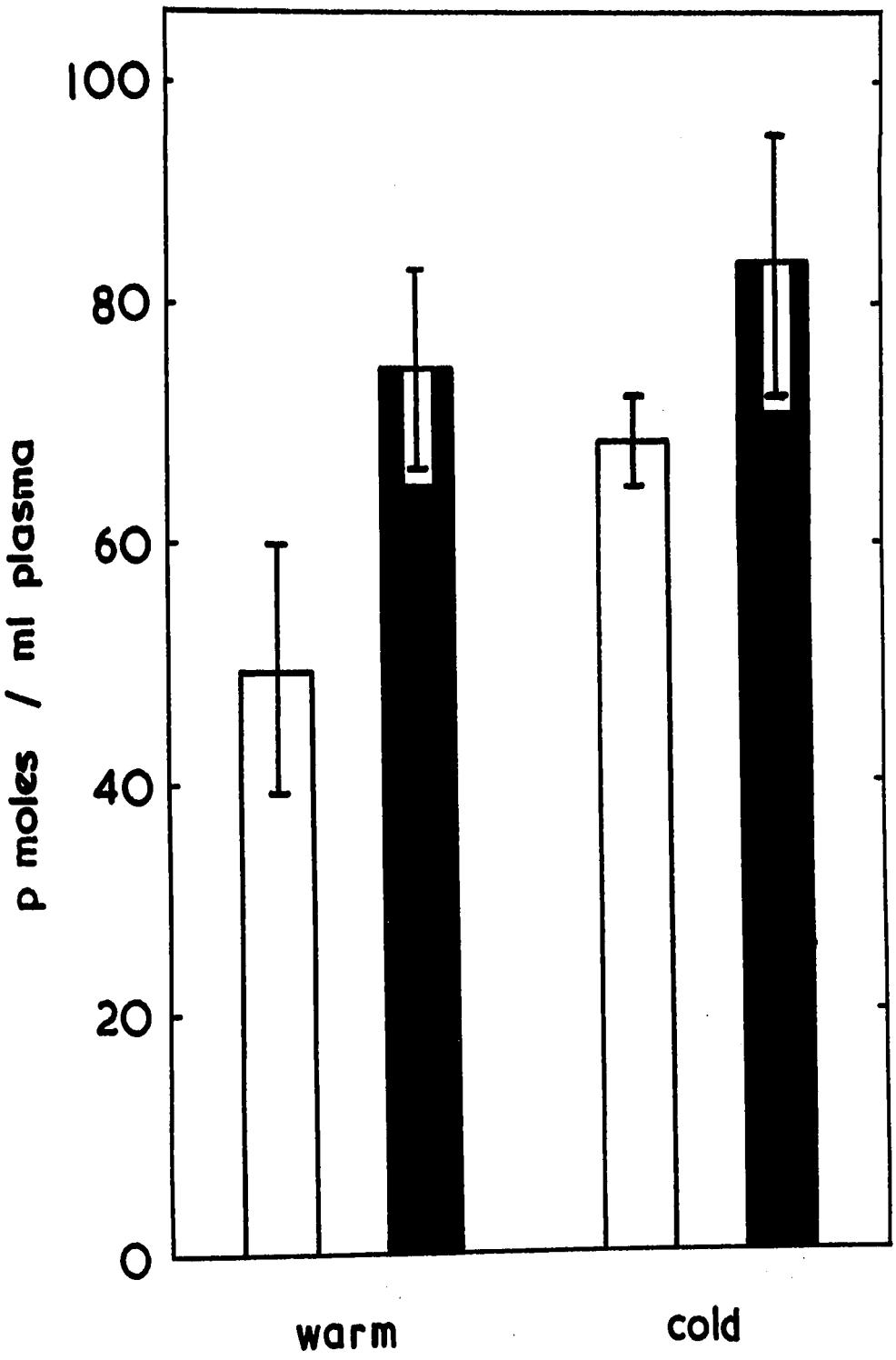

Skeletal muscle
Liver
picomoles of cyclic AMP / mg wet weight
2
1
0
warm
cold
warm
cold

Figure 43: Effects of noradrenaline infusion on the cyclic AMP levels in the plasma of cold and warm acclimated rats.

White bars represent the rats receiving an infusion of saline while the black bars represent the rats receiving an infusion of noradrenaline. There are five rats in each group and they are the same as in figures 41 and 42.

Values are expressed as means ± standard errors. All rats were taken to the laboratory less than two hours before the infusion of saline or noradrenaline.


100
80
60
40
20
0
p moles / ml plasma
warm
cold

the plasma level of cyclic AMP less in cold acclimated rats than in warm acclimated rats.

Another question asked was whether the changes seen in the cyclic AMP levels in the cold acclimated rats could be attributed to an effect of noradrenaline. The answer is no: there are differences between the effects of noradrenaline and the effects of cold acclimation on cyclic AMP levels in cold acclimated rats; the level of cyclic AMP in the brown adipose tissue is not increased by noradrenaline but is increased by cold acclimation; in muscle and liver, noradrenaline infusion increases the cyclic AMP levels but cold acclimation does not. The only points of resemblance are that both noradrenaline and cold acclimation can increase the plasma level of cyclic AMP and that no change is observed in white adipose tissue with noradrenaline or cold acclimation.

Nonshivering thermogenesis (defined as being present in cold acclimated rats exposed to cold or infused with noradrenaline but not in warm acclimated rats at room temperature, in cold acclimated rats at room temperature or in warm acclimated rats infused with noradrenaline) can be concluded to be:

i) not associated with a rise in cyclic AMP concentration in the tissues known to be sites of nonshivering thermogenesis (muscle, brown adipose tissue); a rise in cyclic AMP concentration occurs in the skeletal muscle with noradrenaline infusion but not with cold exposure of cold acclimated rats; a rise in cyclic AMP concentration also occurs in the skeletal muscle of warm acclimated rats infused with noradrenaline in which nonshivering thermogenesis does not occur.

ii) not associated with any increase in the noradrenaline-sensitive adenyl cyclase system (amount of noradrenaline-sensitive adenyl cyclase or sensitivity of the enzyme to stimulation by noradrenaline) in brown adipose tissue or muscle.

It can be concluded that the enhanced capacity for a calorigenic response to noradrenaline and the increased capacity for nonshivering thermogenesis which both occur in cold acclimated rats are <u>not</u> associated with any change in the amount, properties, or operation of the adenyl cyclase system. The adenyl cyclase system does not seem to mediate the calorigenic response to noradrenaline in these animals.

CHAPTER 5: CONCLUSION

A question asked in the introduction was whether the increase in the calorigenic response to catecholamines observed in cold acclimated rats was associated with any increase in the amount of catecholamine -sensitive adenyl cyclase or in the sensitivity of the enzyme to stimulation by catecholamines. No evidence in favour of this was found for brown adipose tissue and skeletal muscle, tissues known to be involved in the process of nonshivering thermogenesis. The catecholamine-sensitive adenyl cyclase system is not involved in the process of nonshivering thermogenesis either in the switching on and off (noradrenaline effect) or in the development of the adaptation. It can be concluded that it is unlikely that changes in the adenyl cyclase system are involved in the development of the capacity for nonshivering thermogenesis or in the operation of the process of nonshivering thermogenesis. No evidence could be obtained even for the participation of the adenyl cyclase system in the calorigenic effect of noradrenaline. If this is correct, this would be the first metabolic effect of noradrenaline which does not involve the mediation of the stimulatory effect by the adenyl cyclase system.

Another question which was asked in the introduction was whether cold acclimation was associated with changes in the adenyl cyclase system.

The transient increases in the amount of adenyl cyclase and in the sensitivity of the enzyme to stimulation by adrenaline

observed in skeletal muscle during the early stages of acclimation to cold are probably associated with shivering and not with cold acclimation.

Some changes in the adenyl cyclase system have been also observed in the brown adipose tissue: there was an increased amount of a different (not noradrenaline-stimulated) adenyl cyclase in the brown adipose tissue of cold acclimated rats; there was also an increase in the levels of cyclic AMP in the brown adipose tissue (as well as in the plasma level and in the urinary excretion of the cyclic nucleotide) of cold acclimated rats. A change that was associated with the adaptive state of cold acclimated rats, and not with the actual operation of nonshivering thermogenesis, was then observed in the brown adipose tissue. The raised concentration of cyclic AMP in the plasma and the increased urinary excretion of cyclic AMP in the urine could both be derived from the brown adipose tissue. Muscle, liver and white adipose tissue would not appear to be major sources for the increased excretion of cyclic AMP but the possible participation of other tissues cannot be excluded. The change in the brown adipose tissue is associated with the adaptive state and not with the switching on and off of nonshivering thermogenesis. The way in which the different adenyl cyclase (observed in the brown adipose tissue of cold acclimated rats) is regulated is unknown. The existence of another hormone-regulated system in the cold acclimated rat may be postulated but the nature of the hormone could not be established in these studies. The function during cold acclimation of the different adenyl cyclase in the brown adipose tissue and

the role of the enzyme and the cyclic AMP it produces in the adaptive process are not known, but it is interesting to note that a special role for the brown adipose tissue (other than heat production) in the process of cold acclimation has been postulated previously on other grounds. This special role has been suggested to be that of an endocrine organ of which the secretion is responsible for the maintenance of the adaptive state of other tissues, particularly skeletal muscle. The changes observed in the adenyl cyclase system of the brown adipose tissue in the cold acclimated rats would be in keeping with a special function for this tissue in the adaptive state, but their relationship to the postulated endocrine function is not at present apparent.

APPENDIX 1

Calculation of adenyl cyclase activity from results obtained by liquid scintillation counting.

The Automatic Quench Correction (counter Beckman LS-250) was set up in such a way that channel A would count tritium with approximately 30% efficiency and that channel B would count C^{14} with 60% efficiency. No tritium counts were present in channel B (< 0.05% of the total dpm of tritium in the vial) and the C^{14} spill over in channel A was 10%.

The following symbols and abbreviations are used in the calculations:

A = cpm present in channel A

B = cpm present in channel B

E_{CA} = % efficiency (cpm/dpm) of C^{14} in channel A

E_{CB} = % efficiency of C^{14} in channel B

E_{HA} = % efficiency of Tritium in channel A

R = % of total cyclic AMP present in the sample recovered in the vial

K_1 = dpm of C^{14} cyclic AMP added to every sample after the incubation

S.A. = specific activity of ATP in dpm/nanomole

Calculation:

$$\text{dpm tritiated cyclic AMP per vial} = \frac{A - [E_{CA} \times \frac{B}{E_{CB}}]}{E_{HA}}$$

$$R = \frac{\text{dpm } C^{14}_{B}}{K_1}$$

$$\text{dpm tritiated cyclic AMP per sample} = \frac{\text{dpm tritiated cyclic AMP per vial}}{R}$$

$$\text{adenyl cyclase activity} = \frac{\text{dpm tritiated cyclic AMP per sample}}{\text{S.A.}}$$

Adenyl cyclase activity is expressed in nanomoles of cyclic AMP produced per sample.

Blank values are subtracted from sample values and the activity of adenyl cyclase is then divided by the time of incubation and by the amount of crude enzyme present (mg tissue or mg protein) so that the final adenyl cyclase activity is expressed in nanomoles of cyclic AMP/min/mg tissue or nanomoles of cyclic AMP produced/min/mg protein.

MODEL 380 PROGRAM TITLE: Adenyl cyclase activity NO. 238

(THIS SPACE FOR INSTRUCTIONS AND NOTES)

E_{CB} stored in register H_{A1}

E_{CA} stored in register H_{B1}

E_{HA} stored in register H_{A2}

K_1 stored in register H_{B2}

S.A. stored in register F_0

No.	Cmd	Code	Comment
00	M	07	
01	1	61	
02	G_1	36	
03	62	62	
04	M	07	
05	2	62	
06	G_1	36	
07	60	60	
08	Print	special oper.	
09	Clear	76	
10	G_1	36	
11	60	60	
12	Clear	76	
13	G_1	36	
14	60	60	
15	SF	13	A
16	3	63	
17	Clear	76	
18	G_1	36	
19	60	60	
20	Clear	76	
21	G_1	36	
22	60	60	
23	enter	41	B
24	RHA	15	
25	1	61	
26	÷ =	47	
27	SF	13	
28	4	64	
29	enter	41	
30	RH_B	14	
31	1	61	
32	X =	46	
33	-	53	
34	RF	17	
35	3	63	
36	+	52	
37	enter	41	
38	RHA	15	
39	2	62	
40	÷ =	47	
41	Print	special oper.	dpm Tr/vial
42	enter	41	
43	RF	17	
44	4	64	
45	÷ =	47	
46	enter	41	
47	RH_B	14	
48	2	62	
49	X =	46	
50	Print	spec. oper.	dpm Tr/sample
51	enter	41	
52	RF	17	
53	0	60	
54	÷ =	47	
55	Print	spec. oper.	μmoles cAMP /sample
56	enter	41	
57	1	61	
58	0	60	
59	0	60	
60	0	60	
61	X =	46	
62	Print	spec. oper.	nmoles cAMP /sample
63	Cl adder R	50	
64	Cl display	76	
65	search	02	
66	1	61	
67			
68			
69			
70			
71			
72			
73			
74			
75			
76			
77			
78			
79			

(SEE OTHER SIDE FOR PROGRAM CODES)

CARD / SHEET NO.

PROGRAM CODE	CODE LISTING FOR PROGRAM CONTROL		
	CP - 1 and 371		CP - 2
	200 thru 360/370/380	362/370-2/380-2	200 thru 362
00			
01	Stop		
02	*Search		
03	*Search & Return		
04	*Return	Same as Column 1	Same as Column 1
05	*Skip if +		
06	*Continue		
07	*Mark		
10	Store Reg. 0	Store Half B	
11	Store Reg. 1	Store Half A	
12	Store Reg. 2	Add Full	Either column as determined by keyboard
13	Store Reg. 3	Store Full	
14	Recall Reg. 0	Recall Half B	
15	Recall Reg. 1	Recall Half A	
16	Recall Reg. 2	Subtract Full	
17	Recall Reg. 3	Recall Full	
20	*Skip if Overflow		Start master programmer at step 00
21	*Control		Start slave programmer No. 1 at step 00
22	*Read 1		Start slave programmer No. 2 at step 00
23	*Read 2	Same as Column 1	Start slave programmer No. 3 at step 00
24	*Write 1		Continue master programmer at next step
25	*Write 2		Continue slave programmer No. 1 at next step
26	*Store Direct		Continue slave programmer No. 2 at next step
27	*Recall Direct		Continue slave programmer No. 3 at next step
30	*Skip if 0		Go to step 00 if W is negative
31			Go to step 10 if W is negative
32	*Store Indirect		Go to step 20 if W is negative
33	*Recall Indirect	Same as Column 1	Go to step 30 if W is negative
34			Go to step 40 if W is negative
35			Go to step 50 if W is negative
36	*Group 1		Go to step 60 if W is negative
37	*Group 2		Go to step 70 if W is negative
40	Print		
41	Enter		
42	$\text{Log}_e X$		
43	e^x		
44	$\sqrt{x}$	Same as Column 1	Same as Column 1
45	X^2		
46	X =		
47	÷=		
50	Clear Right Adder		
51	Recall Right Adder		
52	+ Right Adder		
53	– Right Adder	Same as Column 1	Same as Column 1
54	Clear Left Adder		
55	Recall Left Adder		
56	+ Left Adder		
57	– Left Adder		
60	Numeral 0	Numeral 0 + Reg. 0	
61	Numeral 1	Numeral 1 + Reg. 1	
62	Numeral 2	Numeral 2 + Reg. 2	
63	Numeral 3	Numeral 3 + Reg. 3	Either column as determined by keyboard
64	Numeral 4	Numeral 4 + Reg. 4	
65	Numeral 5	Numeral 5 + Reg. 5	
66	Numeral 6	Numeral 6 + Reg. 6	
67	Numeral 7	Numeral 7 + Reg. 7	
70	Numeral 8	Numeral 8 + Reg. 8	
71	Numeral 9	Numeral 9 + Reg. 9	
72			Either column as determined by keyboard
73			
74			
75	Decimal Point	Decimal Point	
76	Clear Display	Clear Display + Reg. 10	
77	Change Sign	Change Sign + Reg. 11	

*370 & 380 Program functions only.

Printed in U.S.A.
700-0228
6-69

APPENDIX 2

Cycloheximide*, an antibiotic with very little activity against bacteria but very effective against many yeasts, was first isolated in crystalline form from Streptomyces griseus (Leach et al., 1947) and its chemical structure was determined shortly after (Kornfeld et al., 1949). It was then demonstrated (Kerridge, 1958) that cycloheximide at a minimum growth inhibitory concentration, could completely block the synthesis of proteins in cells of Saccharomyces carlsbergensis. It has since been shown that cycloheximide is a potent inhibitor of protein synthesis in other species of yeasts, in Tetrahymena pyriformis in higher plants and in mammals (see review by Sisler and Siegel, 1967).

A single intraperitoneal injection of cycloheximide (10 to 100 mg/kg of body weight) can inhibit the in vivo incorporation of C^{14}-leucine into mouse liver proteins; the effect is at a maximum two hours after the injection but can still be observed after 24 hours (Trakatellis et al., 1965). Table 7 reports some cases (with doses used and duration of the treatment) in which cycloheximide has successfully inhibited protein synthesis in mammalian systems.

The mechanism of action of cycloheximide has been studied in cell-free systems from S. pastorianus (Siegel and Sisler, 1963; Siegel and Sisler, 1964) and from rat liver (Ennis and Lubin,

---

* Cycloheximide is also designated by the following terms: 1-cycloheximide, actidione and naramycin A.

Table 7

Inhibition of protein synthesis by cycloheximide

System inhibited by cycloheximide	Number of intra-peritonial injections	dosage mg/kg body weight	observation time of the rats	Authors
Incorporation of leucine into liver protein of fasted rats	1	0.5 to 10	4 hours	Tornheim et al., 1969
Incorporation of amino acids into regenerating rat liver	3 (every 24 hours)	1.5	3 days	Coggi and Scarpelli, 1970
	7 (every 24 hours)	0.2	7 days	Fiala and Fiala, 1965
Incorporation of amino acids into lipoproteins of rat intestinal lumen	1	1	3 days	Jacobs and Largis, 1969
Incorporation of amino acids into proteins of immature female rat uterus	1	1 to 10	4 hours	Gorski and Axman, 1964
Incorporation of amino acids into rat kidney proteins following injection of folic acid	1	1	6 days	Threlfall and Taylor, 1969

1964). It was concluded that the antibiotic did not inhibit amino acid activation or transfer of activated amino acid to soluble RNA but that it prevented transfer of amino acids from amino-acyl-t RNA into protein. The precise mechanism by which cycloheximide actually prevents that transfer has been the subject of many discussions but has not been elucidated clearly (Sisler and Siegel, 1967).

The LD_{50} of cycloheximide has been determined in different species of animals:

rat, subcutaneous injection, 2.7 mg/kg;

rat, intravenous injection, 2.5 mg/kg;

rat, intraperitoneal injection, not determined;

mouse, intravenous injection, 150 mg/kg;

rabbit, intravenous injection, 17 mg/kg (Whiffen, 1948).

The variation from species to species is very large and difficult to interpret.

Actinomycin, the first antibiotic derived from a Streptomyces, was isolated and crystallized in 1940 (Waksman and Woodruff, 1940). There is now a family of actinomycins known, with related structures (Johnson, 1960). The history of the discovery of the actinomycins and their properties have been reviewed (Woodruff and Waksman, 1960).

The mechanism of action of actinomycin D has been studied in many microbial systems (Slotnick, 1958; Slotnick, 1960) and in higher organisms (Tamaoki and Mueller, 1962; Merits, 1963). The synthesis of all forms of RNA was inhibited by actinomycin D in HeLa cells (Tamaoki and Mueller, 1962) and in rat liver (Merits, 1963). RNA polymerase, the enzyme responsible for the transcription of DNA into RNA is inhibited by actinomycin D.

One of the immediate consequences of the blockade of the synthesis of all classes of RNA is the inhibition of protein synthesis and it has now been shown that actinomycin D can inhibit protein synthesis in many different systems. Actinomycin D can inhibit the in vitro incorporation of labelled amino acids into ribosomal and polysomal fractions of rat liver (Korner and Munro, 1963). Actinomycin D added in vitro to lymphoid cells from hyperimmunized rabbits, can prevent the incorporation of leucine-C^{14} into antibodies (Smiley et al., 1964). Actinomycin D can inhibit the in vitro synthesis of proteins in epithelial cells from calf lens (Papaconstantinou et al., 1966). The induction of phosphopyruvate carboxylase in neonatal rat liver which can be obtained in utero by intraperitoneal injection of cyclic AMP to fetuses can be prevented by simultaneous injection of

actinomycin D (Yeung and Oliver, 1970). There is a lot more evidence in favor of the inhibition of protein synthesis by actinomycin D which cannot be discussed here. There are also a lot of controversial results with the effects of the antibiotic. In numerous cases, the drug did not inhibit the synthesis of proteins but actually induced the synthesis of some proteins (Harris, 1968). For example, treatment of rats with a dose of actinomycin D that permitted survival for a period of five days (150 µg/kg/day) resulted in a marked stimulation of the activities of four hepatic enzymes known to be inducible by cortisol injections: alanine transaminase, serine dehydrase, tyrosine transaminase and tryptophan pyrrolase (Rosen et al., 1964). Actinomycin D can also stimulate the synthesis of proteins in the fiber cells of calf lens while it decreases the synthesis of the same proteins in the epithelial cells of the same preparation (Papaconstantinou et al, 1966).

It is very difficult to inhibit totally the transcription of DNA with actinomycin D and it can always be argued that the template for the protein being studied is resistant to the action of actinomycin D. But the number and the variety of cases in which actinomycin D did not inhibit the synthesis of a given protein makes it difficult to believe that the given proteins are all specified by the small group of genes whose transcription is relatively insensitive to the antibiotic (Harris, 1968). In short term experiments, if the synthesis of a protein goes on in the presence of actinomycin D, it can be said that the template for the given protein is stable (in the sense that

it does not have to be continually replaced by fresh synthesis) because it is still present while the transcription of DNA is blocked. But in a longer term experiment in which both general RNA and protein synthesis have been blocked, the persistence of the increased activity of an enzyme can only with difficulty be attributed to the stability of the template; if all protein synthesis has been inhibited, the template, even if stable cannot have been used for protein synthesis since the latter has been blocked. In that case it can most probably be admitted that the persistence of the increased activity is not due to the synthesis of more enzyme (Harris, 1968).

The toxicity of actinomycin D in mammalian systems was known as early as 1941 (Waksman et al., 1941). Doses not greatly exceeding the LD_{50}, killed within less than 24 hours. Anorexia, diarrhea and disturbances of locomotion were features of chronic poisoning (Waksman et al., 1941; Robinson and Waksman, 1942). In rats weighing between 200 and 300 grams, the LD_{50} are as follows:

intravenous injection, 0.46 mg/kg;

intraperitoneal injection, 0.40 mg/kg;

subcutaneous injection, 0.80 mg/kg;

oral ingestion, 7.2 mg/kg (Philips et al, 1960).

The lethal doses were without effect for five or six hours but thereafter diarrhea began and by the end of the 24 hour period, it became prominent even in rats given sublethal doses. Fatally intoxicated animals were greatly depressed and dyspneic if still surviving after 24 hours. They became moribund,

with slow labored breathing, before they died. Abundant quantities of bloody cloudy fluid were found in the thoracic and abdominal cavities of the rats that survived for more than 24 hours after intraperitoneal injections of sublethal doses of the drug (Philips et al., 1960). Thymus, cervical lymph nodes and spleen all showed a decrease in weight; spleen, bone marrow and intestinal epithelium have a high daily mitotic rate and the number of cells that are renewed every day is higher than in other tissues such as kidney or liver which have a relatively slow daily mitotic rate (Leblond and Walker, 1956). The tissues which respond the most to actinomycin D are those which have a high rate of cell proliferation in adult animals (Philips et al, 1960). These results show how toxic actinomycin D is and how carefully the doses of the drug must be choosen in order to maintain animals in a fairly healthy state during the course of the experiment while inhibiting specifically the process to be studied.

ALTMAN, P.L.: in Blood and other Body Fluids, ed. by D.S. Dittmer, Biological Handbooks, Fed. Amer. Soc. exp. Biol., Washington, D.C., p. 398, 1961.

ALTMAN, P.L., and DITTMER, D.S.: in Biology Data Book, ed. by P.L. Altman and D.S. Dittmer, Biological Handbooks, Fed. Amer. Soc. exp. Biol., Washington, D.C., p. 264, 1964.

APPLEMAN, M.M., and KEMP, R.G.: Biochem. biophys. Res. Commun. 24: 564-568, 1966.

ATKINS, T., and MATTY, A.J.: J. Endocrinol. 51: 67-78, 1941.

AURBACH, G.D., and HOUSTON, B.A.: J. biol. Chem. 243: 5935-5940, 1968.

BALL, J.H., KAMINSKY, N.I., BROADUS, A.E., HARDMAN, J.G., SUTHERLAND, E.W. and LIDDLE, G.W.: Clin. Res. 18: 336, 1970.

BAR, H., and HECHTER, O.: Proc. Nat. Acad. Sci. 63: 350-356, 1969.

BEAVO, J.A., HARDMAN, J.G., and SUTHERLAND, E.W.: J. biol. Chem. 245: 5649-5655, 1970.

BECKER, F.F., and BITENSKY, M.W.: Proc. Soc. exp. Biol. Med. 130: 983-986, 1969.

BEVIZ, A., LUNDHOLM, L., and MOHME-LUNDHOLM, E.: Brit. J. Pharmacol. 34: 198P-199P, 1968.

BEVIZ, A., LUNDHOLM, L., and MOHME-LUNDHOLM, E.: Acta physiol. scand. 81: 145-156, 1971.

BHAGAT, B., and FRIEDMAN, E.: Brit. J. Pharmacol. 37: 24-33, 1969.

BIECK, P., STOCK, K., and WESTERMANN, E.: Life Sci. 5: 2157-2163, 1966.

BIECK, P., STOCK, K., and WESTERMANN, E.: Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. 256: 218-236, 1967.

BIRNBAUMER, L., and RODBELL, M.: J. biol. Chem. 244: 3477-3482, 1969.

BITENSKY, M.W., RUSSELL, V., and ROBERTSON, W.: Biochem. biophys. Res. Commun. 31: 706-712, 1968.

BITENSKY, M.W., RUSSELL, V., and BLANCO, M.: Endocrinology 86: 154-159, 1970.

BOWMAN, W.C., and RAPER, C.: Ann. N.Y. Acad. Sci. 139: 741-753, 1967.

BRADHAM, L.S., HOLT, D.A., and SIMS, M.: Biochem. biophys. Acta 201: 250-260, 1970.

BRANA, H., and CHYTIL, F.: Folia Microbiol. (Prague) 11: 43-1966.

BRAUN, T., and HECHTER, O.: Proc. Nat. Acad. Sci. 66: 995-1001, 1970.

BRECKENRIDGE, B. McL.: Proc. Nat. Acad. Sci. 52: 1580-1586, 1964.

BRECKENRIDGE, B. McL.: Ann. N.Y. Acad. Sci. 185: 10-17, 1971.

BRECKENRIDGE, B. McL., BURN, J.H., and MATSCHINSKY, F.M.: Proc. Nat. Acad. Sci. 57: 1893-1897, 1967.

BRECKENRIDGE, B. McL., and JOHNSTON, R.E.: J. Histochem. Cytochem. 17: 505-511, 1969.

BROADUS, A.E., KAMINSKY, N.I., HARDMAN, J.G., SUTHERLAND, E.W., and LIDDLE, G.W.: J. clin. Invest. 49: 2222-2236, 1970a.

BROADUS, A.E., KAMINSKY, N.I., NORTHCUTT, R.C., HARDMAN, J.G., SUTHERLAND, E.W., and LIDDLE, G.W.: J. clin. Invest. 49: 2237-2245, 1970b.

BROADUS, A.E., HARDMAN, J.G., KAMINSKY, N.I., BALL, J.H., SUTHERLAND, E.W., and LIDDLE, G.W.: Ann. N.Y. Acad. Sci. 185: 50-66, 1971.

BRODIE, B.B., DAVIES, J.I., HYNIE, S., KRISHNA, G., and WEISS, B.: Pharmacol. Rev. 18: 273-289, 1966.

BRODIE, B.B., KRISHNA, G., and HYNIE, S.: Biochem. Pharmacol. 18: 1129-1134, 1969.

BROOKER, G., THOMAS, L.J., and APPLEMAN, M.M.: Biochemistry 7: 4177-4181, 1968.

BROWN, E., CLARKE, D.L., ROUX, V., and SHERMAN, G.H.: J. biol. Chem. 238: 852-853, 1963.

BUTCHER, F.R.: Horm. Metab. Res. 3: 336-340, 1971.

BUTCHER, R.W., BAIRD, C.E., and SUTHERLAND, E.W.: J. biol. Chem. 243: 1705-1712, 1968.

BUTCHER, R.W., HO, R.J., MENG, H.C. and SUTHERLAND, E.W.: J. biol. Chem. 240: 4515-4523, 1965.

BUTCHER, R.W., and SUTHERLAND, E.W.: Pharmacologist 1: 63- , 1959.

BUTCHER, R.W. and SUTHERLAND, E.W.: J. biol. Chem. 237: 1244-1250, 1962.

CASTAGNA, M.: FEBS Letters 8: 289-292, 1970.

CHANG, Y.-Y.: Science 161: 57-59, 1968.

CHAPPEL., J.B., and PERRY, S.V.: Nature (London) 173: 1094-1095, 1954.

CHASE, L.R., and AURBACH, G.D.: Proc. Nat. Acad. Sci. 58: 518-525, 1967.

CHASE, L.R., and AURBACH, G.D.: Science 159: 545-547, 1968.

CHASE, L.R., MELSON, G.L., and AURBACH, G.D.: J. clin. Invest. 48: 1832-1844, 1969.

CHEUNG, W.Y.: Biochem. biophys. Res. Commun. 23: 214-219, 1966.

CHEUNG, W.Y.: Biochemistry 6: 1079-1087, 1967.

CHEUNG, W.Y.: Anal. Biochem. 28: 182-191, 1969.

CHEUNG, W.Y.: Biochem. biophys. Res. Commun. 38: 533-538, 1970.

CHEUNG, W.Y.: Biochim. biophys. Acta 242: 395-409, 1971a.

CHEUNG, W.Y.: J. biol. Chem. 246: 2859-2869, 1971b.

CHEUNG, W.Y., and SALGANICOFF, L.: Nature (London) 214: 90-91, 1967.

COGGI, G., and SCARPELLI, D.G.: Proc. Soc. exp. Biol. Med. 134: 328-331, 1970.

COTTLE, W.H., and CARLSON, L.D.: Proc. Soc. exp. Biol. Med. 92: 845-849, 1956.

COTTLE, W.H., NASH, C.W., VERESS, A.T., and FERGUSON, B.A.: Life Sci. 6: 2267-2271, 1967.

COUNIS, R., and RAULIN, J.: Bull. Soc. Chim. Biol 52: 1393-1405, 1970.

CRAIG, J.W., RALL, T.W., and LARNER, J.: Biochim. biophys. Acta 177: 213-219, 1969.

DANFORTH, W.H., and HELMREICH, E.: J. biol. Chem. 239: 3133-3145, 1964.

DAVOREN, P.R., and SUTHERLAND, E.W.: J. biol. Chem. 238: 3016-3023, 1963.

DEPOCAS, F.: Fed. Proc. 19 suppl. 5: 19-24, 1960a.

DEPOCAS, F.: Can. J. Biochem. Physiol. 38: 107-114, 1960b.

DeROBERTIS, E., ARNAIZ, G.R.D.L., ALBERICI, M., BUTCHER, R.W., and SUTHERLAND, E.W.: J. biol. Chem. 242: 3487-3493, 1967.

DRUMMOND, G.I., and PERROT-YEE, S.: J. biol. Chem. 236: 1126-1129, 1961.

EBADI, M.S., WEISS, B., and COSTA, E.: J. Neurochem. 18: 183-192, 1971.

EKINS, R., and NEWMAN, B.: Acta endocrinol. 64, suppl. 147: 11-36, 1970.

ENNIS, H.L., and LUBIN, M.: Science 146: 1474-1476, 1964.

FAIN, J.N., REED, N., and SAPERSTEIN, R.: J. biol. Chem. 242: 1887-1894, 1967.

FIALA, S., and FIALA, E.: Biochim. biophys. Acta 103: 699-701, 1965.

FISKE, C.H. and SUBBAROW, Y.: J. biol. Chem. 66: 375-400, 1925.

FORN, J., GESSA, G.L., KRISHNA, G., and BRODIE, B.B.: Life Sci. 9, part 1: 429-435, 1970a.

FORN, J., SCHONHOFFER, P.S., SKIDMORE, I.F., and KRISHNA, G.: Biochim. biophys. Acta 208: 304-309, 1970b.

FRANZINI-ARMSTRONG, C.: Proc. Nat. Acad. Sci. 23: 887-895, 1964.

GILL, G.N., and GARREN, L.D.: Proc. Nat. Acad. Sci. 63: 512-519, 1969.

GILL, G.N., and GARREN, L.D.: Biochem. biophys. Res. Commun. 39: 335-343, 1970.

GILL, G.N., and GARREN, L.D.: Proc. Nat. Acad. Sci. 68, 786-790, 1971.

GILMAN, A.G.: Proc. Nat. Acad. Sci. 67: 305-312, 1970.

GIRARDIER, L., and SEYDOUX, J.: J. Physiol. (Paris) 63: 147-186, 1971.

GOLDBERG, A.L., and SINGER, J.J.: Proc. Nat. Acad. Sci. 64: 134-141, 1969.

GOLDBERG, N.D., LARNER, J., SASKO, H., and O'TOOLE, A.G.: Anal. Biochem. 28: 523-544, 1969.

GOLDBERG, N.D., VILLAR-PALASI, C., SASKO, H., and LARNER, J.: Biochim. biophys. Acta 148: 665-672, 1967.

GOLDSTEIN, R.E., SKELTON, C.L., LEVEY, G.S., GLANCY, D.L., BEISER, G.D., and EPSTEIN, S.E.: Circulation 44: 638-648, 1971.

GORDON, R., SPECTOR, S., SJOERDSMA, A., and UDENFRIEND, S.: J. Pharmac. and exp. Ther. 153: 440-447, 1966.

GORMAN, R.E., and BITENSKY, M.W.: Nature (London) 235: 439-440, 1972.

GORSKI, J., and AXMAN, C.: Arch. Biochem. Biophys. 105: 517-520, 1964.

GULYASSY, P.F.: Life Sci. 10, part II: 451-461, 1971.

GULYASSY, P.F., and OKEN, R.L.: Proc. Soc. exp. Biol. Med. 137: 361-365, 1971.

HAHN, P.: Experientia (Basel) 26: 1300, 1970.

HARDMAN, J.G., DAVIS, J.W., and SUTHERLAND, E.W.: J. biol. Chem. 244: 6354-6362, 1969.

HARDMAN, J.G., ROBISON, G.A., and SUTHERLAND, E.W.: Ann. Rev. Physiol. 33: 311-336, 1971.

HARDMAN, J.G. and SUTHERLAND, E.W.: J. biol. Chem. 240: PC3704-3705, 1965.

HARRIS, H.: Nucleus and Cyloplasm, Clarendon Press, Oxford, 1968.

HART, J.S., HEROUX, O., and DEPOCAS, F.: J. appl. Physiol. 9: 404-408, 1956.

HEROUX, O., HART, J.S., and DEPOCAS, F.: J. appl. Physiol. 9: 399-403, 1956.

HIMMS-HAGEN, J.: Pharmacolog. Rev. 19: 367-461, 1967.

HIMMS-HAGEN, J.: J. Physiol. (London) 205: 393-403, 1969.

HIMMS-HAGEN, J.: Adv. Enzyme Regulation 8: 131-151, 1970.

HIMMS-HAGEN, J.: in Catecholamines, Handbuch der experimentellen Pharmakologie edited by E. Muscholl and H. Blaschko, Heidelberg: Springer 33: 363-462, 1972a.

HIMMS-HAGEN, J.: Handbook of Physiology, vol. , Adrenal Medulla, ed. H. Blaschko and A.D. Smith, American Physiological Society (In press), 1972b.

HIMMS-HAGEN, J., BUKOWIECKI, L., BEHRENS, W., and BONIN, M.: Fed. Proc. (In press), 1972.

HIRATA, M., and HAYAISHI, O.: Biochim. biophys. Acta 149: 1-11, 1967.

HSIEH, A.C.L., and CARLSON, L.D.: Amer. J. Physiol. 190: 243-246, 1957.

HSIEH, A.C.L., CARLSON, L.D., and GRAY, G.: Amer. J. Physiol. 190: 247-251, 1957.

HUMES, J.L., ROUNBEHLER, M., and KUEHL, F.A. Jr.: Anal. Biochem. 32: 210-217, 1969.

IMAI, Y., HORWITZ, B.A., and SMITH, R.E.: Proc. Soc. exp. Biol. Med. 127: 717-719, 1968.

JACOBS, F.A., and LARGIS, E.E.: Proc. Soc. exp. Biol. Med. 130: 697-702, 1969.

JANSKY, L.: Acta Universitatis Carolinae-Biologica 1: 1-91, 1965.

JANSKY, L.: Fed. Proc. 25: 1297-1302, 1966.

JANSKY, L. and HART, J.S.: Can. J. Biochem. Physiol. 41: 953-964, 1963.

JANSKY, L. and HART, J.S.: Can. J. Physiol. Pharmac. 46: 653-659, 1968.

JANSKY, L., VOTAPKOVA, Z., and FEIGLOVA, E.: Physiologia Bohemoslovaca 18: 443-451, 1969.

JANSKY, L., ZEISBERGER, E., and DOLEZAL, V.: Nature 202: 397-398, 1964.

JARD, S., and BERNARD, M.: Biochem. biophys. Res. Commun. 41: 781-788, 1970.

JOEL, C.D.: in Handbook of Physiology, Section 5, Adipose Tissue, ed. by A.E. Renold and G.F. Cahill, Jr., pp. 59-85, American Physiological Society, 1965.

JOHANSSON, B.: Metabolism 8: 221-240, 1959.

JOHNSON, A.W.: Ann. N.Y. Acad. Sci. 89: 336-347, 1960.

JUNGAS, R.L.: Proc. Nat. Acad. Sci. 56: 757-763, 1966.

KAKIUCHI, S., and RALL, T.W.: Molec. Pharmacol. 4: 367-378, 1968.

KAKIUCHI, S., and YAMAZAKI, R.: Biochem. biophys. Res. Commun. 41: 1104-1110, 1970.

KAMINSKY, N.I., BROADUS, A.E., HARDMAN, J.G., JONES, D.J. Jr., BALL, J.H., SUTHERLAND, E.W., and LIDDLE, G.W.: J. clin. Invest. 49: 2387-2395, 1970.

KANEKO, T., and FIELD, J.B.: J. Lab. clin. Med. 74: 682-690, 1969.

KERRIDGE, D.: J. gen. Microb. 19: 497-506, 1958.

KHANDELWAL, R.L., and HAMILTON, I.R.: J. biol. Chem. 246: 3297-3304, 1971.

KORNER, A., and MUNRO, A.J.: Biochem. biophys. Res. Commun. 11: 235-238, 1963.

KORNFELD, E.C., JONES, R.G., and PARKE, T.V.: J. Amer. Chem. Soc. 71: 150-159, 1949.

KRISHNA, G., and BIRNBAUMER, L.: Anal. Biochem. 35: 393-397, 1970.

KRISHNA, G., HYNIE, S., and BRODIE, B.B.: Proc. Nat. Acad. Sci. 59: 884-889, 1968b.

KRISHNA, G., WEISS, B., and BRODIE, B.B.: J. Pharmacol. exp. Ther. 163: 379-385, 1968a.

KUEHL, F.A. Jr., PATANELLI, D.J., TARNOFF, J., and HUMES, J.L.: Biol. Reprod. 2: 154-163, 1970.

KUO, J.F., and De RENZO, E.C.: J. biol. Chem. 244: 2252-2260, 1968.

KUO, J.F., and DILL, I.K.: Biochem. biophys. Res. Commun. 32: 333-337, 1968.

KUO, J.F. and GREENGARD, P.: J. biol. Chem. 245: 4067-4073, 1970.

KUWANO, M., and SCHLESSINGER, D.: Proc. Nat. Acad. Sci. 66: 146-152, 1970.

LEACH, B.E., FORD, J.H., and WHIFFEN, B.J.: J. Amer. Chem. Soc. 69: 474, 1947.

LEBLANC, J., and NADEAU, G.: Can. J. Biochem. Physiol. 39: 215-217, 1961.

LEBLANC, J., and POULIOT, M.: Amer. J. Physiol. 270: 853-856, 1964.

LEBLANC, J., and VILLEMAIRE, A.: Amer. J. Physiol. 218: 1742-1745, 1970.

LEBLOND, C.P., and WALKER, B.E.: Physiol. Rev. 36: 255-276, 1956.

LEDUC, J.: Acta physiol. scand. 53: suppl. 183, 1961.

LEVEY, G.S., SKELTON, D.L., and EPSTEIN, S.E.: Endocrinology 85: 1004-1009, 1969a.

LEVEY, G.S., SKELTON, D.L., and EPSTEIN, S.E.: J. clin. Invest. 48: 2244-2250, 1969b.

LOWRY, O., ROSEBROUGH, N.J., FARR, A.L., and RANDALL, R.J.: J. biol. Chem. 193: 265-275, 1951.

LUNDHOLM, L., RALL, T., and VAMOS, N.: Acta physiol. scand. 70: 127-128, 1967.

LYON, J.B., and MAYER, S.E.: Biochem. biophys. Res. Commun. 34: 459-464, 1969.

MAICKEL, R.P., WESTERMANN, E.O., and BRODIE, B.B.: J. Pharmacol. 134: 167-175, 1961.

MANDEL, L.R., and KUEHL, F.A.: Biochem. biophys. Res. Commun. 28: 13-18, 1967.

MANSOUR, T.E., SUTHERLAND, E.W., RALL, T.W., and BUEDING, E.: J. biol. Chem. 235: 466-470, 1960.

MARINETTI, G.V., RAY, T.K. and TOMASI, V.: Biochem. biophys. Res. Commun. 36: 185-193, 1969.

MAYER, S.E., and STULL, J.T.: Ann. N.Y. Acad. Sci. 185: 433-448, 1971.

MEJSNAR, J.: in Abstracts International Symposium on environmental physiology: bioenergetics and temperature regulation, Dublin, p. 70, 1971.

MEJSNAR, J., and JANSKY, L.: in Nonshivering thermogenesis (Prague Symposium, 1970), Academia, pp. 27-38, 1971.

MERITS, I.: Biochem. biophys. Res. Commun. 10: 254-259, 1963.

MONN, E., and CHRISTIANSEN, R.O.: Science 173: 540-541, 1971.

NAIR, K.G.: Biochemistry 5: 150-157, 1966.

NAMM, D.H., and MAYER, S.E.: Molec. Pharmacol. 4: 61-69, 1968.

OLIVER , G., and SCHAFER, E.A.: J. Physiol. (London) 18: 230-276, 1895.

OLIVERIO, A., and STJARNE, L.: Life Sci. 4: 2339-2343, 1965.

PAPACONSTANTINOU, J., STEWART, J.A., and KOEHN, P.V.: Biochim. biophys. Acta 114: 428-430, 1966.

PAUL, M.I., DITZION, B.R., PAUK, G.L., and JANOWSKY, D.S.: Amer. J. Psychiat. 126: 1493-1497, 1970.

PERKINS, J.P., and MOORE, M.M.: J. biol. Chem. 246: 62-68, 1971.

PHILIPS, F.S., SCHWARTZ, H.S., STERNBERG, S.S., and TAN, C.T.C.: Ann. N.Y. Acad. Sci. 89: 348-360, 1960.

PIRAS, R., and STANELONI, R.: Biochemistry 8: 2153-2160, 1969.

POSNER, J.B., HAMMERMEISTER, K.E., BRATVOLD, G.E., and KREBS, E.G.: Biochemistry 3: 1040-1044, 1964.

POSNER, J.B., STERN, R., and KREBS, E.G.: Biochem. biophys. Res. Commun. 9: 293-296, 1962.

POSNER, J.B., STERN, R., and KREBS, E.G.: J. biol. Chem. 240: 982-985, 1965.

RABINOWITZ, M., DESALLES, L., MEISLER, J., and LORAND, L.: Biochim. biophys. Acta 97: 29-36, 1965.

RALL, T.W., and SUTHERLAND, E.W.: J. biol. Chem. 232: 1065-1076, 1958.

RALL, T.W., and SUTHERLAND, E.W.: J. biol. Chem. 237: 1228-1232, 1962.

RALL, T.W., SUTHERLAND, E.W., and BERTHET, J.: J. biol. Chem. 224: 463-475, 1957.

RASMUSSEN, H., and TENEHOUSE, A.: Proc. Nat. Acad. Sci. 59: 1364-1370, 1968.

REED, N., and FAIN, J.N.: J. biol. Chem. 243: 2843-2848, 1968.

REIK, L., PETZOLD, G.L., HIGGINS, J.A., GREENGARD, P. and BARRNETT, R.J.: Science 168: 382-384, 1970.

REIMANN, E.M., BROSTOM, C.O., CORBIN, J.D., KING, C.A. and KREBS, E.G.: Biochem. biophys. Res. Commun. 42: 187-194, 1971.

ROBINSON, H.F., and WAKSMAN, S.A.: J. Pharmacol. exp. Ther. 74: 25-32, 1942.

ROBISON, G.A., BUTCHER, R.W., and SUTHERLAND, E.W.: Ann. N.Y. Acad. Sci. 139: 703-723, 1967a.

ROBISON, G.A., BUTCHER, R.W., and SUTHERLAND, E.W.: Cyclic AMP Academic Press, N.Y. 1971.

ROBISON, G.A., EXTON, J.H., PARK, C.R. and SUTHERLAND, E.W.: Fed. Proc. 26: 1257, 1967b.

ROBISON, G.A., SCHMIDT, M.J. and SUTHERLAND, E.W.: in Role of cyclic AMP in cells function, ed. by P. Greengard and E. Costa, Raven Press, N.Y., pp. 11-30, 1970.

RODBELL, M.: J. biol. Chem. 239: 375-380, 1964.

RODBELL, M.: J. biol. Chem. 242: 5744-5750, 1967.

ROSEN, F., RAINA, P.N., MILHOLLAND, R.J., and NICHOL, C.A.: Science 146: 661-663, 1964.

ROSEN, O.H.: Arch. Biochem. Biophys. 134: 435-441, 1970.

ROSEN, O.M., and ERLICHMAN, J.: Arch. Biochem. Biophys. 133: 171-177, 1969.

ROSEN, O.M., and ROSEN, S.M.: Biochem. biophys. Res. Commun. 31: 82-91, 1968.

ROSEN, O.M., and ROSEN, S.M.: Arch. Biochem. Biophys. 131: 449-456, 1969.

SCHMIDT, M.J., PALMER, E.C., DETTBARN, W.D., and ROBISON, F.A.: Developmental Psychobiology 3: 53-67, 1970.

SCHONHOFER, P.S., SKIDMORE, I.F., KRISHNA, G., and BOURNE, H.R.: Z. analyt. Chem. 252: 182- , 1970.

SCHRAMM, M., and NAIM, E.: J. biol. Chem. 245: 3225-3231, 1970.

SCOTT, N.S., and FALCONER, I.R.: Anal. Biochem. 13: 71-73, 1965.

SELLERS, E.A., SCOTT, J.W., and THOMAS, N.: Amer. J. Physiol. 177: 372-376, 1954.

SERAYDARIAN, K., and MOMMAERTS, W.F.H.M.: J. Cell Biol. 26: 641-656, 1965.

SHUM, A., JOHNSON, G.E., and FLATTERY, K.V.: Amer. J. Physiol. 216: 1164-1169, 1969.

SIEGEL, M.R., and SISLER, H.D.: Nature (London) 200: 675-676, 1963.

SIEGEL, M.R., and SISLER, H.D.: Biochim. biophys. Acta 87: 83-89, 1964.

SISLER, H.D., and SIEGEL, M.R.: in Antibiotics I Mechanism of action, ed. by D. Cottlieb and P.D. Shaw, Springer-Verlag, New York, pp. 283-307, 1967.

SKALA, J., BARNARD, T., and LINDBERG, O.: Comp. Biochem. Physiol. 33: 509-528, 1970b.

SKALA, J., HAHN, P., and BRAUN, T.: Life Sci. 9, part I: 1201-1212, 1970a.

SLOTNICK, I.J.: Antibiotics and Chemotherapy 8: 476-479, 1958.

SLOTNICK, I.J.: Ann. N.Y. Acad. Sci. 89: 342-347, 1960.

SMILEY, J.D., HEARD, J.G., and ZIFF, M.: J. exp. Med. 119: 881-893, 1964.

SMITH, B.M., and MAJOR, P.N.: Life Sci. 10, part I: 1433-1439, 1971.

SMITH, R.E., and HORWITZ, B.A.: Physiol. Rev. 49: 330-425, 1969.

SMITH, R.E., and ROBERTS, J.C.: Amer. J. Physiol. 206: 143-152, 1964.

SOBEL, B.E., DEMPSEY, P.J., and COOPER, T.: Biochem. biophys. Res. Commun. 33: 758-762, 1968.

SOBEL, B.E., DEMPSEY, P.J., and COOPER, T.: Proc. Soc. exp. Biol. Med. 132: 6-9, 1969a.

SOBEL, B.E., HENRY, P.D., ROBISON, A., BLOOR, A.B.C., and ROSS, J. Jr.: Circ. Res. 24: 507-512, 1969b.

SOMERVILLE, A.R., RABOUHANS, M.-L., and SMITH, A.A.: Biochem. J. 120: 11P-12P, 1970.

STEINER, A.L., KIPNIS, D.M., UTIGER, R., and PARKER, C.: Proc. Nat. Acad. Sci. 64: 367-373, 1969.

STREETO, J.M., and REDDY, W.J.: Anal. Biochem. 21: 416-426, 1967.

STRUBELT, O.: in Quantitative Biology, ed. by A. Locker, Springer-Verlag, N.Y., 274-277, 1968.

STRUBELT, O., and SIEGERS, C.-P.: Biochem. Pharmacol. 18: 1207-1220, 1969.

SUTER, E.R.: J. Ultrastr. Res. 26: 216-241, 1969.

SUTHERLAND, E.W., ØYE, I., and BUTCHER, R.W.: Rec. Progr. Horm. Res. 21: 623-646, 1965.

SUTHERLAND, E.W., and RALL, T.W.: J. Amer. Chem. Soc. 79: 3608, 1957a.

SUTHERLAND, E.W., and RALL, T.W.: J. biol. Chem. 232: 1077-1091, 1957b.

SUTHERLAND, E.W., and RALL, T.W.: J. biol. Chem. 232: 1077-1091, 1958.

SUTHERLAND, E.W., and RALL, T.W.: Pharmacol. Rev. 12: 265-299, 1960.

SUTHERLAND, E.W., RALL, T.W., and MENON, T.: J. biol. Chem. 237: 1220-1227, 1962.

TAO, M., and LIPMANN, F.: Proc. Nat. Acad. Sci. 63: 86-92, 1969.

TAMAOKI, T., and MUELLER, G.C.: Biochem. biophys. Res. Commun. 9: 451-454, 1962.

TAYLOR, A.L., DAVIS, B.B., PAWLSON, L.G., JOSIMOVICH, J.B., and MINTZ, D.H.: J. clin. Endocr. 30: 316-324, 1970.

THERRIAULT, D.G., MORNINGSTAR, J.F.Jr., and WINTERS, V.G.: Life Sci. 8, part II: 1353-1358, 1969.

THERRIAULT, D.G., and WINTERS, V.G.: Life Sci. 9, part II: 1053-1060, 1970.

THOMSON, J.F., HABECK, D.A., NANCE, S.L., and BEETHAM, K.L.: J. Cell Biol. 41: 312-334, 1969.

THOMPSON, W.J., and APPLEMAN, M.M.: Biochemistry 10: 311-316, 1971.

THRELFALL, G., and TAYLOR, D.M.: Europ. J. Biochem. 8: 591-596, 1969.

TOMASI, V., KORETZ, S., RAY, T.K., DUNNICK, J., and MARINETTI, G.V.: Biochim. biophys. Acta 211: 31-42, 1970.

TORNHEIM, K., O'DELL, R.O., and PROSKY, L.: Proc. Soc. exp. Biol. Med. 131: 601-605, 1969.

TRAKATELLIS, A.C., MONTJAR, M., and AXELROD, A.E.: Biochemistry 4: 2065-2071, 1965.

VERNIKOS-DANELLIS, J., and HARRIS, C.G.: Proc. Soc. exp. Biol. Med. 128: 1016-1021, 1968.

WAKSMAN, S.A., ROBINSON, H.J., METZGER, H.J., and WOODRUFF, H.B.: Proc. Soc. exp. Biol. Med. 47: 261-263, 1941.

WAKSMAN, S.A., and WOODRUFF, H.B.: Proc. Soc. exp. Biol. Med. 45: 609-614, 1940.

WALTON, G.M., and GARREN, L.D.: Biochemistry 9: 4223-4229, 1970.

WALTON, G.M., GILL, G.N., ABRASS, I.B., and GARREN, L.D.: Proc. Nat. Acad. Sci. 68: 880-884, 1971.

WASTILA, W.B., STULL, J.T., MAYER, S.E., and WALSH, D.A.: J. biol. Chem. 246: 1996-2003, 1971.

WATCHORN, E., and McCANCE, R.A.: Biochem. J. 31: 1379-1390, 1937.

WEISS, B.: J. Pharmacol. exp. Ther. 168: 146-152, 1969.

WEISS, B.: J. Neurochem. 18: 469-477, 1971.

WEISS, B., DAVIES, J.I., and BRODIE, B.B.: Biochem. Pharmacol. 15: 1553-1561, 1966.

WHIFFEN, A.J.: J. Bacteriology 56: 283-291, 1948.

WICKS, W.D.: J. biol. Chem. 244: 3941-3950, 1969.

WILLIAMS, R.H., BARISH, J., and ENSINCK, J.W.: Proc. Soc. exp. Biol. Med. 139: 447-454, 1972.

WILLIAMS, R.H., WALSH, S.A., HEPP, D.K., and ENSINCK, J.W.: Metabolism 17: 653-688, 1968.

WILLIAMSON, J.R., and CORKEY, B.E.: in Methods in Enzymology, vol. 13, ed. by J.M. Lowenstein, p. 437- , 1969.

WOLLENBERGER, A., RISTAV, O., and SCHOFFA, G.: Pflügers Arch. ges. Physiol. 270: 399-412, 1960.

WOODRUFF, H.B., and WAKSMAN, S.A.: Ann. N.Y. Acad. Sci. 89: 287-298, 1960.

YEUNG, D., and OLIVER, I.T.: Biochemistry 7: 3231-3239, 1970.

YOSHIMURA, K., HIROSHIGE, T., and ITOH, S.: Jap. J. Physiol. 19: 176-186, 1969a.

YOSHIMURA, K., HIROSHIGE, T., and ITOH, S.: Jap. J. Physiol. 19: 791-800, 1969b.