NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

THYROXINE 5'-DEIODINASE IN CELLS ISOLATED FROM BROWN ADIPOSE TISSUE OF WARM- AND COLD-ACCLIMATED GUINEA PIGS

by

Stanislaw Adam Kucharczyk

A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Master of Science

Department of Biochemistry
Faculty of Medicine
University of Ottawa

ISBN 0-315-70493-4

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

The long-term objective of this study was to prepare isolated brown adipose tissue (BAT) cells which contain either a high or a low level of thyroxine 5'-deiodinase (T5'D) activity for future use in the study of the role of this enzyme, which produces the thermogenically active thyroid hormone, 3,5,3'-triiodothyronine (T_3), in the regulation of metabolic processes in BAT cells. Cold-acclimation was used to increase T5'D activity in BAT, since it is known to have this effect in rats, hamsters and mice. However, the guinea pig was chosen as experimental animal because this is known to be the only species for which cells isolated from BAT of the cold acclimated animal have a much greater thermogenic response to noradrenaline (NA) than cells from BAT of the warm acclimated animal. T5'D had not hitherto been studied in BAT of the guinea pig, therefore, a first experiment studied the effect of cold acclimation on the activity of this enzyme in BAT of the guinea pig and demonstrated a very large and long-lasting increase in activity. Injection of NA into guinea pigs produced only a very small increase in activity of T5'D in BAT, not a suitable means for obtaining BAT with a high activity of T5'D. BAT of newborn guinea pigs also had a low activity of T5'D, also not a good source of BAT for the purpose of these experiments. In cells isolated from BAT of cold-acclimated guinea pigs the level of T5'D was much higher than in cells from BAT of warm-acclimated guinea pigs. The specific activity of T5'D in cells was greater than that in homogenates from the same tissue, indicating a good maintenance of activity during the preparation of the cells. The activity in the cells decreased slowly during 4 hours incubation in a Krebs Ringer medium, both in cells from cold acclimated guinea pigs and in cells from warm acclimated guinea pigs. Use of Dulbecco's modified Eagle medium prevented the loss of activity during incubation in cells from warm acclimated guinea pigs, indeed increased activity in both cells and homogenate during incubation. It is concluded that BAT cells from cold acclimated and warm acclimated guinea pigs would be a useful model system for studying the role of endogenous T_3 production in regulation of metabolic processes in isolated BAT cells. This is the first time that a high level of activity of T5'D in isolated BAT cells has been achieved and compared directly with that of the intact tissue.

ACKNOWLEDGEMENT

I would like to thank the coincidence for giving me the opportunity to meet a friendly soul, a wonderful woman and a superb supervisor. Finally, must express deep gratitude and admiration to dr. J. Himms-Hagen for her endless patience, without which writing of this thesis would never have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE:

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Thermogenesis

Most mammals maintain a stable body temperature and weight during most of their lifetime, indicating that energy intake and expenditure are adjusted to each other in the long run (LeMagnen, 1983). This is possible only if the expenditure is tightly coupled to the intake of energy. The energy intake is attained through feeding and drinking. On the other hand, energy expenditure is composed of two categories; obligatory thermogenesis and facultative thermogenesis. Obligatory thermogenesis is a result of metabolic reactions in all cells and of processing of food. It occurs in all organs of the body and is responsible for the maintenance of living processes. Facultative thermogenesis can be turned on or off when needed. It occurs only in two organs, skeletal muscle and brown adipose tissue (BAT). In skeletal muscle thermogenesis is associated with voluntary activity or exercise or with thermoregulatory shivering for maintaining body temperature in a cold environment. In BAT, it is also associated with thermoregulation, a process called non-shivering thermogenesis. Non-shivering thermogenesis can be induced by cold exposure (cold-induced thermogenesis), that is, temperature below thermoneutrality for an animal (Himms-Hagen,1986) or in response to food quantity and quality

(diet-induced thermogenesis) (Rothwell and Stock, 1986). A single environmental stimulus can evoke more than one category of facultative thermogenesis. Thus, exposure to cold can evoke both shivering and non-shivering thermogenesis. Sometimes, it may be not easy to measure each separately, since the attempt to eliminate one of them may enhance the contribution of the other. Therefore, the facultative nature of BAT thermogenesis makes it at times somewhat elusive, difficult to measure, and the subject of controversy (Hervey and Tobin,1983; Rothwell and Stock,1983; Maxwell et al.,1987)

Morphology and innervation of BAT

BAT is a distinct organ distributed in discrete depots in the interscapular, intercostal, pericardial and perirenal regions of the body (Smith and Horwitz, 1969). The mature BAT cells account for only 45% of the cells in the tissue, the remaining portion being made up by endothelial, perivascular, and mast cells, fibroblasts and preadipocytes. In rodents, the interscapular BAT is a major depot, and for this reason it is the most frequently used in research. As a rule, brown adipocytes in their active state, in contrast to white adipocytes, contain many triacylglycerol droplets and are referred to as multilocular. In addition, BAT cells are densely packed with large mitochondria, which contribute to the colour of this tissue. They also contain the usual complement of other organelles such as rough and smooth endoplasmic reticulum, Golgi apparatus, free ribosomes, peroxisomes, lysosomes and nucleus. In the quiescent state BAT cells become paler, unilocular with few mitochondria and are then difficult to distinguish from white adipose

tissue cells (Néchad, 1986). BAT has an extensive vasculature and also arteriovenous anastamoses. These morphological characteristics can readily supply a large amount of substrate and oxygen needed in the state of high activity of this tissue. In addition, this feature allows it to act as a convector system for the capture and evacuation of the heat generated in the tissue (Nnodim and Lever, 1988). BAT is a highly innervated tissue (Néchad, 1986; Foster et al., 1982). Two types of sympathetic nerve fibers reach BAT that mediate its growth and activation (Girardier and Seydoux, 1986). Nerves which make contact with brown adipocytes, containing noradrenaline (NA) and nerves making contact with blood vessels, containing NA and neuropeptide Y (NPY) (Norman et al., 1988).

The physiological function of BAT

a) Mechanism of thermogenesis

The physiological function of BAT is thermogenesis. In the activated state there can be an enormous increase in the metabolic rate and the tissue is subject to a very high blood flow rate (Foster and Frydman, 1978 and 1979). The thermogenic function of BAT involves mitochondria, which possess a unique protein located in the inner mitochondrial membrane, capable of uncoupling the electron transport system from oxidative phosphorylation. The presence of this protein, uncoupling protein or UCP, permits a dramatic increase in the inward movement of protons, causing collapse of the proton gradient created by the electron transport system (Nicholls,1976). As a result of the increased proton

permeability of BAT mitochondria the electron transport system is no longer under respiratory control and the rate of the electron flow through the electron transport system and hence substrate oxidation and heat production is at maximum (Nicholls, 1983). Therefore, BAT in its activated state is capable of production of a large amount of heat that is vital to all mammalian species, especially in the immediate postnatal period, during arousal from hibernation and in a cold environment.

b) Control of thermogenesis

Two pathways control the UCP function. The first pathway involves the pH dependent binding of purine nucleotides such as ATP or ADP to UCP, which block the translocation of protons across the inner mitochondrial membrane (Klingenberg, 1988). The second pathway involves the intracellular concentration of fatty acids, which is in turn regulated by the activity of hormone sensitive triacylglycerol lipase. Fatty acids can interact with UCP in such a way as to promote its proton translocating activity (Bukowiecki, 1986).

The rate of thermogenesis is regulated by the sympathetic nervous system. Noradrenaline secreted by the nerve endings interacts with the receptors on BAT cell surface eliciting a response. Interaction with beta receptors initiates c-AMP dependent activation of lipoprotein lipase and the above mentioned hormone-sensitive triacylglycerol lipase. The activation of the latter initiates lipolysis. Free fatty acids released during this process serve as a substrate for mitochondrial dehydrogenases in the conditions of high demand for reduced coenzymes and activate existing membrane UCP. Thus, fatty acids are the major and immediate fuel for noradrenaline-stimulated thermogenesis in BAT, which in its activated state is totally dependent on the oxidation of fatty acids (Bukowiecki, 1986).

Interaction with alpha-1 receptors initiates the phosphatidylinositol- 5 - bisphosphate cycle (Nånberg and Putney, 1986), which brings about opening of some ion channels in the plasma membrane and release of calcium from the cisterns of endoplasmic reticulum and mitochondria. The rapid movement of ions through plasma membranes causes depolarization of membranes and propagation of the stimulatory signal between BAT cells through gap junctions. However, the significance of these events for thermogenesis is unclear (Schneider-Picard et al.,1985). In addition, stimulation of the alpha-1 receptors induces de novo synthesis of thyroxine 5'-deiodinase type II (Jones et al., 1986) an enzyme which converts thyroxine to its active hormonal form, 3',5,3-triiodothyronine. The function of this enzyme will be discussed later on.

Hypertrophy and atrophy of BAT

Prolonged sympathetic stimulation of BAT by cold or diet results in hypertrophy of the tissue, which is mediated both by alpha and beta receptors.This growth of tissue results from the formation of new adipocytes from interstitial cells and from brown preadipocytes (Bukowiecki,et al.,1986). However, growth of BAT is not simply production of more adipocytes of the same type. There is a specific increase in certain components vital for thermogenic function of BAT and reflected as the increase in total and mitochondrial protein content. The overall pattern of changes in BAT protein content varies with the nature of stimulus, that is, cold versus diet. Nevertheless, the most important components that show such adaptive increases include; lipoprotein lipase (Carneheim et al.,1988), thyroxine 5'-

deiodinase (Park and Himms-Hagen,1988), glucose transporters (Greco-Perotto et al.,1987) and UCP (Ricquier et al.,1986). Inversely, quiescence of BAT as a result of reduction in sympathetic nervous system activity produces atrophy of the tissue. This atrophy is largely due to loss of mitochondrial mass and total protein of the tissue.

However, with regard to specific components of activated BAT, different patterns of atrophy occur when an experimental animal (rat) fed a high calorie diet is returned to low calorie diet or cold-acclimated animal to thermoneutrality.

The role of thyroxine 5'-deiodinase and thyroid hormone in the function of BAT

BAT contains thyroxine 5'-deiodinase (type II) that converts thyroxine to its active form 3,5,3'-triiodothyronine. The enzyme is found exclusively in BAT, the brain and the pituitary (Leonard et al., 1983) and differs in its properties from a related enzyme (type I), found in liver and muscle (Silva et al., 1987). The activity of the enzyme is controlled primarily by the sympathetic nervous system activity, increasing up to several hundred times in response to cold exposure (Silva and Larsen, 1983; Kopecký et al., 1986). However, in cold-exposed rats reacclimated to 21-26°C the activity of the enzyme disappears rapidly with the half-life of 2-3 h (Jones et al., 1986; Park and Himms-Hagen, 1988), suggesting total dependence of this enzyme on the continued input from the sympathetic nervous system. In addition, the level of enzyme is increased also by insulin and glucagon (Silva and Larsen, 1986b ; Mills et al., 1987), yet the increase in activity is not as high as in adrenergic stimulation. Its activity is suppressed by thyroxine

and by growth hormone (Silva and Larsen, 1986a). The activity of this enzyme is essential for modulation of thermogenesis in BAT, since noradrenaline -stimulated thermogenesis is decreased significantly in hypothyroid animals (Obregón et al., 1987; Silva, 1988). A large increase in activity of T5'D in cold-exposed rats leads to an increase in production of triiodothyronine in BAT. The rapid increase in the concentration of locally produced triiodothyronine virtually saturates the nuclear receptor within minutes after cold stimulation (Bianco and Sliva,1988). This in turn, significantly amplifies the effect of noradrenaline to increase synthesis of mRNA for UCP (Bianco et al., 1988). Triiodothyronine alone is unable to induce the synthesis of mRNA for UCP in the absence of noradrenaline to any appreciable extent (Silva,1988). Moreover, triiodothyronine generated in BAT by the action of T5'D may not only increase its intracellular concentration but also be the source for blood triiodothyronine (Silva and Matthews,1984; Fernandez et al., 1987).

Diet-induced thermogenesis is not associated with any increase in the activity of T5'D (Kopecký,et al.,1986)

The thermogenic response to noradrenaline in cells isolated from BAT

In vivo studies have revealed that cold-acclimated rats have larger thermogenic response of BAT to cold or noradrenaline than warm -acclimated rats (Kuroshima and Itoh, 1971; Foster and Frydman 1978). An enhanced thermogenic response to cold or noradrenaline has also been reported in cold acclimated guinea pigs (Jansky et al.,1969) .

The brown adipocytes isolated from warm-acclimated rats (Bukowiecki et

al., 1981) and hamsters (Nedergaard and Lindberg,1979) respond to noradrenaline with 10-30 fold stimulation of respiration. However, cells from cold-acclimated rats or hamsters are less responsive and do not differ in their response to noradrenaline from those of warm-acclimated animals (Nedergaard, 1982). In contrast, isolated brown adipocytes of cold-acclimated guinea pigs show response to noradrenaline to be much higher as compared to cells from warm-acclimated guinea pigs (Rafael et al., 1986; Cunningham and Nicholls, 1987).

The experiments reported in this thesis required the study of BAT cells derived from warm-acclimated and cold-acclimated animals. Since isolated brown adipocytes of cold-acclimated rats or mice are obtained in poor yield and do not respond to noradrenaline as expected from their response in vivo. The brown adipocytes from cold-acclimated guinea pigs have an enhanced thermogenic response to noradrenaline compared with brown adipocytes from warm-acclimated guinea pigs. Therefore, the guinea pig was chosen as the experimental animal.

Induction of thyroxine 5'-deiodinase and uncoupling protein in isolated and cultured brown adipocytes.

As in vivo, it has been demonstrated that noradrenaline induction of T5'D activity in isolated brown adipocytes of euthyroid rats is mediated by alpha-1 receptor (Obregón et al., 1987). The induction of T5'D activity in isolated brown adipocytes of guinea pig has not been studied. In cultured brown adipocytes of mice noradrenaline induced T5'D activity, but the activation seemed to be mediated mainly by the beta receptor (Houštěk et al., 1990a), and the reason for this

discrepancy is not clear.

The noradrenaline-induced synthesis of UCP in cultured brown adipocytes of rat, Syrian hamster (Houštěk et al., 1990b) and mouse has been shown to be mediated by beta receptors (Kopecký et al., 1990). In addition, the presence of insulin and thyroid hormone in cultured cells markedly facilitated the noradrenaline response, indicating involvement of both alpha-1 and beta receptors in UCP induction (Rehnmark et al., 1990). The newly synthesized UCP was targeted to the mitochondrial inner membrane (Herron et al., 1990), transforming nonthermogenic mitochondria into thermogenic.

The changes in BAT of new-born guinea pig and during acclimation to cold of adult guinea pig

In neonatal guinea pigs, BAT is present in large quantities (about 5% of body weight) and is thermogenically active (Brück and Wünnnenberg, 1966). However, within 3-4 weeks of age it becomes thermogenically quiescent and atrophied. Histologically the multilocular cells characteristic of BAT are superseded by unilocular cells, which superficially resemble white adipose tissue (Hirvonen et al., 1973), and have a high content of saponifiable triacylglycerols. The cells contain fewer mitochondria, less protein and the changes parallel the disappearance of reactions for mitochondrial enzymes (Huttunen and Kinnula, 1978) and mitochondrial content of UCP (Holloway et al., 1984). However, the exposure of adult guinea pigs to chronic cold leads to losing the appearance of white adipose tissue and regaining that of neonate BAT. This effect of cold-acclimation can be mimicked by continuous infusion of noradrenaline for several

days into animals (Cunningham and Nicholls, 1987). The cold-acclimation is known to increase sympathetic

nervous system activity in guinea pigs (Zeisberger and Roth,1989; Roth et al.,1987,1988). The reactivation of BAT is associated with an increase in total protein content, the synthesis of new mitochondria containing an increased concentration of UCP (Holloway et al., 1984) as well as a selective increase in the concentration of UCP in their mitochondria (Cunningham et al., 1986). The tissue loses weight and stored triacylglycerols (Holloway, et al., 1983 ; Kuroshima et al.,1991).

CHAPTER TWO

STATEMENT OF THE PROBLEM

The long term objective is to develop an in vitro system in which BAT cells contain a high or low level of T5'D and retain good thermogenic responsiveness to noradrenaline so as to be able to study the role of triiodothyronine production by this enzyme in the regulation of metabolic processes in BAT cells.

The best and the simplest way to increase the level of T5'D in BAT is to acclimate rats, hamsters or mice to cold. However, cells isolated from BAT of these commonly used cold-acclimated animals do not exhibit the enhanced thermogenic response to noradrenaline that might be expected (Nedergaard, 1982). Moreover, cold-acclimated mice exhibit only a transient increase in T5'D activity in their BAT during the initial stage of acclimation to cold (Kates and Himms-Hagen, 1990; Eley and Himms-Hagen,1989) and are thus also not a suitable experimental animal. The only species for which enhanced thermogenic responsiveness to noradrenaline by BAT cells isolated from a cold-acclimated animal has been demonstrated is the guinea pig (Rafael et al., 1986; Locke et al., 1982; Cunningham and Nicholls,1987). However, T5'D in BAT of this species has not so far been studied.

Therefore, the first specific objective was to describe the time-course of changes in T5'D in BAT of guinea pigs during acclimation to cold so as to select a suitable duration of cold-acclimation that would result in BAT having a high level of this enzyme.

Since it is not known whether the high level of T5'D activity in BAT of any cold-acclimated animal persists in cells isolated from the tissue, the second specific objective was to isolate cells from BAT of cold-acclimated and warm-acclimated guinea pigs and establish whether the T5'D activity expected to be present at different levels in the tissue is present at similar levels in the isolated cells.

In isolated BAT cells from cold-acclimated guinea pigs the thermogenic response to noradrenaline stimulation is much higher than in warm-acclimated. Therefore, the third specific objective was to check whether the isolated cells from warm- and cold-acclimated guinea pigs show the expected thermogenic responsiveness to noradrenaline.

Since it was not known whether any cold-induced increase in T5'D activity in BAT of guinea pig would be mediated by noradrenaline, as it appears to be in rats (Silva and Larsen, 1986), the fourth specific objective was to find out whether injection of noradrenaline into warm-acclimated guinea pigs would increase the activity of T5'D activity in BAT.

It has been demonstrated on isolated BAT cells of warm-acclimated rats thatT5'D activity is increased by incubation of the cells with noradrenaline (Obregón
et al., 1987). Therefore, the fifth specific objective was to find out whether T5`D activity in BAT cells of warm acclimated guinea pig can be increased by incubation of these cells with noradrenaline.

CHAPTER THREE

MATERIALS AND METHODS

Part 1 MATERIALS

A: ANIMALS

Three weeks-old female Hartley guinea pigs obtained from Charles River Canada, St-Constant, Quebec were used for the experiments. They were immediately housed individually at +28°C. In most of the described experiments the animals were kept in individual cages with free access to food (Prolab Guinea Pig Formula) and water and maintained in controlled lighting schedule of 12:12 light:dark with lights on at 7:30 hours. The experimental procedures usually began after the newly arrived animals had been kept on holding conditions for about a week at room temperature 22-23°C. In one experiment, pregnant guinea pigs were purchased.

B: CHEMICALS

Biochemicals, buffers, acids and other reagents were purchased from Sigma Chemicals and Fisher Scientific. Radiochemical L-(5'-^{125} I) thyroxine with specific activity of 1250 μCi/ μg was purchased from DuPont (NEN). Cation exchange resin (AG 50 W-X2 100-200 mesh) for thyroxine 5'-deiodinase assay was obtained from Bio-Rad. Thyroid hormone-free normal bovine serum was purchased from Biocell Laboratories Calif. Dulbecco Modified Eagle Medium was purchased from GIBCO Laboratories (catalog # 380-2320AJ) and supplemented in our lab with 5 mM D-glucose, 1 mM pyruvic acid, 1 mM ascorbic acid and 20 nM sodium selenite and 4% (w/v) fatty acid free bovine serum albumin.
Collagenase catalog # C 6885, lot 39F68091 was purchased from Sigma.

Part 2 METHODS

A: PROTEIN ESTIMATION

Protein content of BAT homogenates and dispersed BAT cell suspensions was estimated using the Lowry method (Lowry et al.,1951) as modified by Schacterle and Pollack (1973), using bovine serum albumin (Sigma) as the standard. Absorbance readings were made with a Bausch and Lomb Spectronic 20.

B: PREPARATION OF BAT HOMOGENATES

The guinea pigs were killed by decapitation and the interscapular BAT removed immediately. The dissected BAT was promptly cleaned of other adhesive tissue, weighed and placed in ice-cold isolation medium (0.25 mM sucrose, 1 mM HEPES, 1mM EDTA disodium salt pH 7.2) in ratio 1:5 (w/v). The BAT in isolation medium was homogenized for 40-60 s using a Polytron blade homogenizer, set at 4 for power control. After homogenization of BAT, the homogenates were always adjusted with isolation medium to the final volume of 10 mL. Samples of homogenate (0.5 mL) were transferred to microfuge tubes and immediately frozen in liquid nitrogen for later T5'D activity determination. This enzyme is stable in samples of BAT homogenate stored at -80°C for at least four weeks (Kopecký et al. 1986).

C: PREPARATION OF DISPERSED BAT CELLS SUSPENSION FOR THYROXINE 5'-DEIODINASE ASSAY.

Brown adipocytes were isolated from guinea pig interscapular pads as described by Rafael et al. (1986) with some modifications. The tissue was carefully dissected free of connective tissue, chopped finely with scissors and weighed. The pooled tissue (usually from 1-3 animals) was transferred in portions of 1.5 g into 25 mL scintillation vials containing 5 mL of incubation medium. The media used for experiments were either supplemented Dulbecco Modified Eagle Medium (for composition of the medium see Appendix A) or modified Krebs-Ringer solution of the following ion composition (in mM) 142.0 sodium, 5.9 potassium, 1.2 calcium, 1.2 magnesium, 139.0 chloride, 1.2 phosphate, 1.2 sulfate, 9.9 bicarbonate, 30.0 HEPES, 2.8 glucose and 0.64 fatty acid free bovine serum albumin pH 7.4, which had been preequilibrated for 30 min at 37°C in shaking water bath (about 150 cycles per min.). The minced tissue was shaken with the medium for 10 min. Then twelve milligrams of collagenase in 1 mL of medium was added to each vial to get final concentration of 2 mg/mL. The incubation was continued for further 30-40 min with occasional gassing with 5% CO_2 in oxygen. The contents of the vials were then filtered through a 200 μm mesh nylon sieve (Nitex 530) into a 42 mL centrifuge tube and diluted with the collagenase free medium to volume 1:5. The cells were allowed to float under gravity for 10-15 min. and then centrifuged at 300 x g for 3 min at 21°C. The infranatant was removed by aspiration with a syringe and the cell layer was resuspended in 4-5

volumes of medium. Centrifugation and resuspension were repeated two more times. The cell layer was finally diluted with the medium to contain 2-3.0 x 10^6 cells per mL (stock solution) as determined in an improved Neubauer hematocytometer with a depth of 0.1 mm. The brown adipocytes in stock solution were kept at room temperature and used in subsequent experiments. However, slow disintegration of the cells was observed after 4 hrs of incubation as judged by the appearance of fat droplets at the surface of the cell layer.

D: WASHING OF THE ISOLATED CELLS

For the purpose of determination of T5'D II activity in isolated brown adipocytes, the cells (usually 150 000- 200 000) were collected on 3 μm Millipore filters by washing them with 3 mL of bovine serum albumin free buffer containing 250 mM sucrose, 10 mM dithiothreitol and 10 mM HEPES. The filters with washed cells were placed in a microfuge tube and immediately frozen in liquid nitrogen for later T5'D activity determination.

E: PURIFICATION OF COMMERCIAL ^{125}I THYROXINE.

Due to autoradiolysis of ^{125}I thyroxine, the purchased product was purified prior to use. The commercial ^{125}I thyroxine was diluted 1:5 with 0.01 M phosphate buffer pH 7.0 containing 1 mM EDTA disodium salt and passed through a 2 mL column of Sephadex LH-20, prewashed with the same buffer. The eluate containing free ^{125}I was discarded. The column was washed two times with 2mL of water and pure ^{125}I thyroxine was eluted by adding 2mL of ethanol:water (70:30) solution to the column.

F: MEASUREMENT OF THYROXINE 5'-DEIODINASE II ACTIVITY IN BAT

The enzyme activity was measured essentially as described by Visser et al. (1982) and Silva et al. (1987). The measurement of enzyme activity is based on the release of radioactive iodide from ^{125}I labelled thyroxine. The samples, homogenates or cell suspensions, were incubated under nitrogen for 30 min. at 37°C in 200 μL of reaction mixture containing approximately 0.15 nM 125-I thyroxine (tracer, to give about 50 000 c.p.m.) plus 2.32 nM unlabelled thyroxine (carrier) with a total thyroxine concentration circa 2.50 nM, 1 mM EDTA, 10mM dithiothreitol and 1 mM 6-n-propyl-2-thiouracil to inhibit type I thyroxine deiodinase. Each assay tube contained 50-100 μg homogenate protein or 200 000 cells in 200 μL of reaction mixture. Under these conditions the rate of ^{125}I liberation from radiolabelled thyroxine was linearly related to protein or cell content of the reaction mixture (Figures 1 and 2). The reaction was stopped by the addition of 50 μL of ice-cold thyroid hormone free bovine serum that caused the substrate, thyroxine and the reaction product, 3,5,3'-triiodothyronine, to bind to

thyroid hormone binding proteins in the serum. These protein-bound complexes were precipitated with 300 μL of 12% trichloro-acetic acid followed by centrifugation at maximum speed for 5-8 m in a bench Eppendorf micro-centrifuge. Aliquots, i.e. 400 μL of supernatants were loaded on to 2 mL columns of Dowex AG 50 W-X2, 100-200 mesh cation exchange resin, preequilibrated with acetic acid:water (1:10). The resin binds any ^{125}I thyroxine left that may not have been bound to the serum proteins and precipitated. The loaded columns were washed three times with 1 mL of acetic acid: water (1:10) to elute free iodide (I) and the wash was collected in plastic tubes and counted in a Beckman Gamma 5500 counter. A computer programme was used to calculate enzyme activity. Activity was expressed as a specific activity (fmol/mg protein/hr) or total activity (pmol/hr in total tissue proteins) of T_3 released during the reaction. Corrections were made for the decay of ^{125}I (half-life 60 days), background counts, autoradiolysis and counting efficiency of the Gamma counter. Autoradiolysis of ^{125}I thyroxine that had occurred during the assay was measured using tubes where 50 μL of buffer instead of enzyme protein were incubated along with the samples.

G: STATISTICS

Experimental data are expressed as means ± Standard Error. Student t-tests (Bruning and Kintz 1977) were used to assess the significance of the difference between the means. The means were considered significantly different when $P < 0.05$.

Results are presented in tabular and graphical form.

FIGURE ONE: THE INITIAL RATE OF TRIIODOTHYRONINE RELEASE AT INCREASING CONCENTRATION OF BAT HOMOGENATE PROTEIN

BAT homogenates from cold-acclimated guinea pigs were prepared as described in section B and assayed as described in section F. Each point represents the mean of two separate experiments done in triplicate. Values for each point are: 62 ($\pm$6.5); 120 ($\pm$11.7); 246 ($\pm$14.0); 278 ($\pm$16.7); 296 (15.3) fmol T_3 released/hr.

FIGURE 1

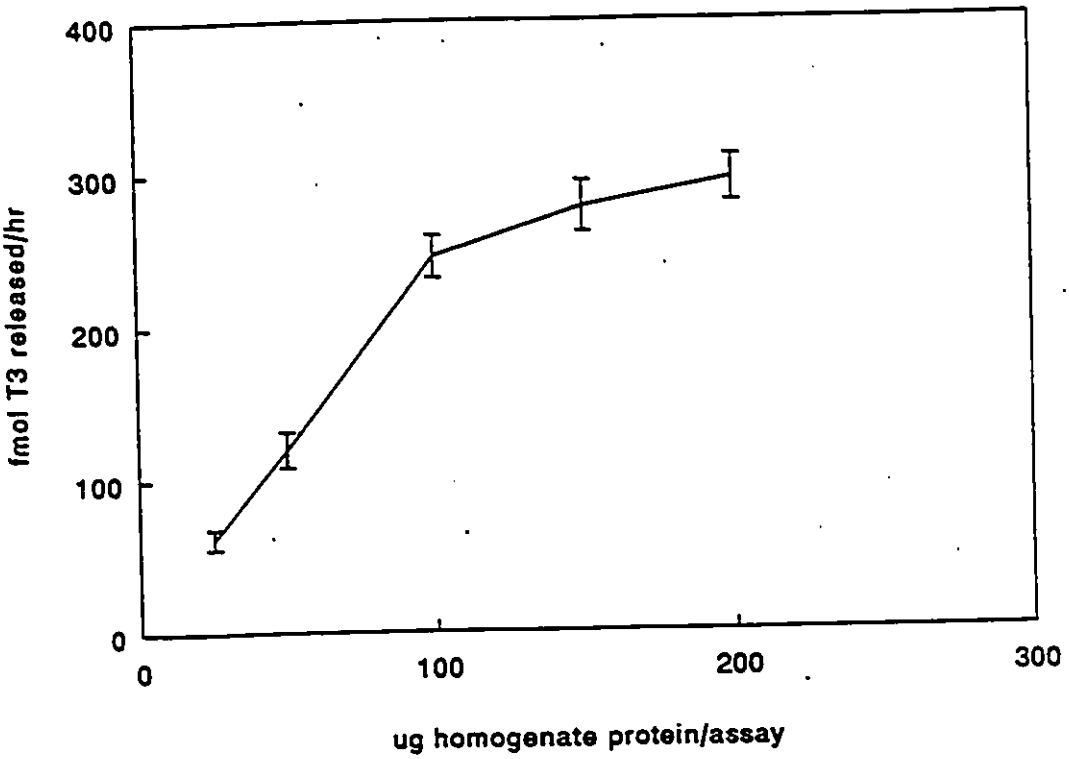
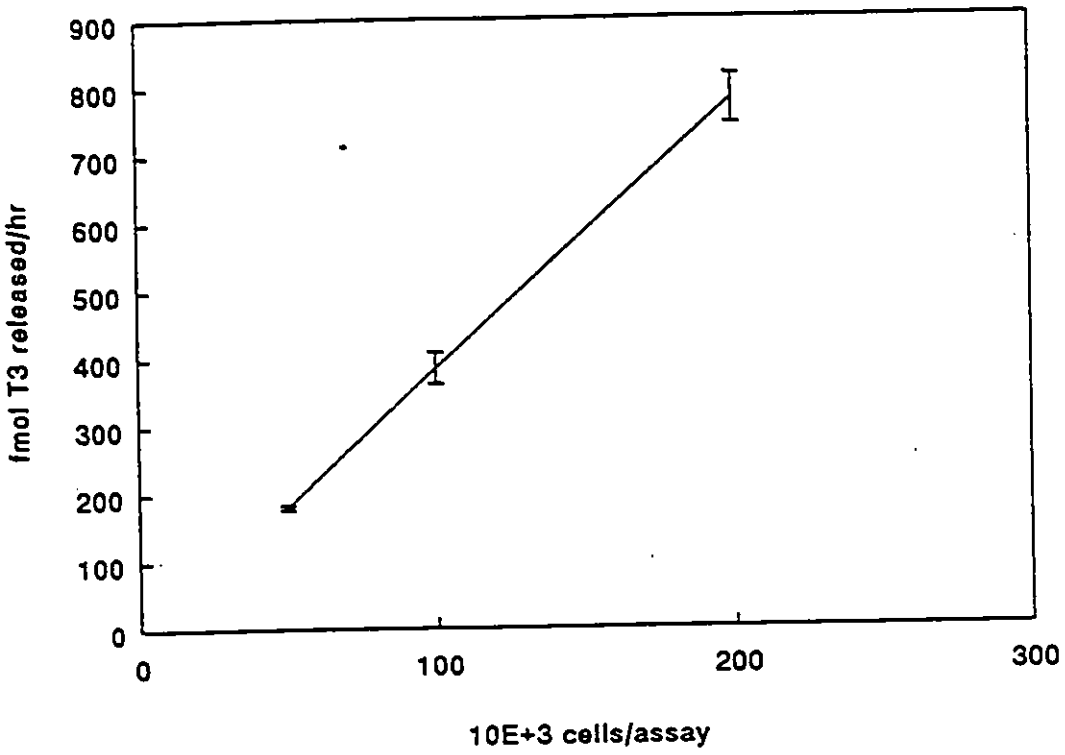

400
300
200
100
0
fmol T3 released/hr
0
100
200
300
ug homogenate protein/assay

FIGURE TWO: THE INITIAL RATES OF TRIIODOTHYRONINE RELEASE BY INCREASING NUMBERS OF CELLS.

BAT cells from cold-acclimated guinea pigs were prepared as described in section C and D and assayed as described in section F. Each point represents the mean of three separate experiments done in triplicate. Values for each point are: 180 ($\pm$3.5); 385 ($\pm$23.5); 780 ($\pm$36.6) fmol T_3 released/hr.

FIGURE 2


900
800
700
600
500
400
300
200
100
0
fmol T3 released/hr
0
100
200
300
10E+3 cells/assay

CHAPTER FOUR

RESULTS

THE TIME COURSE OF CHANGES IN THYROXINE 5'-DEIODINASE ACTIVITY IN BAT OF GUINEA PIGS DURING ACCLIMATION TO COLD.

Recent studies have demonstrated that there are species differences in the control of T5'D activity in BAT during acclimation to cold. Thus, rats and Syrian hamsters exposed to acute cold maintain a high level of T5'D activity as long as they remain in the cold. However, as the total activity of the enzyme in both species shows a slow progressive increase, the specific activity of the enzyme declines within one or two days in the cold (Kopecký et al., 1986, Park and Himms-Hagen 1988). On the other hand, acute cold exposure of mice stimulates an increase in activity of this enzyme but its level returns to normal after about two weeks in the cold, in spite of continuing stimulation of the tissue (Eley and Himms-Hagen 1989). The following experiment was undertaken to assess which of the above mentioned patterns of the control of T5'D activity expression applies to BAT of the guinea pig in long term exposure to cold.

METHODS:

Guinea pigs that were initially at 22°C were divided randomly into two groups and weighed. One group of animals was placed in a cold room at +4°C, another at +28°C. Initially four guinea pigs were weighed and killed by decapitation after half day of exposure to cold. Afterwards, four guinea pigs cold exposed and two guinea pigs warm exposed were weighed and killed as above after 1, 3, 14 and 28 days. Their interscapular BAT was removed and homogenized. Samples of homogenate (0.5 mL) were immediately frozen in liquid nitrogen and stored at -80°C for later determination of protein and T5'D activity as described in Materials and Methods.

RESULTS:

The initial body weight did not differ between warm and cold acclimated animals. After thermal acclimation of three days, the body weights of warm acclimated animals were significantly higher than those of cold acclimated animals (Figure 3). BAT weight of warm acclimated guinea pigs after three days of exposure to +28°C showed a steady increase, indicating accumulation of lipids in the tissue. However, cold acclimation of guinea pigs caused rather a decrease in BAT weight, suggesting depletion of tissue lipids (Figure 4).

Total protein content of BAT (Figure 5) in warm acclimated guinea pigs changed little during the 28 days of experiment. In cold acclimated guinea pigs,

total BAT protein content increased significantly by 28 days (Figure 5) and this trend and was inversely correlated with BAT weight of cold acclimated animals (compare Figure 4).

BAT thyroxine 5'-deiodinase activity is shown in Figures 6 and 7. Warm acclimated guinea pigs after three days of exposure to 28°C showed an abrupt decrease in both total and specific activity of T5'D (five and seven fold respectively) and afterwards the activity of the enzyme remained at a very low level for the time spanning the experiment. Acclimation of guinea pigs to cold resulted in a progressive and very large increase in the activity of T5'D (Figure 6 and 7). By day twenty eight of the experiment both total and specific activity reached the highest values without any trend to decline. The total and specific activity increased sixty and forty five fold respectively, relative to the average basal level at +28°C.

The increase in T5'D activity occurred more rapidly and to much greater extent than did total BAT protein (compare Figure 5 and 7)

FIGURE THREE: EFFECT OF AMBIENT TEMPERATURE ON BODY WEIGHT.

Animals were housed individually in cages at +4°C or +28°C for up to 28 days. The number of animals used for each determination was 4 for those at 4°C and 2 for those at 28°C. Values represent means ± S.E.

SYMBOLS:

* Significant difference compared to those housed at +28°C

FIGURE 3

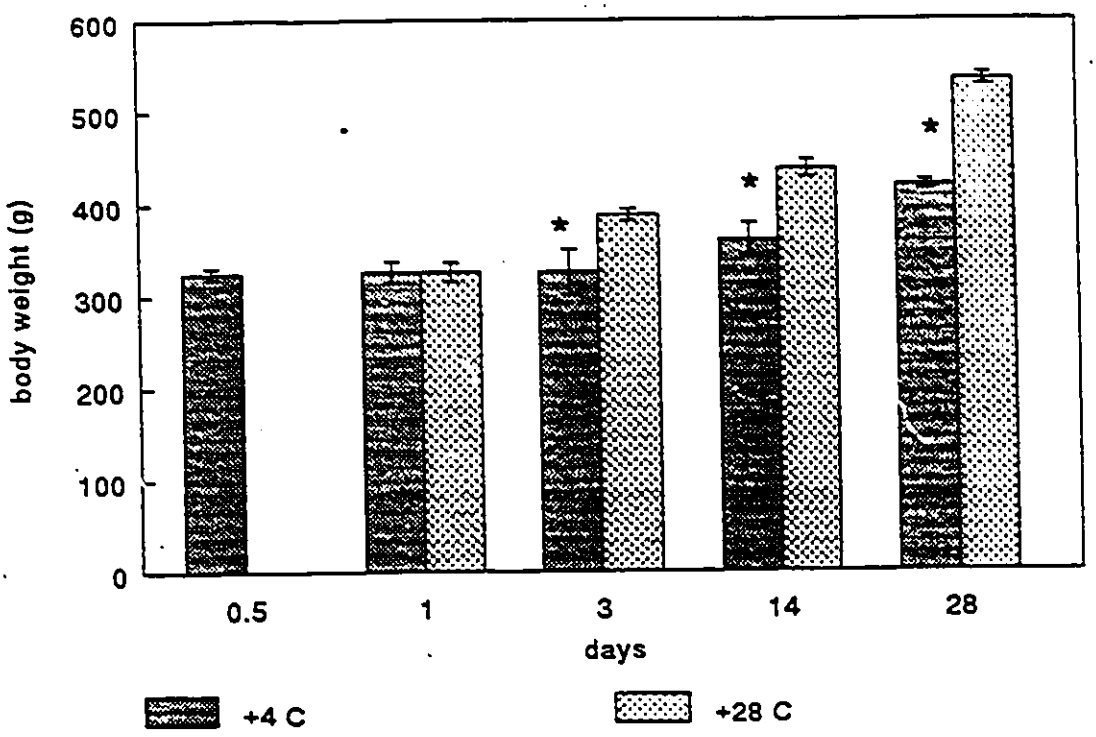


FIGURE FOUR: EFFECT OF AMBIENT TEMPERATURE ON WET WEIGHT OF INTERSCAPULAR BAT.

Experimental conditions as in Figure 3. Values are means ± S.E.

SYMBOLS:

* Significant difference compared to those housed at +28°C

FIGURE 4

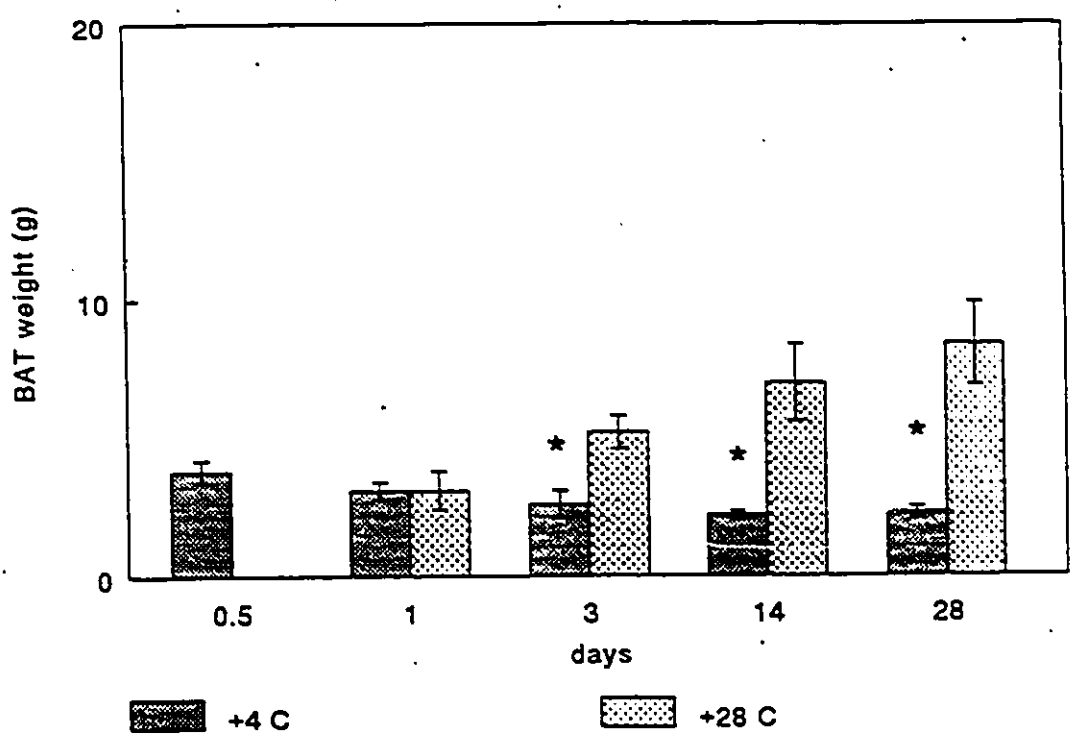


FIGURE FIVE: EFFECT OF AMBIENT TEMPERATURE ON TOTAL PROTEIN CONTENT OF INTERSCAPULAR BAT.

Experimental conditions as in Figure 3. Values are means ± S.E.

SYMBOLS:

* Significant difference compared to those housed at +28°C

FIGURE 5

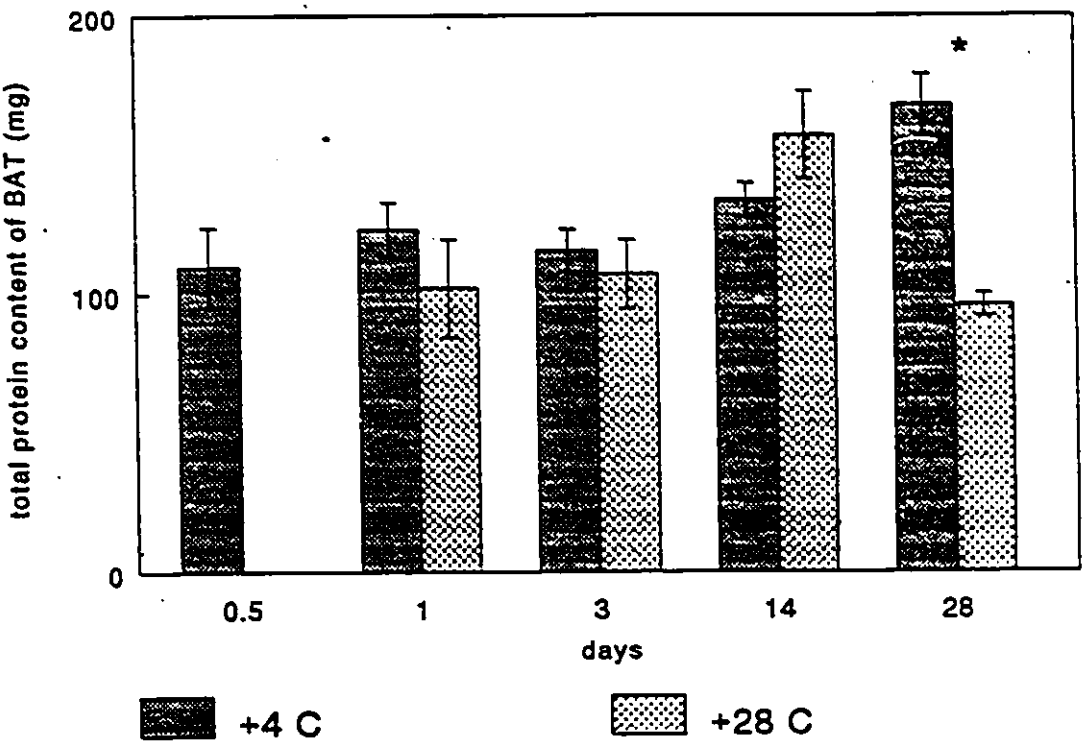

200
100
0
total protein content of BAT (mg)
*
0.5
1
3
14
28
days
+4 C
+28 C

FIGURE SIX: EFFECT OF AMBIENT TEMPERATURE ON THE SPECIFIC ACTIVITY OF THYROXINE 5'-DEIODINASE IN BAT.

Experimental conditions as in Figure 3. Values are means ± S.E.
S.E. bars are too small to be seen for warm acclimated animals.
Values for warm acclimated animals are: 287 (±45); 41 (±1.9); 35 (±1.2) and 58 (±3.5) fmol T_3 released/mg protein/hr

SYMBOLS:

* Significant difference compared to those housed at +28°C

FIGURE SEVEN: EFFECT OF AMBIENT TEMPERATURE ON THE TOTAL ACTIVITY OF THYROXINE 5'-DEIODINASE IN BAT

Experimental conditions as in Figure 3. Values are means ± S.E.
S.E. bars are too small to be seen for warm acclimated animals.
Values for warm acclimated animals are: 15.0 (±1.0); 4.3 (±0.3); 4.2 (±0.5) and 5.1 (±0.1) pmol T_3 released/hr

SYMBOLS:

* Significant difference compared to those housed at +28°C

FIGURE 6

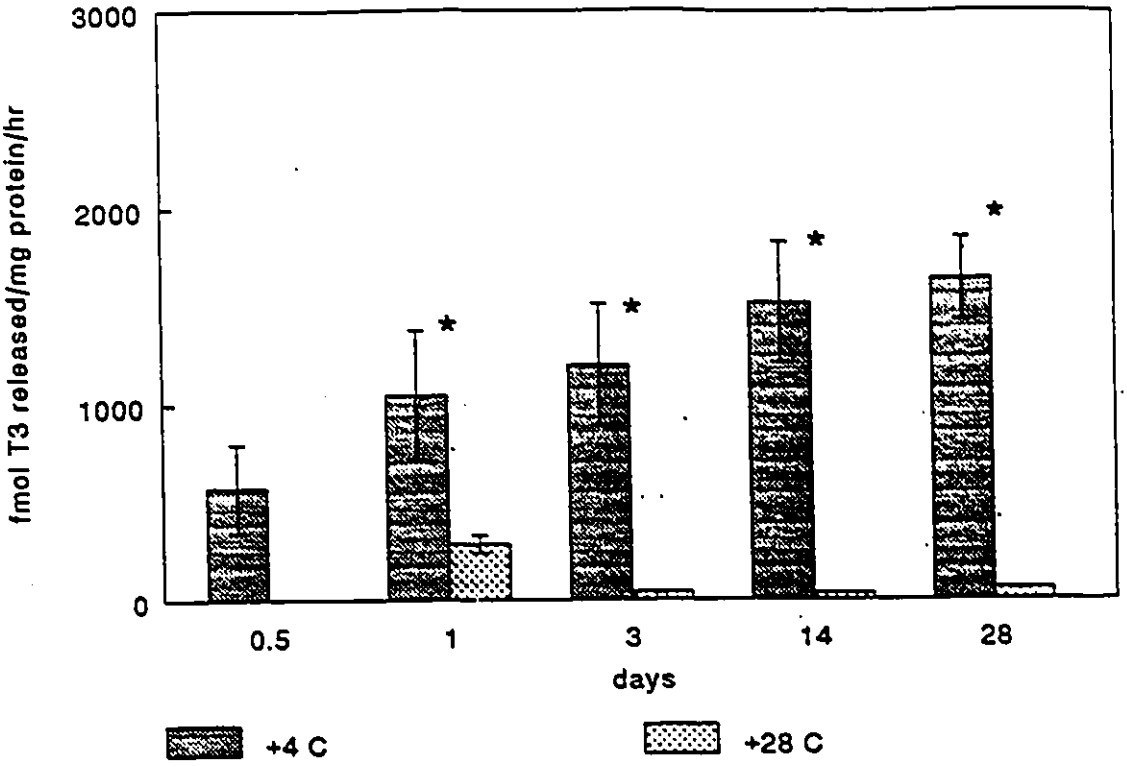
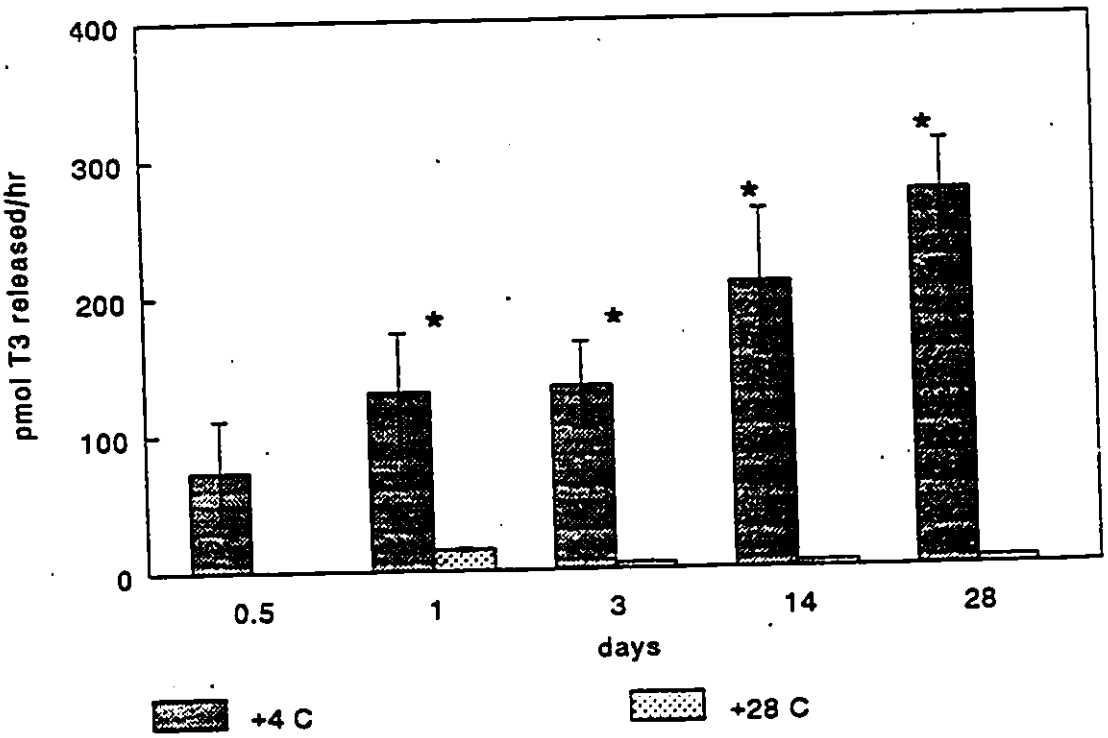


FIGURE 7



TIME COURSE OF CHANGES IN THYROXINE 5'-DEIODINASE ACTIVITY IN PUPS OF GUINEA PIG DURING POSTNATAL PERIOD.

In the previous experiment it was shown that cold stimulated activation of BAT was accompanied by a high level of T5'D activity.

There was some indication that activity of the enzyme might be higher in younger guinea pigs.

The guinea pig, which belongs to precocious animals, is born with highly innervated, well developed BAT (Huttunen and Kinnula, 1979). In this animal the thermogenic activity of BAT is maximal at birth as shown by a high GDP binding (Rafael and Heldt 1976) and UCP level (Ricquier et al., 1979), which are highest during the first day of life and rapidly decrease towards adulthood.

The purpose of this experiment was to assess whether in the absence of sympathetic stimulation of BAT (i.e. temperature close to 28°C) activity of T5'D in very young guinea pigs is higher than that of adult or/and whether changes in T5'D activity parallels decreasing thermogenic activity of BAT.

METHODS:

Pregnant guinea pigs were housed individually at +28°C. Near term, the guinea pigs were checked every two or three hours and the delivery of pups was recorded. The new-born guinea pigs remained with their dams in cages until they were sacrificed. The pups whose time of delivery was known to be ± an hour were sacrificed on the first or third day of experiment, whereas those with less precisely known time of delivery were sacrificed later, that is on day 7,14 and 35 of the experiment. No more than two siblings were used for determination of each time-point. Both female and male pups were used. The pups were weighed and killed by decapitation, the interscapular BAT dissected weighed and homogenized. Homogenate aliquots (0.5mL) were immediately frozen in liquid nitrogen and stored at -80°C for determination of protein and T5'D activity as described in Materials and Methods.

RESULTS:

Mean body weight and interscapular BAT weight in guinea pig pups (Table 1) increased progressively during 35 days of the experiment. However, the tissue weight relative to body weight did not change (Table 1). In contrast, protein concentration of BAT relative to tissue weight dropped five fold. BAT thyroxine 5'-deiodinase activity values throughout the experiment are shown in Table 1 and figures 8 and 9. There was no significant change in total T5'D activity, however when expressed as specific activity it reached statistical significant difference for the thirty fifth day of the experiment (Table 1 and figure 8), which coincided with a decrease in protein concentration in the BAT (Table 1).

TABLE ONE: THE CHANGES OF SOME PHYSIOLOGICAL DATA DURING POSTNATAL PERIOD IN GUINEA PIG PUPS.

The pups were housed with their dams at +28°C. Values represent means ± S.E. Four pups were used for each determination.

Symbols:

* Significant difference compared to those sacrificed at day one.

TABLE 1

Days at +28°C	Body weight (g)	BAT weight (g)	BAT protein mg/g tissue	T5'D fmol/mg protein/hr.	T5'D pmol/hr
1	117.7 ±3.8	2.05 ±0.35	62.4 ±5.9	102.1 ±5.5	13.1 ±0.75
3	113.4 ±3.2	2.10 ±0.24	55.7 ±13.7	137.3 ±24.5	14.7 ±0.80
7	180.4 ±16.9*	2.75 ±0.35	39.2 ±8.25	115.2 ±19.0	12.1 ±1.25
14	240.6 ±10.9*	3.63 ±0.25*	20.8 ±6.4*	124.0 ±28.0	12.5 ±0.42
35	425.6 ±23.8*	7.58±0.79*	13.4 ±2.3*	165.5 ±14.3*	15.3 ±0.90

FIGURE EIGHT: SPECIFIC ACTIVITY OF THYROXINE 5'-DEIODINASE IN BAT OF GUINEA PIG PUPS.

The pups were housed with their dams in cages at +28°C. Four pups were used for each determination. Values are mean ± S.E.

SYMBOLS:

* Significant difference compared to those sacrificed at day one.

FIGURE NINE: TOTAL ACTIVITY OF THYROXINE 5'-DEIODINASE IN BAT OF GUINEA PIG PUPS.

Experimental conditions as in Figure 8. Values are mean ± S.E.

FIGURE 8

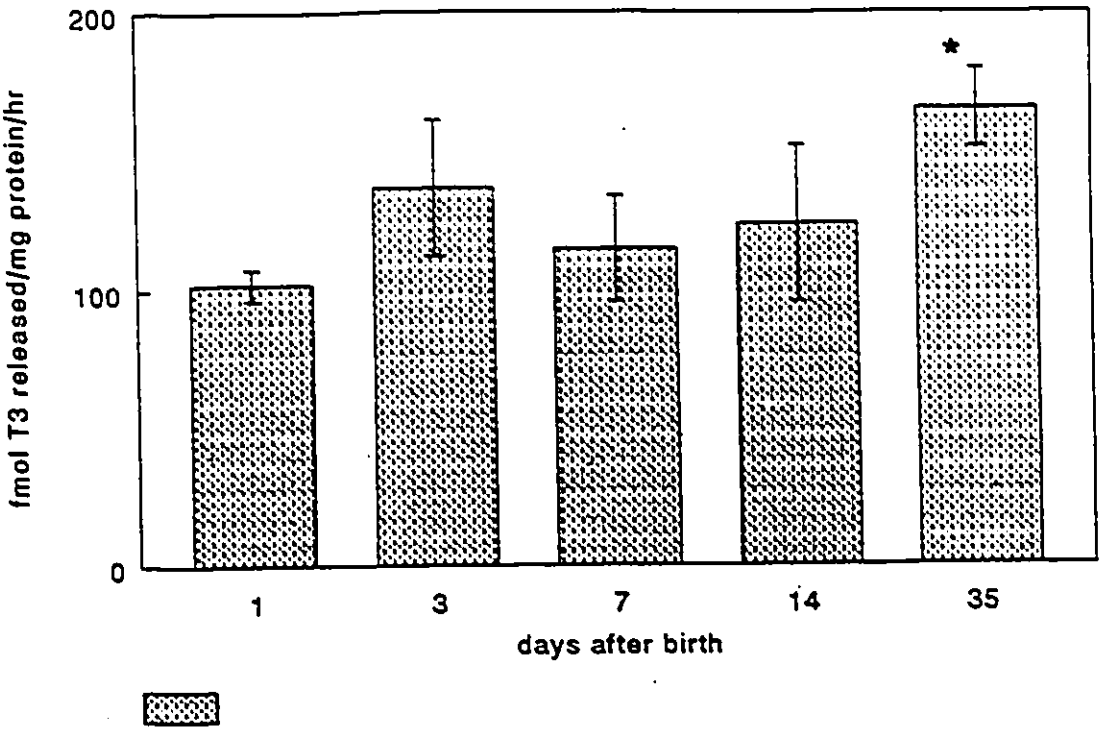
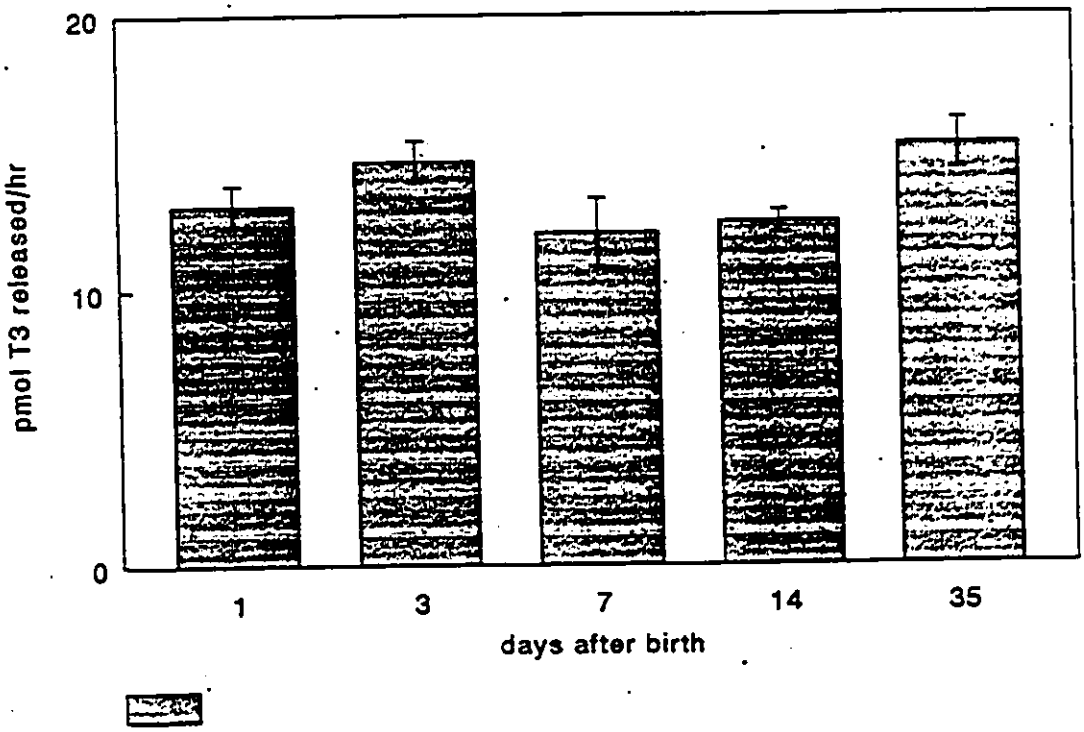


FIGURE 9



THYROXINE 5'-DEIODINASE ACTIVITY IN DISPERSED BAT CELLS FROM WARM AND COLD ACCLIMATED GUINEA PIGS: EFFECT OF MEDIUM.

In my first experiment I have demonstrated that cold acclimation of the guinea pig is associated with a very large increase in BAT T5'D activity. The initial purpose of this experiment was to find out whether a high level of T5'D activity in cold acclimated animal is maintained in dispersed BAT cells and how long it persists and whether BAT cells obtained from warm acclimated animal retain any T5'D activity.

METHODS:

Guinea pigs were divided randomly into two groups and placed for one week at +4°C or +28°C. After a week of acclimation they were used for experiments. Usually 1-2 guinea pigs were used for each preparation of dispersed BAT cells as described in section C of Materials and Methods. The isolated BAT cells were used immediately for the experiments. For the purpose of experiments, the cells from the stock solution were diluted to 1 000 000 cells/mL with Krebs-Ringer solution or with Dulbecco Modified Eagle Medium and incubated for 4 hours in a water bath at +37°C with gentle shaking. At the beginning of

incubation, designated as zero time, and afterwards at two hour intervals, 200 000 cells were taken from the incubation vial, filtered an frozen as described in section D of Materials and Methods and used for T5'D activity determination within 24 hours. BAT homogenates were prepared in Modified Eagle Medium or in Krebs-Ringer solution as described in section B of Materials and Methods and incubated in thc same conditions as dispersed BAT cells for T5'D activity determination.

RESULTS:

Figure 10 illustrates the effect of acclimation conditions on T5'D activity in dispersed BAT cells prepared in Krebs-Ringer solution. At zero time, activity of the enzyme in cells of cold acclimated animals was 12 times higher than that of warm acclimated animals. During the course of incubation, T5'D activity gradually declined and the loss in activity by the fourth hour both for warm and cold acclimated animals was 50%, maintaining the same ratio.

In order to assess the approximate relative level of T5'D activity in dispersed BAT cells, homogenates of BAT from the same cold-acclimated guinea pigs were prepared in Krebs-Ringer solution and incubated in the same conditions as the cells.

Figure 11 illustrates changes in T5'D activity in BAT homogenates and dispersed BAT cells from cold-acclimated guinea pigs. For comparative reasons, the enzyme activity both for homogenates and dispersed cells was expressed in terms of protein concentration. Since preparation of the dispersed cells takes a

much longer time than that of homogenates, Figure 11 shows T5'D activity at -3 hrs, that is immediately after preparation of homogenates and at -1.5 hr before zero time of incubation for the dispersed cells. The largest decrease in T5'D activity occurred during the first -1.5 hr. (45%) and afterwards activity declined progressively so that by the fourth hour of incubation homogenates retained only 25% of the original activity. In dispersed BAT cells T5'D specific activity was very high relative to the homogenates; at zero time it surpassed more than two times that of homogenates. The T5'D activity in dispersed BAT cells decreased during incubation in parallel with homogenates (Figure 11).

The much higher activity of the enzyme in the dispersed cells as compared to homogenates could be explained by lower content of balance proteins such as connective tissue proteins after preparation of the cells and removal of cells other than brown adipocytes that do not contain T5D activity.

The activity of T5'D in dispersed BAT cells in Krebs -Ringer solution from warm acclimated animals was low and disappeared rapidly (Figure10), presumably due to degradation or inactivation. Therefore, I employed Dulbecco Modified Eagle Medium in an attempt to slow down the loss in activity of the enzyme. Figure 12 shows the effect of the employed media on changes in T5'D activity in dispersed BAT cells from warm-acclimated animals, prepared either in Krebs-Ringer solution or in Dulbecco Modified Eagle Medium. At zero time the activity of T5'D for both media was approximately the same. During the time of incubation, dispersed BAT cells prepared in Krebs-Ringer solution lost their T5'D activity comparably to the previous experiment (Figure 10). However, the cells prepared in Modified Eagle Medium not only maintained the activity of the enzyme but contrary to any expectations increased it to such an extent that by the fourth hour of incubation

T5'D activity almost doubled, relative to zero time (Figure 12).

Figure 13 depicts the same experiment but activity of T5'D is expressed here in terms of protein content of the dispersed BAT cells. Again, there was a clear decreasing and increasing trend in T5'D activity for the respective medium, however, the differences in activity of T5'D for the time intervals were not statistically significant and this was the result of large variations in protein content amongst samples as seen by large error bars.

The increase of T5'D activity in BAT dispersed cells prepared in Modified Eagle Medium was surprising, therefore I prepared BAT homogenates not in the medium described in section B Materials and Methods but in Modified Eagle Medium to find out whether activity of T5'D would increase during the course of incubation. Figure 14 illustrates changes in T5'D activity in BAT homogenates. The activity of T5'D at -3.0 hrs. that is, immediately after preparation of BAT homogenates and at -1.5 hr. before zero time of incubation for the dispersed cells is shown on the graph. During the first -3.0 hrs. of incubation there was slow but progressive trend in the increase of T5'D activity. The biggest increment in T5'D activity occurred between 2 and 4 hrs of incubation (Figure 14). Comparing the above results with those on dispersed cells, the main finding is that the increase in T5'D activity in dispersed BAT cells was not coincidental, secondly, when T5'D activity in dispersed cells was expressed in terms of specific activity it was comparable to activity for homogenates. Furthermore, the increase in T5'D activity was not immediate but delayed in time.

FIGURE TEN: EFFECT OF AMBIENT TEMPERATURE ON THYROXINE 5'-DEIODINASE ACTIVITY IN DISPERSED BAT CELLS

The animals were housed for one week at +4°C or +28°C. Dispersed BAT cells were prepared in Krebs-Ringer solution. Values represent means ± S.E. for three separate experiments. For warm acclimated animals S.E. bars are too small to be seen. Values for the cells from warm-acclimated animals are: 62.3 (±5.0); 46.4 (±8.4); 27.6 (±6.2) fmol T3 released/1E+6 cells/hr

SYMBOLS:

* Significant difference compared to cells from warm-acclimated guinea pigs.

& Significant difference compared to those cells incubated 4 hrs.

FIGURE 10

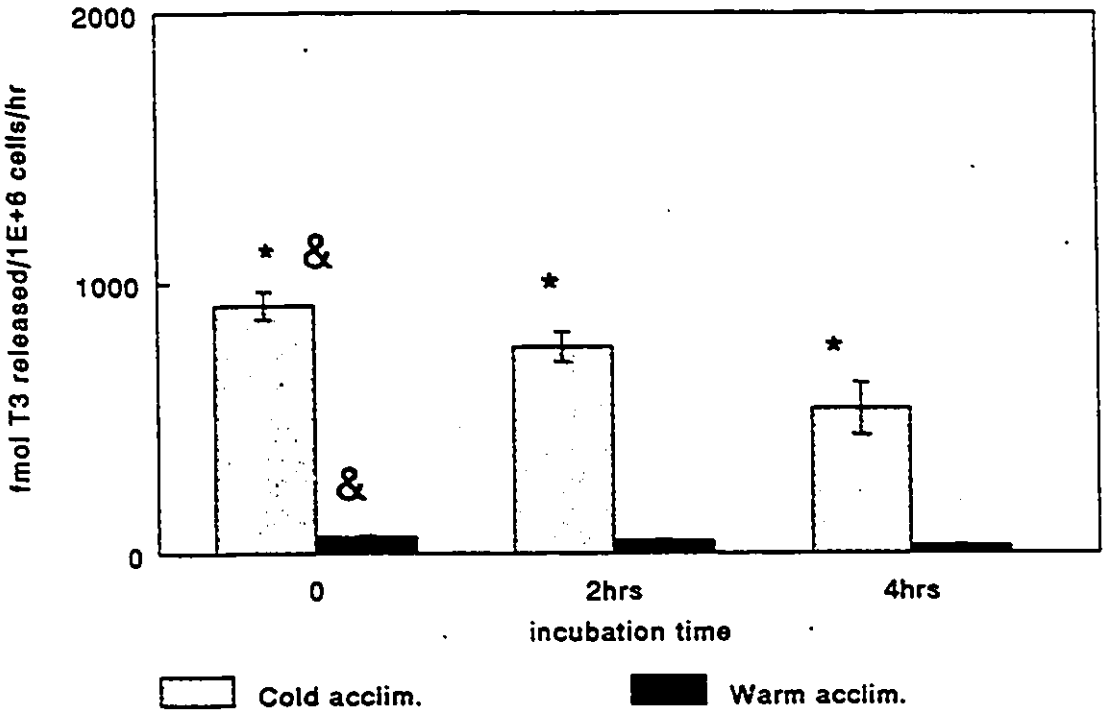

2000
1000
0
fmol T3 released/1E+6 cells/hr
* &
&
*
*
0
2hrs
4hrs
incubation time
Cold acclim.
Warm acclim.

FIGURE ELEVEN: TIME COURSE OF CHANGES IN THYROXINE 5'-DEIODINASE ACTIVITY IN BAT HOMOGENATES AND DISPERSED BAT CELLS

The animals were housed one week at +4°C. BAT homogenates and dispersed brown adipocytes were prepared in Krebs-Ringer solution and incubated at +37°C. Values are means ± S.E. for three separate experiments.

SYMBOLS:

* Significant difference compared to homogenates at -3.0 hr.

& Significant difference compared to cells at 0 hrs.

FIGURE11

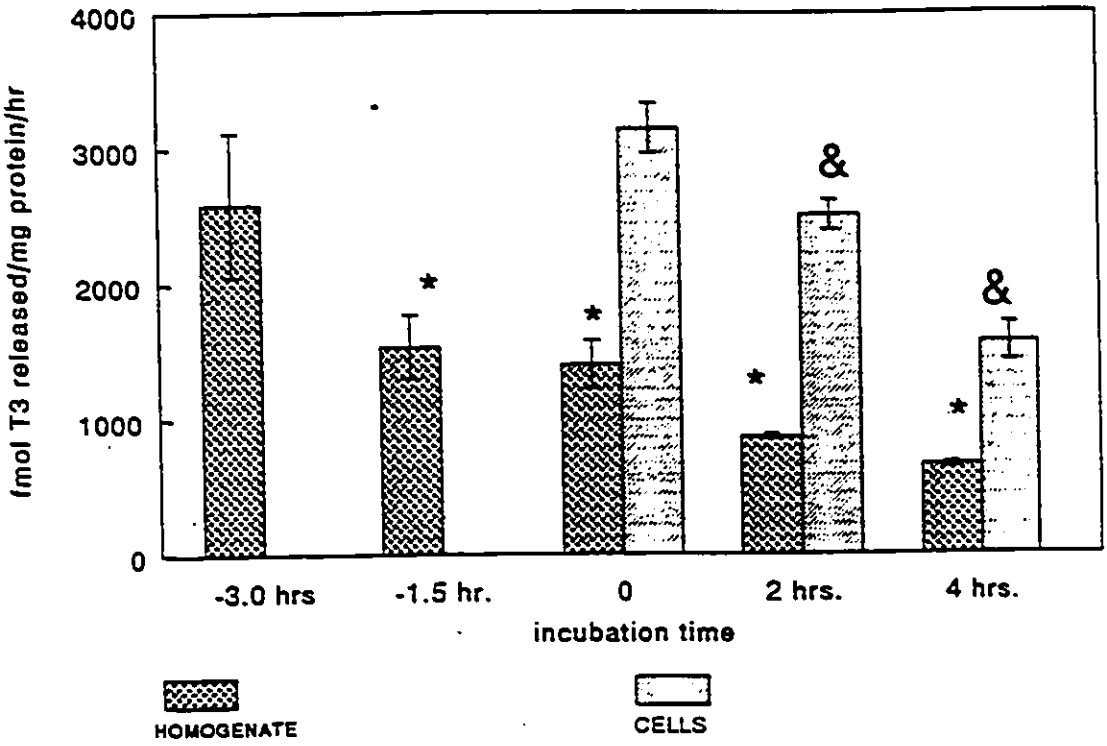

4000
3000
2000
1000
0
fmol T3 released/mg protein/hr
-3.0 hrs
-1.5 hr.
0
2 hrs.
4 hrs.
incubation time
*
&
HOMOGENATE
CELLS

FIGURE TWELVE : TIME COURSE OF CHANGES IN THYROXINE 5'-DEIODINASE ACTIVITY IN DISPERSED BAT CELLS

The animals were housed one week at +28°C. The dispersed BAT cells were prepared either in Dulbecco Modified Eagle Medium or in Krebs-Ringer solution and incubated at +37°C. Values are means ± S.E. for three separate experiments.

SYMBOLS:

DMEM - Dulbecco Modified Eagle Medium

K-R - Krebs-Ringer solution

* Significant difference compared to cells incubated 4 hrs. in Modified Eagle Medium

& Significant difference compared to cells incubated 4 hrs. in Krebs-Ringer solution

FIGURE THIRTEEN:TIME COURSE OF CHANGES IN SPECIFIC ACTIVITY OF THYROXINE 5'-DEIODINASE IN DISPERSED BAT CELLS

Experimental conditions as in Figure 12. Values are mean ± S.E.

FIGURE12

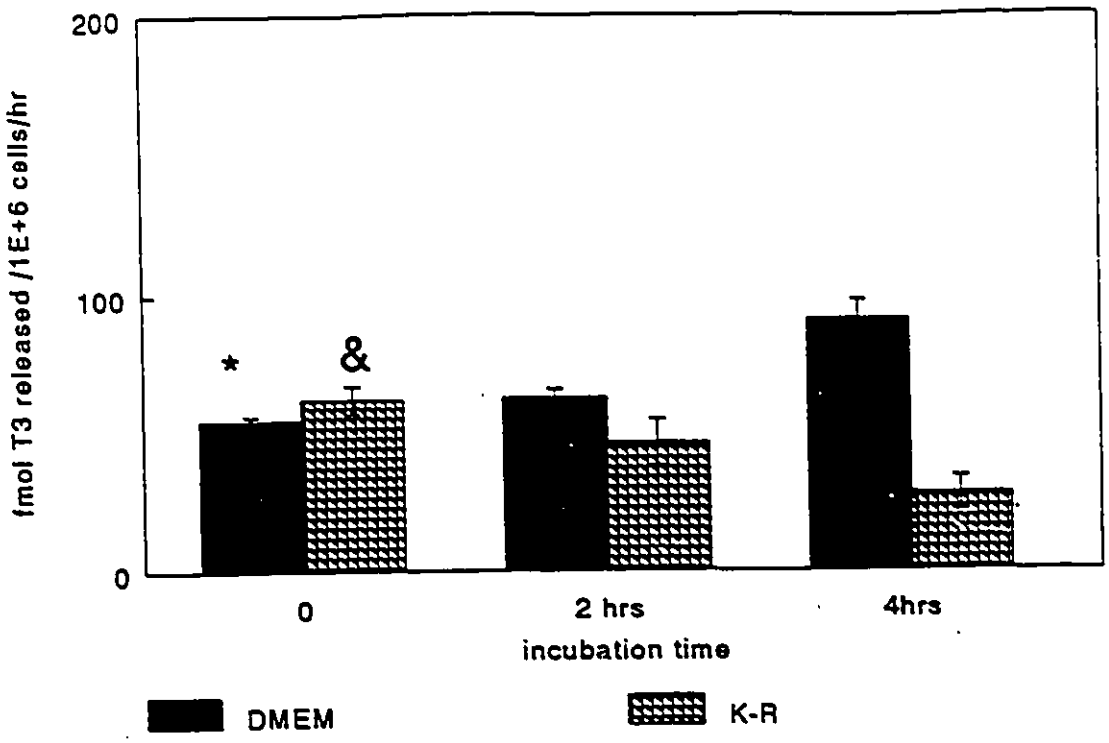


FIGURE13

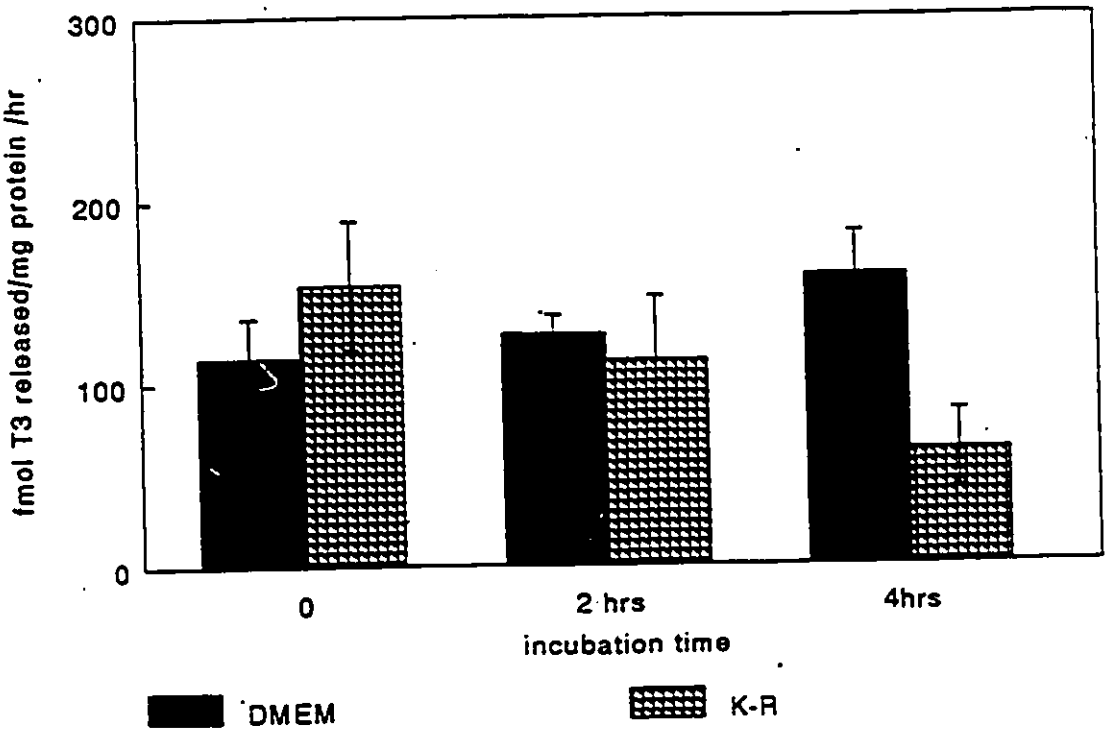


FIGURE FOURTEEN: TIME COURSE OF CHANGES IN THYROXINE 5'-DEIODINASE ACTIVITY IN BAT HOMOGENATES

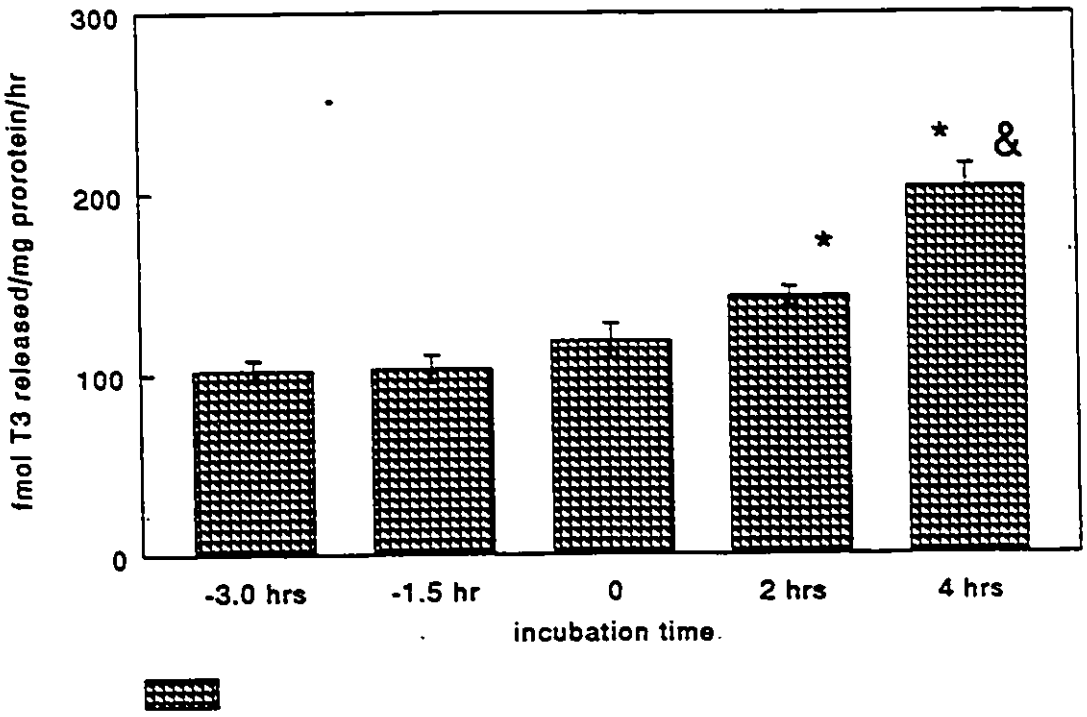
The animals were housed one week at +28°C. BAT homogenates were prepared in Modified Eagle Medium and incubated at +37°C. Values are means ± S.E. for three separate experiments.

SYMBOLS:

* Significant difference compared to homogenates at -3 hrs.

& Significant difference compared to homogenates at 2 hrs.

FIGURE 14


300
200
100
0
fmol T3 released/mg proprotein/hr
*
*
&
-3.0 hrs
-1.5 hr
0
2 hrs
4 hrs
incubation time

NORADRENALINE STIMULATION OF THYROXINE 5'-DEIODINASE ACTIVITY IN DISPERSED BROWN ADIPOCYTES AND IN BAT OF INTACT GUINEA PIGS.

Cold acclimation is known to restore BAT of the adult guinea pig to a highly thermogenic state (Holloway et al., 1984). In the rat this effect is mediated by the action of noradrenaline on alpha-1 and beta receptors. This state, as I have shown, is accompanied by a high activity of T5'D. In the rat the induction of activity of this enzyme is thought to be mediated by an action of noradrenaline on alpha-1 receptors (Silva and Larsen, 1983). The objective of this experiment was to find out whether in the guinea pig noradrenaline could increase T5'D activity, either in vivo or in isolated cells.

METHODS:

After a week of acclimation at +28°C, guinea pigs were used for the experiments. The dispersed BAT cells were prepared in Dulbecco Modified Eagle Medium as described in section C Materials and Methods. To vials containing diluted cells (1 000 000 cells/mL), noradrenaline hydrochloride was added in concentration of 0.1, 0.5, 3.0, 6.0, 10.0, 20.0 μM and the cells were incubated in water bath for 4 hrs. at +37°C with gentle shaking. One hour before the end of incubation 2 mM dithiothreitol was added to each vial to activate T5'D. The

controls were incubated without noradrenaline and represent basal level. At the end of incubation, the vials were placed on ice. 200 000 cells were taken from each vial, filtered and frozen as described in section C Materials and Methods and used for T5'D activity determination the next day.

For the in vivo experiment on intact guinea pigs, the animals remained two weeks at room temperature, 22°C. On the day of experiment, at 10 AM, one group of animals was injected subcutaneously, under sterile conditions, with 100 μg noradrenaline hydrochloride per 100 g of body weight, and another, control group with 0.9% saline. This dose of noradrenaline was used on the basis of previous experiments on rats (Silva and Larsen,1986). After 3, 6 and 9 hrs. of exposure, three guinea pigs from the noradrenaline treated group and two from the control group were killed by decapitation. BAT homogenates were prepared as described in section C Materials and Methods and used for T5'D activity within a week.

RESULTS:

Figure 15 shows effect of varying concentrations of noradrenaline added on activity of T5'D in dispersed BAT cells. Stimulation of T5'D activity in response to the increasing concentration of noradrenaline added was dose related in a range of 0.1-0.5 μM. The dispersed BAT cells did respond to higher concentrations of noradrenaline but the pattern of the response was irregular. Different preparations of BAT cells responded variously to the same concentration of noradrenaline added, as can be assessed from large error bars (Figure 15).

The experiment illustrated in Figure 16 shows response of BAT T5'D to subcutaneous injection of 100 μg/100 g B.W. noradrenaline. Noradrenaline induced an increase in T5'D activity that was apparent only after 9 hours of exposure.

Some indication of a time of day effect on T5'D activity was seen. The basal, unstimulated level of T5'D was lower at 19:00 hr than at 13:00 hr.

FIGURE FIFTEEN: EFFECT OF NORADRENALINE STIMULATION ON THYROXINE 5'-DEIODINASE ACTIVITY IN DISPERSED BAT CELLS

The animals were housed for one week at +28°C. The dispersed BAT cells were prepared in Dulbecco Modified Eagle Medium and incubated with shown concentrations of noradrenaline or without for four hours at +37°C.
Values are means ± S.E. for three separate experiments.

FIGURE 15

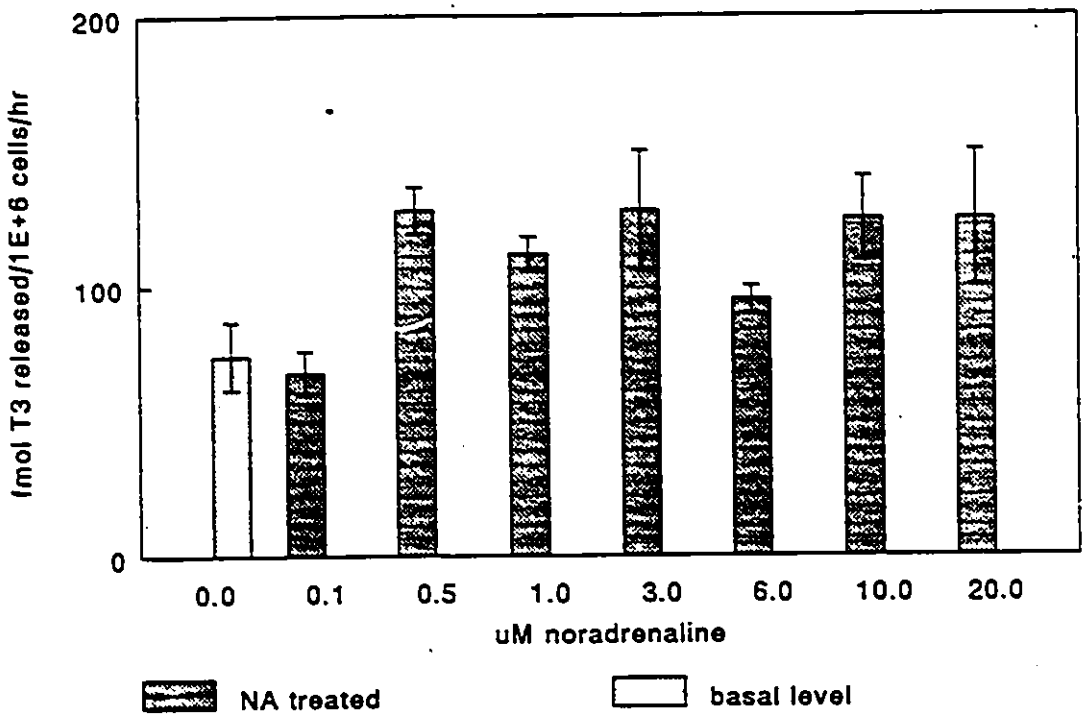

200
100
0
fmol T3 released/1E+6 cells/hr
0.0
0.1
0.5
1.0
3.0
6.0
10.0
20.0
uM noradrenaline
NA treated
basal level

FIGURE SIXTEEN: EFFECT OF NORADRENALINE STIMULATION ON BAT THYROXINE 5'-DEIODINASE ACTIVITY IN INTACT GUINEA PIGS

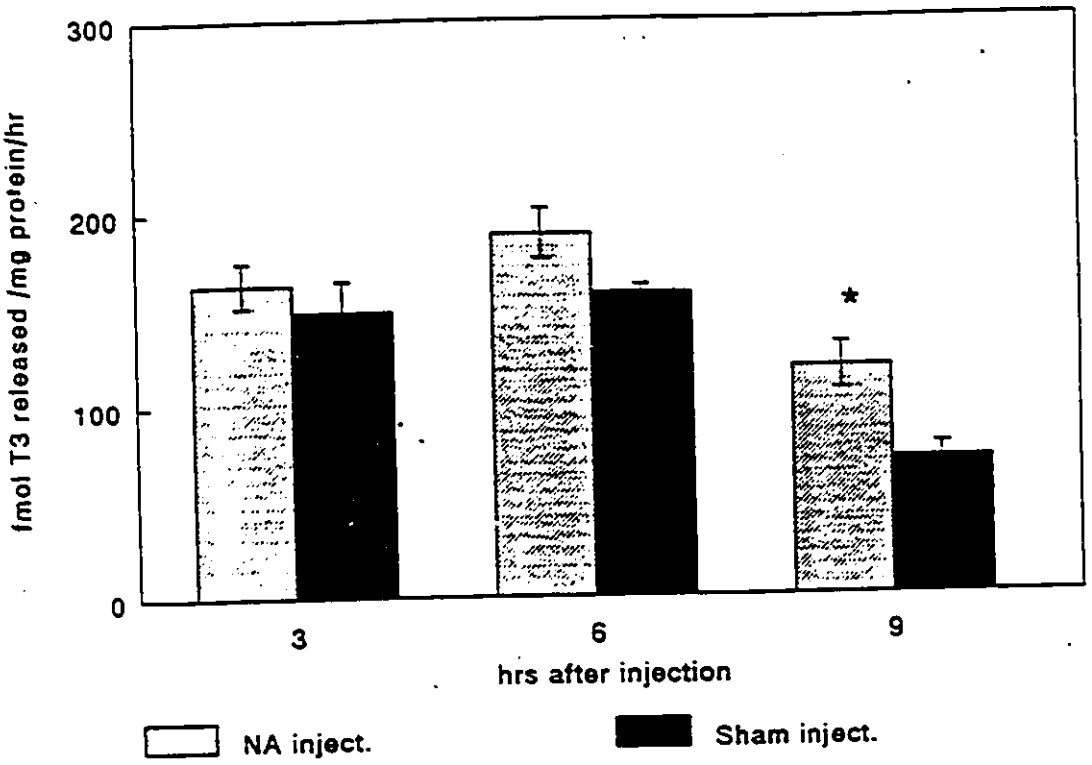
The animals were housed at +22°C. Noradrenaline, 100 μg/100 g B.W. was injected subcutaneously at time zero and BAT tissue was obtained from the animals after 3, 6 and 9 hrs. of exposure.

Three guinea pigs noradrenaline injected and two guinea pigs sham injected were used for each time point. Values are means ± S.E.

SYMBOLS:

* Significant difference compared to those controls at 9 hrs.

FIGURE 16


300
200
100
0
fmol T3 released /mg protein/hr
3
6
9
hrs after injection
*
NA inject.
Sham inject.

NORADRENALINE STIMULATED OXYGEN CONSUMPTION IN DISPERSED BROWN ADIPOCYTES FROM COLD AND WARM ACCLIMATED GUINEA PIGS

In the previous experiment the response to noradrenaline of stimulation of T5'D activity in dispersed BAT cells was poor. Therefore, the following experiment was undertaken to assess whether dispersed BAT cells from warm and cold acclimated animals maintain thermogenic responsiveness to noradrenaline.

METHODS:

After two weeks of acclimation at +4°C or +28°C, one guinea pig was used for each preparation of dispersed BAT cells in Dulbecco Modified Eagle Medium as described in section C Materials and Methods. The dispersed BAT cells obtained from a cold or warm acclimated animal were diluted to one million per mL and used for oxygen consumption experiments within two hours. Oxygen consumption was measured in oxygen-saturated Dulbecco Modified Eagle Medium with a Clark type oxygen electrode (Model 53, Yellow Springs Instruments). Before each experiment, the cells were preincubated in 3 mL chamber by gassing them for 5-6 minutes with 5% carbon dioxide in oxygen. Respiration of the dispersed BAT cells was measured for 7-10 minutes periods, 3-4 minutes after noradrenaline addition when rates of oxygen uptake became linear.

RESULTS:

Figure 17 illustrates noradrenaline stimulated oxygen consumption in dispersed BAT cells from warm and cold acclimated animals. The basal oxygen consumption per million cells obtained from warm acclimated guinea pig was 4.4 nmol/min. Addition of 10 μM noradrenaline to the cell suspension increased oxygen consumption to 8.3 nmol/min, that is, two fold. The dispersed BAT cells prepared from a second warm acclimated guinea pig did not respond to 10 μM noradrenaline and their basal oxygen consumption did not differ from the base line. The basal oxygen consumption per million cells prepared from a cold acclimated guinea pig was 12.4 nmol/min, which was three fold that of the warm acclimated guinea pig. 10 μM noradrenaline stimulated oxygen consumption was 63.9 nmol/min and was increased six fold in comparison to that of basal cold acclimated and fifteen fold of warm acclimated guinea pig BAT cells.

Dose-response relationships between stimulation of oxygen consumption and concentrations of noradrenaline employed are shown in Figure 18. The most effective noradrenaline concentrations seems to be about 1 μM. A higher concentration (10 μM) blunted the response. Although 100 μM noradrenaline stimulated oxygen consumption, the rise was not high in comparison to the effect caused by 1 μM concentration.

FIGURE SEVENTEEN: EFFECT OF AMBIENT TEMPERATURE ON OXYGEN CONSUMPTION IN NORADRENALINE STIMULATED DISPERSED BAT CELLS

The animals were housed two weeks either at +4°C or +28°C. The dispersed BAT cells were prepared in Dulbecco Modified Eagle Medium.
10 μM noradrenaline were added to a suspension containing one million cells/mL obtained from warm or cold-acclimated guinea pig.
Values represent single measurements for warm acclimated and two measurements for cold acclimated animals.

FIGURE 17

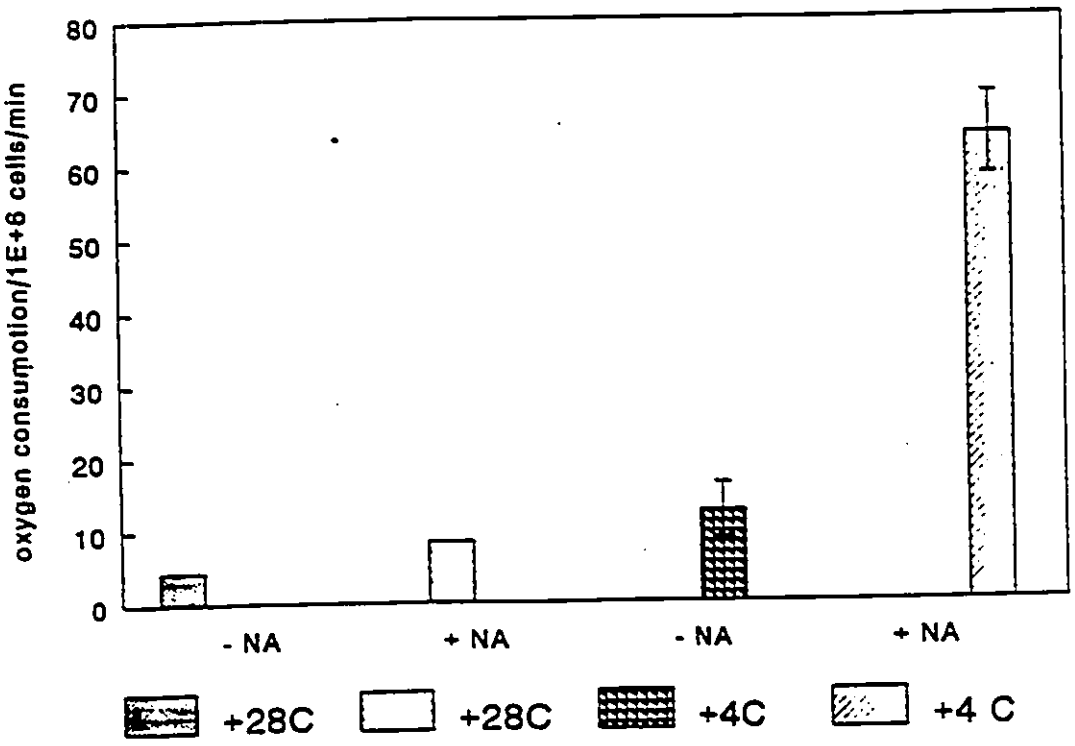
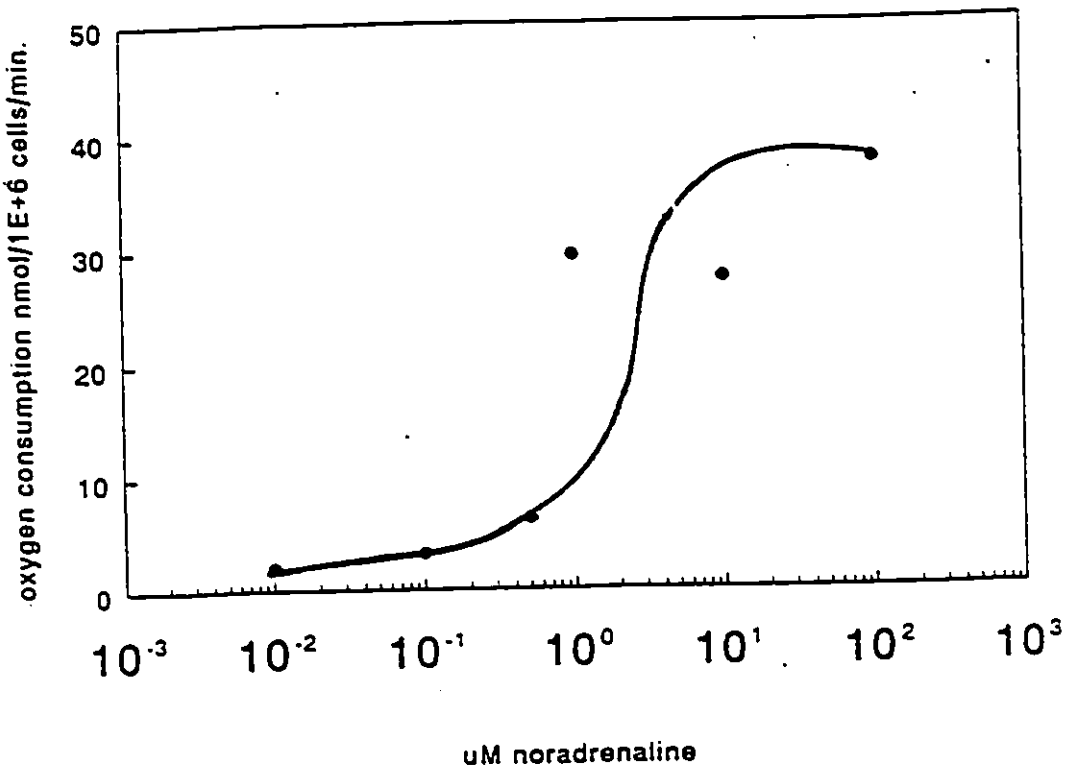

oxygen consumption/1E+6 cells/min
80
70
60
50
40
30
20
10
0
- NA
+ NA
- NA
+ NA
+28C
+28C
+4C
+4 C

FIGURE EIGHTEEN: DOSE-RESPONSE CURVE OF NORADRENALINE STIMULATED OXYGEN CONSUMPTION IN DISPERSED BAT CELLS

The animal was housed two weeks at +4°C. The dispersed BAT cells obtained from one cold-acclimated guinea pig were prepared in Dulbecco Modified Eagle Medium. To one million cells per mL, the shown concentrations of noradrenaline were added.

Values represent single measurements for each noradrenaline concentration.

FIGURE 18


oxygen consumption nmol/1E+6 cells/min.
50
40
30
20
10
0
10-3
10-2
10-1
100
101
102
103
uM noradrenaline

CHAPTER FIVE

DISCUSSION

The principal finding in the experiment on the effect of ambient temperature on BAT T5'D activity is that the pattern of T5'D activity expression in cold acclimated guinea pigs did not follow that of mouse and rat. In contrast to mouse, T5'D activity did not have any tendency to decline during exposure to cold and contrary to rat, both total and specific activity of the enzyme continued to increase throughout the experiment so that total activity of T5'D surpassed significantly that of rat (Park and Himms-Hagen,1988). BAT total protein content paralleled well with the changes of T5'D activity in cold and warm acclimated animals. In spite of the increase in the total protein content of BAT of cold acclimated animals, the specific activity of the enzyme increased progressively, indicating that de novo synthesis of the new T5'D protein in stimulated BAT overbalances synthesis of other proteins associated with growth of the tissue. As compared to rat, continuous and very large increase in T5'D activity in cold acclimated guinea pigs suggests the different mode of stimulation by noradrenaline or/and the much higher activation of sympathetic nervous system by cold in this species. Moreover, the generation of triiodothyronine in BAT under these conditions may not only modulate the BAT thermogenic state itself but also could be the source of

triiodothyronine for other hormone sensitive organs (Silva and Larsen,1985). The physiological role of T5'D in guinea pig could be its involvement in producing and maintaining the hyperthyroid state of other organs, since Zeisberger and Roth (1989) reported recently an increased concentration of T_4 and T_3 in the blood of cold acclimated guinea pigs. Moreover, the resting metabolic rate of a young cold-acclimated guinea pig at 32°C is increased by 40-45% (Brück and Wünnenberg,1966). A similar role for BAT T5'D in increasing the resting metabolic rate of cold-acclimated Syrian hamsters Sigurdson and Himms-Hagen (1988) correlated with an increase in serum T_3 concentration (Kopecký et al.,1986; Sigurdson and Himms-Hagen,1988). To test this hypothesis the effects of elevated serum triiodothyronine level on such organs as skeletal muscle and WAT, known to be hormone sensitive in the rat (Segal, 1989), should be determined in vivo in guinea pigs.

The significant decrease in T5'D activity within three days after rising the ambient temperature only 6°C, that is from room temperature 22°C to 28°C, underscores the importance of adrenergic activity in the expression of T5'D and confirms the findings of others with rats (Silva and Larsen, 1983).

Despite tissue growth and the increase in protein content, BAT weight decreased in cold acclimated guinea pig. The decrease in BAT weight in cold acclimated guinea pigs as a result of lipid depletion confirms earlier reports (Holloway et al.,1984) and stands in contrast to rat, in which cold acclimation increases BAT weight and lipid content (Trayhurn, 1979). Apparently, in cold adapted guinea pig lipogenesis is not stimulated by cold to such an extent as in rat (Goubern and Porter, 1986) and the rate of lipolysis exceeds that of lipogenesis. Since fatty acids are the main substrate for thermogenesis in BAT, the cold acclimated guinea pig must utilize external resources, that is VLDL derived from

liver or free fatty acids from WAT. Therefore, for comparative reasons, it would be useful to determine the activity of lipogenic enzymes, such as lipoprotein lipase, and triglyceride turnover in the cold acclimated guinea pigs.

Despite a high UCP level (Ricquier et al.,1979) and a high thermogenic activity of BAT (Brück and Wünnenberg,1966) in the immediate postnatal period in guinea pigs, the total activity of T5'D was low in the new-born guinea pig and it did not change as the animal underwent developmental changes. The increase in specific activity of the enzyme in the late period of the experiment (at the age of five weeks, figure 8) could be the result of an atrophy of BAT, that is loss of the proteins associated with the high thermogenic state of BAT, whereas the T5'D protein remains at a steady level. The low activity of the enzyme and the atrophy of the tissue in new-born guinea pigs exposed to a thermoneutral environment signify the dependence of BAT growth and activation on adrenergic stimulation.

What induces high level of UCP in the new born guinea pig is not clear. The T5'D activity in the prenatal period in the guinea pig has not been investigated. However, in rat (Iglesias, et al.,1987) and in ruminant (cow) (Giralt et al., 1989) the activity of T5'D in fetal BAT reaches a very high level in the late period of gestation, falls suddenly at birth and remains low afterwards. The physiological role of the high T5'D activity in fetal brown fat in these species is not defined. Triiodothyronine generated in fetal brown fat could be involved in the prenatal induction of the expression of the uncoupling protein gene and the differentiation of the mitochondrion characteristic of BAT, since it has been reported that in bovine fetus, the maximal T5'D level overlaps the moment when the mRNA coding for the UCP first appears (Casteilla et al., 1989). How very high T5'D activity in fetal BAT is induced, when the sympathetic system activity is expected to be absent or low (Sundin and Cannon, 1980) poses another problem. Possibly, growth

hormone (Iglesias et al.,1987) or insulin (Silva and Larsen, 1986) may be involved in the induction as both hormones affect T5'D activity. Whether the observation on the rat and the cow could be extended to the guinea pig remains to be determined.

A very high level of T5'D activity in dispersed BAT cells, as compared to BAT homogenates obtained from cold-acclimated animals, indicates a good yield in the preparation and the persistence of the enzymatic activity in the isolated cells.

The uniform loss of T5'D activity in dispersed BAT cells from warm and cold acclimated guinea pigs during incubation in Krebs-Ringer solution implies that the rate of inactivation or/and degradation is independent of the initial level of T5'D activity.

The increase in T5'D activity in dispersed BAT cells prepared from warm-acclimated guinea pigs during incubation in Dulbecco Modified Eagle Medium is disturbing. Clearly the composition of the medium affected enzyme activity. The observed increase in T5'D activity could be the result of continuous protein synthesis in dispersed BAT cells or /and the activation of the existing enzyme by one of the components of the medium. Since the increase in activity of the enzyme was delayed in time and the biggest increment in activity occurred after two hours of incubation it is probable that activation of the enzyme activity by one of the components of the medium took place. It would be of interest to identify this component. Because of a very recent report (Arthur, et al.,1990) describing thyroxine 5'-deiodinase type I as a selenium dependent protein, I included sodium selenite in the medium. It is conceivable that T5'D of BAT is also a strongly selenium-dependent enzyme. However, isolated BAT cells and homogenates would have to possess the biologically active system(s) capable of incorporating inorganic selenium to significantly increase T5'D activity.

The conditions for noradrenaline stimulation of T5'D activity in dispersed BAT cells proved to be complex since the dispersed cells responded differently to the same concentrations of noradrenaline, nevertheless the response was dose dependent in a range of noradrenaline concentration (0.1-0.5 μM, Figure 15). The variations in the basal activity of the enzyme in different preparations of the cells and the increase in activity during incubation could be the part of problem. The relative insensitivity of dispersed BAT cells to higher than 0.5 μM of noradrenaline may be result of intrinsic properties of guinea pig BAT cells. However, in contrast to guinea pig, T5'D activity in rat isolated BAT cells was maximal for 1-3 μM of noradrenaline added (Obregón et al., 1987). In the light of the above data it seems that the expression of T5'D activity in isolated BAT cells of the guinea pig obeys different rules but at present there is no evidence to support such a possibility.

As expected, in vivo the guinea pigs responded to noradrenaline injection but BAT T5'D activity increased modestly as compared to the increase in the rat (Silva and Larsen, 1986). The persistence of the noradrenaline effect and the progressive increment in enzyme activity up to nine hours after noradrenaline injection suggest a long-lasting process in response to this stimuli. This could be the result of a slow fractional turnover rate of the enzyme or/and a long-lived mRNA coding for the T5'D protein. To examine whether this assumption is correct the effect of cycloheximide and actinomycin D on noradrenaline-induced increase in BAT T5'D activity should be tested. The different values of T5'D activity for sham injected guinea pigs in Figure 16 most probably represent circadian rhythm of the enzyme since the experiment lasted nine hours, from 10 AM to 7PM. However, there is no available data on the changes in the activity of T5'D during the 24 hrs cycle in guinea pigs.

The capacity for thermogenesis measured as oxygen consumption in dispersed BAT cells from warm and cold acclimated guinea pigs confirmed the reports of others (Locke et al., 1982, Cunningham and Nicholls, 1987) that the respiratory response to noradrenaline in brown adipocytes from cold-acclimated guinea pig is much higher as compared to warm controls. The lack of a respiratory response to noradrenaline stimulus in the second BAT cell preparations of the warm-acclimated guinea pig illustrates individual differences between animals and a much lower capacity for thermogenesis. This agrees with the variability in responsiveness of BAT cells preparations to noradrenaline seen by others (Locke et al., 1982). The lower respiratory response of dispersed BAT cells from warm-acclimated animals to noradrenaline could be the result of the decrease in mitochondrial UCP content (Cunningham and Nicholls,1987).

The large increase in the respiratory response to noradrenaline in brown adipocytes from cold-acclimated guinea pigs contrasts with that of rat brown adipocytes, which do not differ in their respiratory response to noradrenaline (Nedergaard, 1982). However, the noradrenaline stimulated respiration of cells from the cold-acclimated rat is comparable to the stimulated respiration of cells from the cold-acclimated guinea pig. The lack of increased respiration in response to the noradrenaline stimulus in cells from the cold-acclimated rat, as compared to warm controls, could be the result of the fact that rat reared at 25°C still retains much of its mitochondrial UCP in the BAT and the dispersed BAT cells are able to unmask the existing UCP (Desautels and Himms-Hagen,1979), and therefore remain partially cold-acclimated. The warm-acclimated guinea pigs does not retain any of its mitochondrial UCP and is truly warm-acclimated.

CONCLUSIONS

Thyroxine 5'-deiodinase activity increases rapidly and to a very high level in BAT of the guinea pig during acclimation to cold. The pattern differs from that previously observed in other laboratory animals (rat, Syrian hamster, mouse) in that both specific and total activities remain at a very high level for as long as the guinea pig remains in the cold (up to 28 days).

It is possible to prepare isolated brown adipocytes from BAT of cold-acclimated guinea pigs which retain a high level of T5'D activity and a good thermogenic responsiveness to noradrenaline. These cells differ from those isolated from BAT of warm-acclimated guinea pigs in possessing a much higher activity of T5'D and a much greater thermogenic response to noradrenaline. They should be a useful model system for studying the role of endogenous T3 production in the regulation of metabolic processes in BAT cells. This is the first time that the activity of T5'D in isolated cells has been compared with that of the intact tissue in cold-acclimated animals.

The effect of noradrenaline, as it is known in the rat and the presumed mediator of the cold-induced increase in T5'D activity in the guinea pigs, to increase T5'D activity in isolated cells or in intact guinea pigs was much weaker than that of cold. To produce cells with a high level of T5'D activity for study in vitro, the incubation of BAT cells with noradrenaline to increase their T5'D activity is not as effective as the acclimation of the guinea pig to cold.

REFERENCES

Arthur, J.,R., Nicol, F., Beckett, G.,J. (1990)
Hepatic iodothyronine 5`-deiodinase. The role of selenium.
Biochem. J. 272:537-540

Bianco, A.C. and Silva,J.E. (1988)
Cold exposure rapidly induces virtual saturation of brown adipose tissue nuclear T-3 receptor.
Am. J. Physiol. 255:E496-E503

Bianco, A,C., Sheng,X., Silva, J.E. (1988)
Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by mechanism not requiring protein synthesis.
J Biol. Chem. 263: 18168-18175

Bukowiecki, L.J., Follea, N., Lupien,J. and Paradis, A (1981)
Metabolic relationships between lipolysis and respiration in rat brown adipocytes.
J.Biol.Chem. 256:12840-12848

Bukowiecki,L.J. (1986a)
Lipid metabolism in brown adipose tissue. In Brown Adipose Tissue, Trayhurn,P and Nicholls, D.G. (eds.) pp. 105-121, London: Arnold

Bukowiecki,L.J., Géloën, A, and Collet A.J. (1986b)
Proliferation and differentiation of brown adipocytes from interstitial cells during cold acclimation.
Am.J. Physiol. 250:C880-887

Brück, K. and Wünnenberg,K. (1966)
Influence of ambient temperature in the process of replacement of non-shivering by shivering thermogenesis during post-natal development.
Fed. Proc. 25: 1332-1337

Bruning,J.L., Kintz,B.L. (1977)
Computational handbook of statistics. (2 nd edition)
Scott, Foresman and Co., Gelview, Illinois, London.

Carneheim,C., Nedergaard, J. and Cannon,B. (1988)
Cold induced beta-adrenergic recruitment of lipoprotein lipase in brown fat is due to increased transcription.
Am. J. Physiol. 254:E155-161

Casteilla,L., Champigny,O., Bouillaud,F., Robelin,L., and Ricquier,D.(1989)
Sequential changes in the expression of mitochondrial protein m-RNA during the development of brown adipose tissue in bovine and ovine species.
Biochem. J. 257: 665-671

Cunningham, S.A. and Nicholls,D.G. (1987)
Induction of functional uncoupling protein in guinea pig infused with noradrenaline.
Biochem. J. 245:485-491

Desautels, M. and Himms-Hagen, J (1979)
Role of noradrenaline and protein synthesis in the cold-induced increase in purine nucleotide binding by rat brown adipose tissue mitochondria.
Can. J. Biochem. 57:968-976

Eley,J. and Himms-Hagen,J. (1989)
Attenuated response to cold in brown adipose tissue of goldthioglucose-obese mice.
Can.J.Physiol.Pharmacol. 67:116-121

Fernandez, J.A., Mampel, T., Villarroya,F. and Iglesias, R. (1987)
Direct assessement of brown adipose tissue as a site for systemic triiodothyronine production in rat
Biochem. J. 243:281-284

Foster,D.O., Depocas,F., Zaror-Behrens,G. (1982)
Unilaterality of the sympathetic innervation of each pad of rat interscapular brown adipose tissue.
Can. J. Physiol. Pharmac. 60: 747-754

Foster,D.O. and Frydman, M.L. (1978)
Nonshivering thermogenesis in rat. Measurement of blood flow with micro-spheres point to adipose tissue as the dominant site of the calorigenesis induced by noradrenaline.
Can. J. Physiol. Pharmac. 56:110-122

Foster, D.O. and Frydman, M.L. (1979)
Tissue distribution of cold-induced thermogenesis in concious warm- and cold-acclimated rats reevaluated from changes in tissue blood flow: The dominant role of brown adipose tiisue in the replacement of shivering by nonshivering thermogenesis.
Can. J. Physiol. Pharmac. 56:257-270

Giralt,M., Martin,I., Mampel,T., Villarroya,F., Iglesias,R. and Vinas,O. (1988)
Evidence for a differential physiological modulation of brown fat iodothyronine 5'-deiodinase activity in the perinatal period.
Biochem. Biophys. Res. Comun. 156: 493-498

Giralt,M., Casteilla, L., Vînas,O., Mampel, T., Iglesias, R., Robelin, J., and Villarroya, F. (1989)
Iodothyronine 5'-deiodinase activity as an early event of prenatal brown-fat differentiation in bovine development.
Biochem. J. 259:555-559

Girardier,L. and Seydoux,J. (1986)
Neural control of brown adipose tissue thermogenesis. In Brown Adipose Tissue pp.122-151, Trayhurn,P. and Nicholls, D.G. (ads) Edward Arnold,London.

Goubern, M. and Portet, R. (1986)
Modulation of fatty acids synthesis in vivo in brown adipose tissue, liver and white adipose tissue of cold acclimated rats.
Ann.Nutr. Metab. 30:380-385

Greco-Perotto, R., Zaninetti,D., Assimacopoulos-Jeannet, F., Bobbioni,E and Jeanrenaud, B (1987a)
Stimulatory effect of cold adaptation on glucose utilization by brown adipose tissue. relationship with changes in glucose trnasport system.
J.Biol. Chem. 262:7732-7736

Greco-Perotto,R. Assimacopoulos-Jeannet, R and Jeanrenaud,B (1987b)
Insulin modifies the properties of glucose transporters in rat brown adipose tissue.
Biochem. J. 247:63-68

Hervey,G.R. and Tobin,G. (1983)
Luxuskonsumption, diet-induced thermogenesis and brown fat: A critical review.
Clin.Sci. 64:7-18

Heron, D., Rehnmark,S., Néchad, M., Loncar,D., Cannon,B and Nedergaard, J. (1990)
Norepinephrine-induced synthesis of the uncoupling protein thermogenin (UCP) and its mitochondrial targeting in brown adipocytes differentiated in culture.
FEBS Lett. 268:296-300

Himms-Hagen,J.(1986)
Brown adipose tissue and cold acclimation. In Brown Adipose Tissue, pp.214-268, Trayhurn, P. and Nicholls,D.G. (eds.), Edward Arnold, London.

Holloway, B.R., Davidson, R.G., Freeman, S., Wheeler,H. and Stribling,D.(1984)
Post-natal development of interscapular brown adipose tissue in the guinea pig: Effect of environmental temperature.
Int. J. Obesity. 8:259-303

Holzer,P. (1988)
Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene related peptide and other neuropeptides.
Neuroscience 24:739-768

Houštěk,J., Pavelka, S., Baudyšová, M. and Kopecký, J (1990a)
Induction of type II iodothyronin 5'-deiodinase and mitochondrial uncoupling protein in brown adipocytes differentiated in cell culture.
FEBS Lett. in press

Houštěk, J., Kopecký, J., Baudyšová, M., Jaňiková,D., Pavelka,S. and Klement, P. (1990b)
Differentiation of brown adipose tissue and biogenesis of thermogenic mitochondria in situ and in cell culture.
Biochim. Biophys. Acta 1018:243-247

Huttunen, P. and Kinnula, V. (1979)
Effects of catecholamine treatment as well as activities of the adipose tissue in a guinea pig (Cavia Porcellulus)
Comp. Biochem. Pharmacol. 63C, 13-16

Iglesias,R., Fernandez,J.A., Mampel,T., Obregon, M.J. and Villarroya,F. (1987)
Iodothyronine 5'-deiodinase activity in rat brown adipose tissue during development.
Biochim. Biophys. Acta 923: 233-240

Janský, L., Bartuňková, R., Kočkova, J., Mejsnar, J. and Zeisberger, E. (1969)
Interspecies differences in cold adaptation and nonshivering thermogenesis.
Fedn. Proc. 28:1053-1058

Jones,R. Henschen,L. Mohell,N and Nedergaard,J. (1986)
Requirement of gene transcription and protein synthesis for cold and norepinephrine-induced stimulation of thyroxine 5'-deiodinase activity in rat brown adipose tissue.
Biochm. Biophys. Acta 889: 366-373

Klingenberg,M. (1988)
Nucleotide binding to uncoupling protein. Mechanism of control by protonation.
Biochemistry 27:772-779

Kopecký,J., Baudyšová,M., Zanotti,F., Javníková, D.,Pavelka,S. and Houštěk,J (1990)
Synthesis of mitochondrial uncoupling protein in brown adipocytes differentiated in cell culture.
J. Biol. Chem. 265:22204-22209

Kopecký,J., Sigurdson,L., Park, I.R.A. and Himms-Hagen,J (1986)
Thyroxine 5'-deiodinase in hamster and rat brown adipose tissue; effect of cold and diet.
Am.J. Physiol. 251:E1-E7

Kuroshima,A. and Yahata,T. (1985)
Effect of food restriction or cold adaptability of rats.
Can. J. Physiol. Pharmacol. 63:68-71

Le Magnen,J. (1983)
Body energy balance and food intake: a neuroendocrine regulatory mechanism.
Physiol. Rev. 63:314-386

Leonard,J.L., Mellen,S.A., Larsen, P.R. (1983)
Thyroxine 5'-deiodinase activity in brown adipose tissue.
Endocrinology 112:1153-1155

Locke, R.,M., Rial,E. and Nicholls, D.,G.
The acute regulation of mitochondrial proton conductance in cells and mitochondria from brown fat of cold adapted and warm adapted guinea pig.
Eur. J. Biochem. 129:381-387

Lowry,O.H., Rosenbrough,N.J., Farr, A.L., Randall,R.J. (1951)
Protein measurement with Folin phenol reagent.
J.Biol. Chem. 193:265-275

Maxwell, G.M., Nobes,S. and Bates,D.J.(1987)
Diet-induced thermogenesis in cafeteria-fed rats: A myth?
Am.J Physiol. 253:E264-E270

Mills,I., Raasmaja,A., Moolten,N., Lemack,G., Silva,J.E. and Larsen,P.R.(1989)
Effect of thyroid status on catecholamine stimulation of thyroxine 5'-deiodinase in brown adipocytes.
Am. J. Physiol. 256:E74-E79

Mills,I., Barge,R.M., Silva, J.E. and Larsen, P.R. (1987)
Insuline stimulation of iodothyronine 5'-deiodinase in rat brown adipocytes.
Bichem. Biophys. Res. Commun. 143:81-86

Nånberg,E. and Putney, J. Jr.(1986)
Alpha-1 adrenergic activation of brown adipocytes leads to an increased formation of inositol phosphates.
FEBS Letters 195:319-322

Néchad, M. (1986)
Structure and development of brown adipose tissue. In Brown Adipose Tissue, pp.1-30, Trayhurn,P. and Nicholls,D.G. (eds.) Edward Arnold,London.

Nedergaard, J. (1982)
Catecholamine sensitivity in brown fat cells from cold-acclimated hamsters and rats.
Am. J. Physiol. 242: C250-C257

Nedergaard, J. and Lindberg, O. (1979)
Norepinephrine-stimulated fatty acid release and oxygen consumption in isolated hamster brown fat cells. Influence of buffers, albumin, insulin and mitochondrial inhibitors.
Eur. J. Biochem. 95:139-145

Nicholls,D. (1976)
Hamster brown adipose tissue mitochondria. Purine nucleotide control of the ion conductance binding site.
Eur. J. Biochem. 62:223-228

Nicholls, D. (1983)
The thermogenic mechanism of brown adipose tissue
Biosci. Rep. 3:431-441

Nnodim, J.O. and Lever, J.D. (1988)
Neural and vascular provisions of rat interscapular brown adipose tissue.
Am. J. Anat. 182:283-292

Norman,D., Mukherjee,S., Symons,D. Jung,R.T. and Lever, J.D. (1988)
Neuropeptides in interscapular and perirenal brown adipose tissue in the rat: a plurality of innervation.
J.Neurocytol. 17:305-311

Obregón, M-J., Mills, I., Silva, J.E. and Larsen, P.R.
Catecholamine stimulation of iodothyronine 5'-deiodinase activity in rat dispersed brown adipocytes.
Endocrinology 120:1069-1072

Obregón,M-J, Pitamber,R., Jacobsson,A., Nedergaard,J., Cannon,B. (1987)
Euthyroid status is essential for the perinatal increase in thermogenin mRNA in brown adipose tissue of rat pups.
Biochem. Biophys. Res. Commun. 148:9-14

Park,I.R.A. and Himms-Hagen,J (1988)
Neural influences on trophic changes in brown adipose tissue during cold acclimation.
Am.J. Physiol. 255:R874-R881

Rafael,J., Fesser,W. and Nicholls,D.G. (1986)
Cold adaptation in guinea pig at level of isolated brown adipocyte.
Am. J. Physiol. 250:C228-C235

Rafael, J. and Heldt, H.W. (1976)
Binding of guanine nucleotides to the outer surface of the inner membrane of guinea pig brown fat mitochondria in correlation with the thermogenic activity of the tissue.
FEBS Lett. 63: 304-308

Rehnmark, S., Néchad,M., Herron,D., Cannon,B. and Nedergaard, J. (1990)
alpha- and beta-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture.
J. Biol. Chem. 265: 16464-16471

Ricquier, D., Bouillaud,F., Toumelin, P (1986)
Expression of uncoupling protein mRNA in thermogenic brown adipose tissue. Evidence for a rapid beta adrenoreceptor mediated and transcriptionally regulated step during activation of thermogenesis.
J. Biol. Chem. 26: 13905-13910

Roth, J., Zeisberger, E. and Schwandt, H-J. (1987)
Changes in peripherial metabolism of catecholamines in guinea pig during thermal adaptation.
J. therm. Biol. 12:39-44

Roth, J., Zeisberger, E. and Schwandt, H-J. (1988)
Influence of increased catecholamine levels in blood plasma during cold adaptation and intramuscular infusion on thresholds of thermoregulatory reactions in guinea pigs.
J. Comp. Physiol. B. 157:855-863

Rothwell, N.J. and Stock, M.J. (1986)
Brown adipose tissue and diet-induced thermogenesis. In Brown Adipose Tissue, pp.262-298, Trayhurn, P. and Nicholls, D,G. (eds.) Edward Arnold, London.

Rothwell, N.J. and Stock,M.J. (1983)
Luxuskonsumption, diet-induced thermogenesis and brown fat: The case in favour.
Clin. Sci. 64:19-23

Schacterle,G.P., Pollack,R.L. (1973)
A simplified method for the quantitative assay of small amounts of protein in biological material.
Anal. Biochem. 51:654-655

Schneider-Picard,G., Coles, J.A. and Girardier,L. (1985)
Alpha and Beta-adrenergic mediation of changes in metabolism and Na/K exchange in rat brown fat.
J. Gen. Physiol. 86:169-188

Segal, J. (1989)
A rapid, extranuclear effect of 3,5,3'-triiodothyronine on sugar uptake by several tissues in rat in vivo. Evidence for physiological role for the thyroid hormone action at the level of plasma membrane.
Endocrinology 124: 2755-22764

Silva, J.E. (1988)
Full expression of uncoupling protein gene requires the concurrence of norepinephrine and triiodothyronine.
Mol. Endocrinol. 2:706-713

Silva,J.E. and Larsen, P.R. (1983)
Adrenergic activation of triiodothyronine production in brown adipose tissue.
Nature Lond. 305:712-713

Silva,J.E. and Larsen, P.R. (1985)
Potential of brown adipose tissue type II thyroxine 5'-deiodinase as a local and systemic source of triiodothyronine in rats.
J. Clin. Invest. 76: 2296-2301

Silva,J.E. and Larsen,P.R. (1986a)
Hormonal regulation of iodothyronine 5'-deiodinase in rat brown adipose tissue
Am.J. Physiol. 251: E631-E643

Silva, J.E. and Larsen, P.R. (1986b)
Interrelationships among thyroxine, growth hormone and the sympathetic nervous system in the regulation of thyroxine 5'-deiodinase in rat brown adipose tisuue.
J. Clin. Invest. 77: 1214-1223

Silva,J.E., Mellen,S., Larsen,P.R. (1987)
Comparison of kidney and brown adipose tissue iodothyronine 5'-deiodinase.
Endocrinology 121:650-656

Silva, J.E. and Matthews,P (1984)
Thyroid hormone metobolism and the source of plasma triiodothyronine in 2-week-old rats: Effect of thyroid status
Endocrinology 114:2394-2405

Smith,R. and Horwitz, B. (1989)
Brown fat and thermogenesis
Physiol.Rev. 49:330-425

Sundin,U. and Cannon,B. (1980)
GDP binding to the brown fat mitochondria of developing and cold adapted rats.
Comp. Biochem. Physiol. 65B: 463-471

Trayhurn, P. (1979)
Fatty acid synthesis in vivo in brown adipose tissue, liver and white adipose tissue of the cold acclimated rat.
FEBS Lett. 104:13-16

Visser,T.M., Leonard,J.L., Kaplan,M.M. and Larsen, P.R. (1982)
Kinetic evidence suggesting two mechanisms for iodothyronine 5'-deiodination in rat cerebral cortex.
Proc. Natl. Acad. Sci. USA 79:5080-5084

Zeisberger, E. and Roth, J. (1989)
In: Living in the cold II. pp 435-444, Eds: Malan. A., Canguilhem, B.
Colloque INSERM/John Libbey Eurotext. Ltd.

APPENDIX A

The composition of Dulbecco Modified Eagle Medium (mg/L)
(GIBCO catalog # 380-2320 AJ)

Inorganic salts:

$CaCl_2$	200.0	NaCl	4750.0
$Fe(NO_{3)3}.\ 9H_2O$	0.1	$NaHCO_3$	3700.0
KCL	400.0	$NaHPO4.H_2O$	125.0
$MgSO_4$	200.0		

Other Components:

D-glucose	1000.0
Phenol red	15.0
HEPES	5958.0
Sodium pyruvate	110.0

Amino Acids:

L-Arginine	84.0	L-Leucine	105.0
L-Cystine	48.0	L-Lysine.HCl	146.0
L-Glutamine	584.0	L-Methionine	30.0
Glycine	30.0	L-Phenylalanine	66.0
L-Histidine.HCl.H_2O	42.0	L-Serine	42.0

(Composition of the medium- continued)

L-Isoleucine	105.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0

Vitamins:

Ca-pantothenate	4.0	Niacinamide	4.0
Choline Chloride	4.0	Pyridoxal.HCl	4.0
Folic acic	4.0	Riboflavin.	4.0
Inositol	7.2	Thiamine.HCl	4.0

Other components added to the medium in our lab.

ascorbic acid	176.0
glucose	1000.0
sodium pyruvate	110.0

fatty acid free bovine serum albumine 4% (w/v)

APPENDIX B

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BAT	Brown Adipose Tissue
BW	Body weight
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-2-hydroxylethylpiperazine-N'-ethanesulfonic acid
NA	Noradrenaline
PTU	6-propyl-2-thiouracil
S.E.	Standard error of the mean
T5'D	Thyroxine 5'-deiodinase
T_3	3,5,3'-Triiodothyronine
T_4	Thyroxine
TCA	Trichloroacetic acid
TRIS	Tris(hydroxymethyl) aminomethane
UCP	Uncoupling protein
WAT	White Adipose Tissue