THYROID HORMONE METABOLISM IN BROWN ADIPOSE TISSUE OF LEAN AND GENETICALLY OBESE (OB/OB) MICE

by

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ABSTRACT

The defective brown adipose tissue (BAT) function of the genetically obese (ob/ob) mouse is believed to be associated with refractoriness to the effect of 3,5,3'-triiodothyronine (T_3) on thermogenic responsiveness to noradrenaline, which might be due to an abnormality in the recently discovered enzyme, thyroxine 5'-deiodinase (T5'D), in BAT. This enzyme produces T_3 from thyroxine (T_4) within BAT and its activity is controlled by noradrenaline. Thus, the initial hypothesis was that BAT of the ob/ob mouse might be in a state of intracellular hypothyroidism secondary to a defective functioning of T5'D. The results of the experiments in this thesis show that ob/ob mice fail to increase BAT T5'D activity in response to acute cold-exposure. However, treatments such as adrenalectomy and gradual acclimation to cold allow a normal increase in BAT T5'D activity in the ob/ob mouse.

In contrast to the defective response of BAT of the ob/ob mouse to cold, the response of BAT T5'D to diet and injected noradrenaline is normal. The response of BAT T5'D to noradrenaline is mediated by both α_1- and β-adrenergic receptors in lean and ob/ob mice. Since the response of BAT T5'D activity of the ob/ob mouse to injected NA is normal, it seems likely that the lack of response to endogenous NA during cold-exposure is due to the presence in the ob/ob mouse, but not the lean mouse, of some excessive inhibitory influence during acute exposure to cold. A likely candidate for this inhibitory influence is suggested to be corticosterone.

Future directions that research on T5'D activity in BAT may take might include an investigation of the potential function of this enzyme in man. The present study of BAT and BAT T5'D activity in a primate species represents a first step in that direction.

DEDICATION

To my

Mother,

Father,

and to Robert

for all your love and support.

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ABBREVIATIONS

ad lib	Ad libitum
ADP	Adenosine diphosphate
ADX	Adrenalectomy
ATP	Adenosine triphosphate
ACTH	Adrenocorticotropic hormone
BAT	Brown adipose tissue
B_0	Total binding
bw	Body weight
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CoA	Coenzyme A
DIT	Diet-induced thermogenesis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid

GDP	Guanosine diphosphate
GH	Growth hormone
GTG	Gold thioglucose
HEPES	N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid
Km	Michaelis Menten Coefficient
KOH	Potassium hydroxide
L:D	Hours of light:hours of darkness
MOPS	(3-[N-Morpholino] propanesulfonic acid)
NA	Noradrenaline
NaOH	Sodium hydroxide
NATO	Noradrenaline turnover
NCS	NCS tissue solubilizer
NCM	Nitrocellulose membrane
NSB	Non-specific binding
NST	Nonshivering thermogenesis
ob/ob	Genetically obese mouse
PAGE	Polyacrylamide gel electrophoresis
PPO	2,5 diphenyloxazole
PTU	6-propyl-2-thiouracil

PVN	Paraventricular nucleus
RIA	Radioimmunoassay
r^2	Squared linear correlation coefficient (Pearson r)
S.C.	Subcutaneous
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
T_3	3,5,3'- Triiodothyronine
rT_3	3,5',3'- Triiodothyronine
T_2	3,3'- diiodothyronine
T_4	Thyroxine
TCA	Trichloroacetic Acid
TES	N-tris [Hydroxymethyl] methyl-2-aminoethane Sulfonic Acid
TETRAC	Tetraiodothyroacetic acid
THFS	Thyroid Hormone free serum
TRIAC	3.3,5'-Triiodothyroacetic acid
TRIS	Tris (hydroxymethyl) aminomethane
TSH	Thyroid stimulating hormone
Tween-20	Polyoxyethylene sorbitan monolaurate
Tx	Thyroidectomized
UCP	Uncoupling protein
V	Volts
VMH	Ventromedial hypothalamus
WAT	White adipose tissue

INTRODUCTION

HISTORY:

Brown adipose tissue (BAT) was first described in the interscapular area of the marmot (Muris [Marmota] alpinus) (Gesner, 1551; Velschi, 1670). The function of BAT was first associated with arousal from hibernation and BAT was therefore termed "hibernating gland". However, as the existence of BAT became known in many nonhibernating mammals, (Rowlatt et al., 1971) the exact function remained obscure. It was not until this century and most recently the past 25 years that a thermoregulatory function of BAT was seriously examined (Smith and Horwitz, 1969; Smith and Hock, 1963).

Nonshivering thermogenesis (NST), the cold induced increase in heat production occurring in the absence of shivering, was shown to be important in the thermoregulatory response of rats to cold (Cottle and Carlson, 1956; Héroux et al., 1956) but for many years the site of NST was thought to be in organs other than BAT. The work of Foster and Frydman (1978a, 1978b, 1979) using radioactive microspheres demonstrated that BAT was responsible for 60-70 % of the increase in metabolic rate caused by acclimation of rats to cold. Thus BAT is now recognized to be the major effector of NST (Foster 1984, 1986).

Thermogenesis in BAT is also activated by ingestion of a palatable "cafeteria" diet, referred to as diet-induced thermogenesis (DIT) (Rothwell and Stock, 1979). Rothwell and Stock (1981) also used radioactive microspheres to measure the noradrenaline stimulated increase in blood flow to BAT in rats fed a cafeteria diet and thus demonstrated that BAT was the effector of DIT.

DEFINITIONS:

In order to more fully comprehend what is meant by nonshivering thermogenesis and the other terms of thermogenesis used in this thesis, I will briefly describe a few of the more commonly used terms (See Himms-Hagen et al., 1989 and Himms-Hagen, 1989 for more detail).

Overall energy expenditure is the sum of two categories, obligatory and facultative thermogenesis. Obligatory thermogenesis includes energy expenditure essential to the life of all cells and essential for endothermy. Obligatory thermogenesis occurs in every organ of the body, is controlled primarily by thyroid hormones, and corresponds roughly to the resting metabolic rate. Obligatory thermogenesis also includes the energy expenditure associated with the ingestion, digestion and processing of food, and the energy cost of growth.

Facultative thermogenesis, in contrast, represents an auxiliary heat source that can be rapidly turned on or off depending on the needs of each particular species. Facultative thermogenesis occurs principally in only two organs, skeletal muscle and BAT. Skeletal muscle is the major effector of exercise-induced thermogenesis (voluntary), cold-induced shivering thermogenesis (involuntary) and is controlled by motor nerves. Facultative thermogenesis in BAT is controlled by noradrenaline (NA) secreted from sympathetic nerves (Cottle and Cottle, 1970) in response to cold (cold-induced nonshivering thermogenesis (CINST)) and by diet (diet-induced thermogenesis (DIT)). Prolonged stimulation of BAT by NA causes growth of the tissue. Thus, BAT is used as an auxiliary heat source to produce heat in response to cold and diet. Therefore if heat is

produced by another mechanism, BAT will not be activated to produce heat. This point can be emphasized by the work of Arnold and coworkers (reviewed in Arnold and Richard, 1987) who combined exercise training with cold exposure and were able to partially (Arnold et al., 1986) or completely (Arnold and Richard, 1987) eliminate the effect of cold to induce BAT growth. Thus when studying thermogenesis in BAT it is very important to note that heat from other sources (in this case muscle) may in fact suppress BAT thermogenesis. For this reason it is very important to note the temperature at which the animals are housed, how many animals per cage (social thermoregulation), the type of diet animals are fed and if the animals are pregnant or lactating. The use of anesthetics can also inhibit BAT thermogenesis (Foster, 1974), thus care must be taken in designing experiments when anesthetics must be used.

BAT LOCATION AND MORPHOLOGY:

The total amount of BAT found in rodents varies between 1-2 % of total body weight and is found in patches often in contact with blood vessels (see Néchad, 1986 for review). Interscapular BAT, the most studied depot in rats and mice, comprises about one third of the total BAT. Other depots are found in the subscapular, axillary, perirenal areas and around the aorta and heart. In other species, primates and man, the axillary depot is the most abundant and very little BAT is found in the interscapular area.

Brown adipocytes arise from mesenchymal cells which are associated with blood vessels (see Néchad, 1986 for review). These cells

proliferate at the same time as the blood vessels. Small lipid inclusions develop in the cytoplasm as the cells form preadipocytes. The preadipocytes increase the size and number of their mitochondria and progressively lose their ability to divide. At this point the mature brown adipocytes are multilocular (many lipid droplets) and are packed with many mitochondria. It has been shown that by the end of its differentiation (at birth, for the rat), BAT is functionally innervated (Schneider-Picard and Girardier, 1982) which indicates that the sympathetic nerve fibers grow into the tissue as the tissue is differentiating. Brown adipocytes are connected to each other by gap junctions, which may provide electrical coupling between them (Schneider-Picard et al., 1984). In the fully active tissue the mature brown adipocytes occupy approximately 70 % of the total tissue volume but only 40 % of the total number of cells in interscapular BAT (Bukowiecki et al., 1982). The remainder of the tissue is made up of various cell types: endothelial cells, perivascular mesenchymal cells, preadipocytes, mast cells, schwann cells and fibroblasts, all of which are small in size and generally difficult to detect at the light microscopic level of resolution (Néchad, 1986).

When BAT is inactive the cells accumulate triglyceride and the multilocular droplets fuse to become unilocular These inactive brown adipocytes look like white adipocytes but are usually smaller in size and contain more mitochondria. White and brown fat are two distinct tissues, although they both originate from the same embryonic layer, the mesoderm. They have distinctly different precursor cells which are committed to develop differently (Néchad et al., 1983).

Upon sympathetic nerve stimulation these inactive brown adipocytes will once again become multilocular and the mitochondria will proliferate. If the sympathetic nerve stimulation is prolonged, BAT will grow. This growth involves the division of precursor endothelial cells and preadipocytes with a consequent hyperplasia of the tissue and thus an increased capacity for thermogenesis (Bukowiecki et al., 1982).

VASCULATURE AND INNERVATION:

Anatomical studies of the vascular connections of the various BAT depots have shown that they are served by vessels derived from the major arteries and veins (Smith and Roberts, 1964). Thus BAT supplies heat both by direct contact with an organ and by returning warmed blood to the heart for distribution throughout the body. In interscapular BAT, the thoracodorsal veins lie in close apposition to the thoracodorsal arteries forming a plexus which allows a countercurrent heat exchange. Simultaneously the unpaired Sulzer's vein allows the modulation of the temperature increase in the plexus by rapidly draining heat into the thorax towards the spinal cord and heart (Smith and Roberts, 1964; Nnodim and Lever, 1988). Thus a very small amount of BAT can export a considerable amount of heat. The rapid on-off control of this heat production is controlled by the very rich sympathetic innervation of BAT (Flaim et al., 1976).

The most studied depot in rats and mice, interscapular BAT, consists of two symmetrical pads, each supplied by five intercostal nerves and one nerve which enters along the thoracodorsal artery. The origin of these nerves arises from the middle and inferior cervical

ganglia and the first five thoracic ganglia (Girardier and Sedoux, 1986). Contrary to earlier reports, there is no cross-innervation between the symmetrical pads (Foster et al., 1982a, 1982b; Park and Himms-Hagen, 1988; Himms-Hagen 1988, 1989). The nerves supplying the blood vessels contain in addition to noradrenaline, neuropeptide Y, substance P and calcitonin gene-related peptide (CGRP) (Cannon et al., 1986; Norman et al., 1988). Noradrenaline does not directly cause vasodilation in BAT, in fact through α-adrenergic receptors, NA can cause vasoconstriction (Foster and Depocas, 1980). Foster and Depocas (1980) have suggested the possible release of an as yet unidentified vasodilator agent during thermogenesis. CGRP is known to be a potent vasodilator (Brain et al., 1985; for review see Breimer et al., 1988). It is possible that the nerves supplying the blood vessels could release a vasodilating peptide such as CGRP but further research is necessary before we understand the mechanism of vasodilation and increased blood flow.

MECHANISM OF THERMOGENESIS:

β-ADRENERGIC RECEPTOR-MEDIATED PATHWAYS

Activation of the sympathetic nervous system (SNS) (by cold or diet) via the hypothalamus causes the release of noradrenaline from the sympathetic nerve terminals in BAT (for review see Nicholls and Locke, 1984; Cannon and Nedergaard, 1985; Himms-Hagen, 1988, 1989). Noradrenaline then binds to β-adrenergic receptors. activating adenylate cyclase. thus increasing levels of cyclic AMP (Bégin-Heick and Heick, 1984). Cyclic AMP stimulates the activity of a protein

kinase that phosphorylates hormone-sensitive triacylglycerol lipase to produce the active form of this enzyme (Bukowiecki, 1984). The activated enzyme enhances the breakdown of triglycerides stored in the multilocular droplets to free fatty acids which serve as fuel for the increased thermogenesis and activate the proton conductance pathway. Fatty acyl-CoA derivatives enter the mitochondria where they undergo β-oxidation with the resulting acetyl-CoA entering the tricarboxylic acid cycle. The electron transport chain produces an electrochemical proton gradient which is usually coupled to the synthesis of ATP. However, BAT mitochondria have a low capacity for oxidative phosphorylation, instead the electrochemical proton gradient is dissipated by allowing protons to leak back into the mitochondria, producing heat rather than storing the energy as ATP (Nicholls, 1979; Nicholls, 1983; Nicholls et al., 1986). Since BAT metabolism is not controlled by the level of ATP, but by SNS stimulation, electron transport and metabolic pathways proceed at a maximum rate, no longer restricted by a proton gradient and by the amount of work being done by the cell. In BAT, the electrochemical gradient is dissipated by a mitochondrial inner membrane protein of molecular weight 32,000 Daltons (Nicholls, 1979; Nicholls et al., 1978; Heaton et al., 1978). This protein has been named uncoupling protein (UCP) but is also known as thermogenin and nucleotide binding protein.

When BAT is in a thermogenically inactive state, the uncoupling is inhibited by the binding of purine nucleoside di or triphosphates (ATP and ADP) to unique sites on UCP. In order for UCP to function as a proton carrier, the purine nucleotides are removed. One possible

mechanism for this is a small increase in intracellular pH, since the binding of purine nucleotides to UCP is known to be pH dependent (Klingenberg, 1984, 1988). Free fatty acids produced from the cAMP mediated lipolysis of multilocular triglyceride fat droplets can also activate UCP, most probably via a conformational change which lowers the membrane potential at which conductance occurs (Rial et al., 1983; Bukowiecki et al., 1981; Nicholls et al., 1986; LaNoue et al., 1986).

α-ADRENERGIC RECEPTOR-MEDIATED PATHWAYS

In addition to the β-receptor mediated response to noradrenaline described above, an increasing body of evidence has implicated a role for α-adrenergic receptors in BAT. In vitro approximately 20 % of the NA stimulated increase in oxygen consumption is due to α-receptor mediated reactions (Mohell et al., 1983). This component involves an ATPase type of control mechanism rather than the β-adrenergic receptor uncoupling mechanism.

In vivo there is a potentiating effect of α_1-adrenergic receptor stimulation on thermogenesis resulting from β-receptor pathways (Foster, 1985; Ma and Foster, 1984). Noradrenaline induces a very rapid and transient depolarization of BAT mediated mainly by α_1-adrenergic receptors, followed by a brief hyperpolarization and a subsequent prolonged depolarization, mediated by β-adrenergic receptors (Girardier and Seydoux, 1986, Schneider-Picard et al., 1985). The gap junctions between brown adipocytes (Schneider-Picard et al., 1984) probably provide a means for the propagation of the depolarization throughout BAT.

throughout BAT.

The phosphatidylinositol bisphosphate (PIP_2) cycle is activated through α_1-adrenergic receptors in BAT and increases the production of inositol trisphosphate (IP_3) and diacylglycerol (DG) (Mohell et al., 1984, 1987; Nånberg and Putney, 1986). IP_3 increases Ca^{2+} release from intracellular stores (Connolly et al., 1984). The source of Ca^{2+}, (mitochondria or endoplasmic reticulum) is not known (Nedergaard et al., 1986). The increase in Ca^{2+} activates a Ca^{2+}-activated cation channel which caused a transient efflux of K^+ (Nånberg et al., 1985; Nedergaard et al., 1986; Siemen and Reuhl, 1987). An influx of Na^+ also occurs with the increase in intracellular Na^+ concentration causing a further release of Ca^{2+} (Nedergaard et al., 1986; Connolly et al., 1984). It is possible that the efflux of K^+ and the influx of Na^+ stimulates the activity of Na^+ K^+-ATPase to restore the normal distribution of these ions. An increase in Na^+ K^+-ATPase activity is a part of the α_1-adrenergic agonist thermogenic response in BAT (Mohell et al., 1987; Nånberg et al., 1984).

A second possible function of the α_1-adrenergic pathway in BAT metabolism could be the initiation of the trophic response of BAT to prolonged stimulation by NA. Berridge (1984) has suggested a role for DG, Ca^{2+}, Na^+, IP_3 and protein kinase C in proliferative responses in other cell types. Similar mechanisms could also function in BAT.

FUEL FOR THERMOGENESIS:

Fatty acids, derived from the lipolysis of triglycerides as described above are the fuel for thermogenesis. Glucose has been found to be only a minor substrate for thermogenesis (Ma and Foster, 1986).

prolonged periods of stimulated thermogenesis. The stores of triacylglycerol can support thermogenesis for only a short period of time, therefore, the fuel supply for thermogenesis must come ultimately from the food that is consumed. Food intake is known to double or even triple in cold stimulated mice (Trayhurn, 1981).

Rats and mice fed a high carbohydrate diet are capable of large increases in lipogenesis in BAT when exposed to cold (Goubern and Portet, 1986; Trayhurn, 1981). The increase in activity of lipogenic enzymes during hypertrophy of BAT in the cold (Buckley and Rath, 1987) is probably required before high rates of lipogenesis can occur. BAT is unique in that both lipogenesis and lipolysis can occur simultaneously. Insulin is known to mediate lipogenesis at many levels; mobilization of glucose transporters to the plasma membrane of BAT cells (Greco-Perotto et al., 1987), increase in the uptake of glucose (Ma and Foster, 1986) and an increase in activity of lipogenic enzymes (Buckley and Rath, 1987; McCormack and Denton, 1977).

A number of researchers have found a rapid cold induced increase in lipoprotein lipase in BAT even when lipogenesis is stimulated (Cannon et al., 1986b; Carneheim et al., 1984 and Goubern and Portet, 1981). This suggests that BAT also uses blood lipids from VLDL, even when lipogenesis is occurring at a rapid rate.

The activity of lipoprotein lipase is controlled through β-adrenergic receptors (Carneheim et al., 1984). The increase in lipoprotein lipase activity in BAT of cold exposed rats is not due to an activation of a pre-existing enzyme nor due to an increased half-life of functional enzyme. Rather the rate of lipoprotein lipase gene

transcription is related to the cellular level of cAMP and this increase in lipoprotein lipase mRNA leads to an increased rate of enzyme synthesis and therefore to an increase in activity (Carneheim et al., 1988).

A high fat diet is known to suppress lipogenesis in rats and mice (Trayhurn, 1981; Goubern and Portet, 1986). It is supposed that the direct use of dietary lipid in the form of chylomicrons is the fuel for thermogenesis in this situation although the direct evidence for this hypothesis is lacking and further research is necessary.

UNCOUPLING PROTEIN:

Lin and Klingenberg (1980, 1982) have isolated the uncoupling protein from rat and hamster BAT and a sensitive RIA has been developed (Cannon et al., 1982; Lean et al., 1983; see Ricquier and Bouillaud, 1986 for a review). The RIA has shown UCP to be unique to BAT and undetectable in liver, white adipose tissue and muscle (Cannon et al., 1982; Afong et al., 1985). The availability of antibodies to UCP has also allowed the development of immunohistochemical techniques to identify UCP in BAT (Cadrin et al., 1985).

Recently cDNA sequences of UCP have been cloned for rat (Bouillaud et al., 1985; Ridley et al., 1986a), and mouse (Jacobsson et al., 1985), and a genomic fragment for human UCP (Bouillaud et al., 1988). The complete sequence has been established for both hamster (Aquila et al., 1985) and rat UCP (Bouillaud et al., 1986; Ridley et al., 1986a). UCP has 306 amino acids and there is about 92 % homology between rat and hamster UCP (Aquila et al., 1985; Ridley et al., 1986a; Bouillaud

et al., 1986. The uncoupling protein has six hydrophobic regions that span the inner mitochondrial membrane (Aquila et al., 1985; Klingenberg et al., 1980) and show considerable homology to the ADP-ATP translocase (Aquila et al., 1985) and the phosphate carrier (Runswick et al., 1987).

The UCP does not possess a specific N-terminal sequence that directs import into the mitochondria from the cytosol where it is made (Freeman et al., 1983, 1985; Bouillaud et al., 1986; Ridley et al., 1986a). The C-terminal region projects on the outer surface of the inner mitochondrial membrane and contains the nucleotide binding site (Eckerskorn and Klingenberg, 1987) and it is the primary antigenic determinant of the protein (Ridley et al., 1986b). Control of the proton carrier function of UCP is primarily mediated through the external C-terminal peptide (Fernandez M. et al., 1987; Kopecky et al., 1987; Nicholls et al., 1986; Ricquier and Bouillaud, 1986).

The complete gene sequence of BAT UCP has recently been elucidated (Bouillaud et al., 1988a). The transcription unit spans 8.4 Kb and contains 6 exons and 5 introns. The presence of two polyadenylation sites explains the two mRNAs for rat UCP previously detected in Northern blot analysis of rat BAT RNA (Bouillaud et al., 1985). Conversely, human UCP has only one mRNA of which the size corresponds to the larger mRNA of rodents (Bouillaud et al., 1988b).

The RIA of UCP is used to determine the total amount of UCP available for thermogenesis. The concentration of UCP determines the thermogenic capacity of BAT thermogenesis (Rial and Nicholls, 1984) but does not indicate the actual activity of BAT thermogenesis which is

measured by GDP-binding. The measurement of GDP-binding to isolated BAT mitochondria indicates the thermogenic activity of BAT in the intact animal. GDP is used *in vitro* because it does not enter the mitochondria, unlike ADP which is transported by the adenine nucleotide translocase. GDP-binding to isolated BAT mitochondria does not quantitate the amount of UCP but it does give a sensitive index of whether BAT has been stimulated or not. For example, cold acclimated rats have high levels of BAT UCP in the cold. When placed in a thermoneutral environment BAT thermogenesis is rapidly switched off (GDP-binding to isolated BAT mitochondria decreases) but the level of BAT UCP remains high for at least 12 hours (Park and Himms-Hagen, 1988).

Thus the rapid appearance or disappearance of GDP-binding sites on BAT mitochondria without any change in the amount of UCP reflects masking or unmasking of sites. This phenomena was first reported ten years ago (Desautels *et al.*, 1978; Himms-Hagen, 1989). Unmasking is found when animals are cold-exposed, injected with noradrenaline and the effect was rapidly reversible (Desautels *et al.*, 1978; Desautels and Himms-Hagen 1979, 1980). These older studies used densitometry of SDS page to estimate the amount of UCP. More recent studies using the RIA for UCP and GDP-binding to isolated BAT mitochondria have confirmed the earlier findings. Unmasking is seen as early as 20 minutes after cold-exposure (Swick and Swick, 1988) and remasking is seen 3 hours after cold acclimated rats were returned to 27°C (Peachey *et al.*, 1988). Unmasking is also found in response to noradrenaline (Peachey *et al.*, 1988) during arousal from hibernation (Horwitz *et al.*, 1985)

during arousal of restricted-fed mice from torpor (Himms-Hagen, 1985) and as a daily rhythm in cafeteria-fed rats (Rothwell et al., 1983b) and mice (Eley and Himms-Hagen, 1988). The level of GDP-binding also varies with the number of mice housed per cage (Jennings et al., 1986).

The mechanisms involved in the acute activation of UCP and the unmasking of binding sites are unknown. Possibilities include conformational changes in UCP, alterations in its oligomeric structure (Milner et al., 1988; Peachey et al., 1988), and mitochondrial swelling (Nedergaard and Cannon, 1987). Two studies however, have shown that unmasking was not simply due to mitochondrial swelling (Swick and Swick, 1986; Milner and Trayhurn, 1988) and the exact mechanism still remains unknown.

HYPOTHALAMIC REGULATION OF BAT THERMOGENESIS

It is thought that the hypothalamus is able to recognize changes in environmental temperature (cold) and changes in composition of diet (diet). The details of the exact location of these control centers and the connections to BAT thermogenesis are not fully elucidated as yet but many researchers are studying the problem (see Himms-Hagen et al., 1989 and Himms-Hagen 1989 for a review).

Electrical stimulation of the ventromedial hypothalamus (VMH) increases the thermogenic activity of BAT (Freeman and Wellman, 1987; Holt et al., 1987; Minokoshi et al., 1986) and denervation of the sympathetic nerves to BAT prevents the response (Minokoshi et al., 1986). Freeman and Wellman (1987) have shown that stimulation of the paraventricular nucleus (PVN) also activates BAT thermogenesis.

However, Holt et al. (1987) were unable to show an increase in BAT thermogenesis in response to electrical stimulation of the PVN.

Glucose injection into the VMH increases the firing rate of sympathetic nerves in BAT (Sakaguchi and Bray, 1987a). Insulin, however, suppresses the firing rate of BAT sympathetic nerves when injected into the VMH (Sakaguchi and Bray, 1987b). Thus, it seems likely that the hypothalamus is able to recognize changes in the diet.

Injection of corticotropin releasing factor (CRF) into the PVN is able to stimulate BAT thermogenesis and this effect is prevented by denervation of the sympathetic nerves in BAT (Lefeuvre et al., 1987).

The PVN is also involved in the control of thyroid status (see Thyroid Hormone section), food intake (Leibowitz, 1986) and in the control of ACTH (Liposits et al., 1987). It is thought that the suppressive effect of glucocorticoids on BAT thermogenesis (Tokuyama and Himms-Hagen, 1987) may also be mediated by the PVN.

Thus, the hypothalamic regulation of BAT thermogenesis is complex and further research is needed before we clearly understand the mechanisms involved.

THYROID HORMONES

SYNTHESIS AND RELEASE

Thyroid function is normally regulated via the hypothalamus and the pituitary (see Greenspan and Rapoport, 1986, for a review). Thyroid-releasing hormone (TRH) is secreted from the hypothalamus to the anterior pituitary and causes increased synthesis and release of

thyroid stimulating hormone (TSH). TSH then stimulates thyroid hormone production and release from the thyroid gland.

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) are synthesized on the glycoprotein matrix, thyroglobulin in the follicular cells of the thyroid gland. The follicular cells are surrounded by a rich vasculature and are innervated mostly by sympathetic nerves (Melander et al., 1975). The thyroid gland also contains C cells which produce calcitonin.

Thyroglobulin is a large protein with a molecular weight of 660,000 Daltons and contains approximately 110 tyrosine residues. The relative number of tyrosines in thyroglobulin is not higher than in many other proteins. The propensity of thyrogobulin for iodination is determined by its proximity to a very efficient iodination mechanism in the thyroid cell as well as by its tertiary structure which facilitates iodination. Iodine is taken up from the blood by the thyroid gland by active transport requiring ATP and O_2 and thyroglobulin is iodinated to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). The exact mechanism whereby two DIT's form T_4 and DIT and MIT forms T_3 is not clear. Several non-enzymatic and enzymatic pathways have been proposed. The newly formed thyroid hormones are stored in the thyroid gland as peptide-linked amino acids in thyroglobulin. Since they are released from the gland into the blood stream as the free amino acids, it is apparent that secretion must be preceded by proteolysis of the thyroglobulin (Dunn, 1984).

Thyroid stimulating hormone (TSH) has many effects on thyroid hormone synthesis and secretion. TSH stimulates the production of

thyroglobulin mRNA, synthesis of the protein and endocytosis of the glycoprotein into the follicular lumen. TSH also stimulates the iodide transport mechanism and the iodination of thyroglobulin. The secretion of T_4 and T_3 from the thyroid gland is stimulated by TSH by stimulating the proteolysis of the hormones from thyroglobulin by activating thiol proteases (Dunn, 1984). Direct stimulation of the nerves innervating the thyroid can also cause release of T_4 and T_3 (Green, 1987). Muraki et al., (1982) have shown that catecholamines act via β-adrenergic receptors to suppress TSH stimulated release of T_4. Thus, the secretion of thyroid hormone is complex.

When T_4 and T_3 are released into the circulation, they are almost instantaneously and completely bound to serum carrier proteins (see Robbins and Bartalena, 1986, for review). Thyroid binding globulin (TBG) binds the majority of T_4 and T_3 with the greatest affinity. thyroid binding pre-albumin (TBPA) has an intermediate affinity and albumin has the lowest affinity but the highest capacity for binding thyroid hormones.

The mean daily rate of T_4 delivery into the circulation of euthyroid man is 90 ± 9 μg and is closely regulated. T_4 is the major secretory product of the thyroid gland and usually less than 1% of the intrathyroidal T_4 pool is mobilized to meet this daily requirement (Greenspan and Rapoport, 1986).

3,5,3'-Triiodothyronine (T_3), the biologically active thyroid hormone (Surks and Oppenheimer, 1977), is secreted by the thyroid gland at about 1/20 the rate of T_4. Most circulating T_3 is therefore derived from the deiodination of T_4 to T_3 in non-thyroidal tissues (Engler and

Burger, 1984). Serum T_4 and T_3 levels are measured by RIA (see Meinhold, 1986 for a review).

THE HYPOTHALAMIC-HYPOPHYSEAL-THYROID AXIS

High levels of thyroid releasing hormone (TRH) exist in various parts of the hypothalamus including the paraventricular nucleus (PVN). Electrical stimulation of this nucleus causes the release of TSH, whereas its destruction decreases plasma TSH and thyroid hormones (Sheward et al., 1985; Aizawa and Greer, 1981). Thus TRH causes the release of TSH. Thyroid hormones exert a negative feed back control on the hypothalamic release of TRH into hypophysial stalk circulation (Rondeel et al., 1988).

Thyroid hormones also regulate the expression of the TRH gene since hypothyroid rats have increased and hyperthyroid rats have decreased TRH mRNA in the PVN (Segerson et al., 1987; Dyess et al., 1988). TSH secretion is also under negative feed back control of thyroid hormone, and there is strong evidence that T_4 must be converted to T_3 within the anterior pituitary to inhibit secretion (Larsen et al., 1979).

THYROID HORMONE METABOLISM

Less than 20% of the total serum T_3 is produced in the thyroid gland. The remaining 80-90% is derived from the outer (phenolic) ring monodeiodination of T_4 in the peripheral tissues, a reaction that is referred to as 5'-deiodination. By 5'-deiodination T_4 is converted to T_3 and reverse T_3 (3,5',3'-triiodothyronine) (rT_3) is converted to 3,3'-T_2. A second pathway involves monodeiodination of the tyrosyl or

inner ring, a process termed 5-deiodination. By 5-deiodination T_4 is converted to rT_3 and T_3 is converted to 3,3'-T_2. Since the 5'-deiodinating pathway produced T_3, the active thyroid hormone, the 5'-deiodinating pathway is considered to be the activating pathway whereas the 5-deiodination is considered to be an inactivating pathway.

There are at least three different iodothyronine deiodinases (for a review see Leonard and Visser, 1986; Köhrle et al., 1987). These enzymes are categorized on the basis of the type of reaction they catalyze and the susceptibility to inhibition by 6-propyl-2-thiouracil (PTU). Type I iodothyronine deiodinase is a nonselective enzyme that deiodinates both rings and is inhibited by PTU. Type II iodothyronine deiodinase is a true 5'-deiodinase since it only catalyzes phenolic ring deiodination producing T_3 from T_4. Enzyme Type III deiodinates only the tyrosyl ring and is, therefore, a true 5-deiodinase. Both Type II and Type III deiodinases are not inhibited by PTU.

TYPE I DEIODINASE

The Type I deiodinase enzyme is present in the thyroid gland, liver, and kidney (Chopra, 1977; Kaplan and Utiger, 1978; Erickson et al., 1981). The Type I enzyme requires sulfhydryl groups as cofactors, obeys ping-pong kinetics, K_m values for T_4 are in the μM range and the enzyme is inhibited by PTU (Visser, 1979; Leonard and Rosenberg, 1980). Under euthyroid conditions the Type I deiodinase is the major mechanism for the production of circulating T_3 (Silva et al., 1984), the enzyme is most active in the conversion of rT_3 into 3,3'-diiodothyronine (Leonard and Rosenberg, 1980). The enzyme is an intrinsic membrane

protein, therefore, the solubilization and purification of this enzyme have been very difficult. Recently, Yamauchi et al. (1987) and Boado et al. (1988) have cloned, near full length cDNA for the Type I enzyme. The predicted amino acid sequence showed a 99% identity with protein disulfide isomerase (PDI). PDI is known to participate in the breakage and reformation of disulfide bonds, thus PDI should act as a 5'-deiodinase in its reduced state.

TYPE II THYROXINE 5'-DEIODINASE (T5'-D)

The Type II enzyme differs from the Type I enzyme mainly because the activity is not inhibited by PTU. The Type II enzyme has not been purified and it is not known if the differences in enzymatic properties reflect separate enzyme polypeptides. Both Type I and Type II activities are found in microsomal fractions (Kaplan and Shaw, 1984).

Type II T5'D activity has been found in the CNS (cerebral cortex) (Visser et al., 1982), pituitary gland (Silva et al., 1978), placenta (Kaplan and Shaw, 1984), pineal gland (Tanaka et al., 1986) harderian gland (Guerrero et al., 1987) and in BAT (Leonard et al., 1983; Silva and Larsen, 1983). The Type II enzyme also requires sulfhydryl groups as cofactor but the enzyme follows sequential kinetics (Visser et al., 1982). The apparent Km values for T_4 in the T5'D reaction range between 0.5 and 2 nM which is near physiological levels of T_4. Thus, the Type II T5'D has a greater affinity for T_4 than the Type I enzyme (Visser et al., 1982; Kaplan and Shaw, 1984).

Since changes in the availability of substrate could have a significant impact on T_3 production and ultimately intracellular T_3

levels, the brain and the pituitary have evolved additional mechanisms to maintain intracellular T_3 within narrow limits. Thyroidectomy is known to increase Type II T5'D activity in cerebral cortex, pituitary and BAT (Leonard et al., 1983, 1984; Silva and Larsen, 1983). In BAT T5'D activity is stimulated by NA and cold stress (Silva and Larsen, 1983). The adrenergic mechanisms by which BAT T5'D activity is stimulated will be discussed in detail in chapter 3 of this thesis.

The Type II T5'D functions as a major local source of T_3 to be bound to nuclear T_3 receptors, unlike the Type I T5'D which produces T_3 mainly for circulating T_3 (Silva and Larsen, 1986c).

TYPE III IODOTHYRONINE DEIODINASE:

The Type III enzyme activity is found in the CNS and placenta. The enzyme follows sequential type enzyme kinetics and the Km for T_4 is 100 nM. T_3 is a preferred substrate for the inner ring deiodination since the Km is lower (40 nM). The Type III enzyme has not been purified and it is not known if this enzyme activity represents a separate polypeptide (Leonard and Visser, 1986).

NONDEIODINATIVE PATHWAYS OF THYROID HORMONES:

Thyroid hormones can be conjugated at the hydroxyl and amino group of the iodothyronines to glucuronic acid or sulfuric acid. Conjugation occurs mainly in the liver and kidney. Conjugated iodothyronines in bile are hydrolyzed by intestinal flora and deconjugated iodothyronines are reabsorbed by the intestine via the enterohepatic cycle. Thus thyroid hormones are eliminated in the urine and feces.

Oxidative deamination of the alanine side chain produced tetraiodothyroacetic acid (tetrac) and triiodothyroacetic acid (triac). These derivatives can also be conjugated and eliminated and urinary iodide has been shown to be the major degradation product (Burger, 1986).

CELLULAR MECHANISMS OF THYROID HORMONE ACTION:

Thyroid hormones regulate the basal metabolic rate by regulating O_2 consumption of almost all metabolically active tissues (obligatory thermogenesis). The exceptions are adult brain, testes, uterus, lymph nodes and spleen. Thyroid hormones are critically important in fetal development, particularly of the neural and skeletal systems. The development, growth and differentiation of normal cells depends on the presence of thyroid hormone and other growth factors (Tata, 1980). The mechanisms by which thyroid hormones influence these metabolic functions are complex and are not fully elucidated.

The interaction of thyroid hormones in target cells resulting in the final expression of its effects involves several binding proteins or specific receptors. These include sites on the plasma membrane, in the cytosol, on the mitochondria and in the nucleus (next section).

Plasma membrane binding sites for T_4 and T_3 have been described for liver and kidney (Gharbi-Chichi and Torresani, 1981). These binding sites could affect the activity of various transport mechanisms such as uptake of sugars, amino acids and Ca^{2+}, and they could also function as a specific transport of the thyroid hormones system within the plasma membrane (Müller and Seitz, 1984).

Cytosolic thyroid hormone-binding proteins have been described in brain, liver, kidney and heart (Osty et al., 1988; Oppenheimer and Schwartz, 1985). The physiological significance of cytosolic binding sites may relate to the storage and distribution of thyroid hormones between the cellular compartments (Oppenheimer and Schwartz, 1985).

Specific binding sites with high affinity and low capacity for thyroid hormones have been described for mitochondria isolated from liver, kidney, intestine, lung, heart and skeletal muscle and white adipose tissue (Müller and Seitz, 1984; Sterling, 1989). There is a general agreement that thyroid hormones increase mitochondrial activity but the exact mechanisms involved are not clear (Horst et al., 1989).

REGULATION OF GENE EXPRESSION BY THYROID HORMONE:

In the 60s Tata and co-workers showed that thyroid hormone administration increased the rate of RNA synthesis in rat liver suggesting that T_3 and T_4 might act by controlling gene expression. High affinity low capacity nuclear receptors have been found in all thyroid responsive tissues including brain, liver, kidney, and a variety of cultured cell lines (Samuels et al., 1988). The nuclear receptors have a 10 fold higher affinity for T_3 than for T_4, a molecular weight of 50 - 55,000 Daltons and bind to linker DNA regions in chromatin (Jump et al., 1981). In contrast to the successful isolation of the estradiol, progesterone, glucocorticoid, and vitamin D receptors by standard protein fractionation techniques, similar efforts by several groups have resulted in only the partial purification of the nuclear T_3 receptor (Oppenheimer et al., 1987). Recently a number of

investigators have reported that the product of the cellular oncogene c-erb-A gene has characteristics strongly suggestive of a role as a T_3 receptor (Weinberg et al., 1986; Sap et al., 1986; Samuels et al., 1988; Oppenheimer et al., 1987). Studies of the activation and regulation of this gene product may be a useful tool in studying the action of T_3 at the nuclear level.

Thyroid hormone stimulates the accumulation of mRNAs which encode for specific proteins such as growth hormone (Samuels et al., 1988), malic enzyme and other lipogenic enzymes (Goodridge, 1983), Na^+K^+ ATPase (Ismail-Beigi, 1988) and for several genes that encode hepatic proteins of unknown function such as Spot 14 (Oppenheimer et al., 1989). Identification of a consensus sequence specific for the binding of the thyroid hormone receptor would be an ideal way of determining whether the effect of thyroid hormone is direct or indirect; such an objective has not as yet been achieved (Oppenheimer et al., 1989).

A linear relationship has been found between the number of nuclear receptors occupied by T_3 and the intensity of the biological response for growth hormone and TSH; however a more complex relationship has been found in rat liver for malic enzyme and mitochondrial α-glycerophosphate dehydrogenase (Silva and Larsen, 1986c) and Spot 14 (Mariash, 1989). Thus, translational and post-translational regulatory steps may be important in T_3 regulation of malic enzyme, mitochondrial α-glycerophosphate dehydrogenase (Silva and Larsen, 1986c) and Na^+K^+-ATPase (Ismail-Beigi et al., 1989) and S14 (Mariash, 1989).

There are many unanswered questions in the mechanism of action of thyroid hormones. The precise molecular events at the level of the

gene require further research before we understand the cellular effects of thyroid hormones.

THYROID HORMONES AND BAT METABOLISM

Thyroid hormone is required for the thermogenic response of BAT to noradrenaline. Thyroidectomized rats maintained at low ambient temperatures will die within hours unless exogenous T_3 or T_4 is administered (Sellers et al., 1974). The noradrenaline content of BAT in thyroidectomized rats is normal (Kennedy et al., 1977) but they are unable to activate a thermogenic response. Treatment with low doses of T_4, however, permits a normal thermogenic response to cold (Triandafillou et al., 1982; Fellenz et al., 1982). It was therefore concluded that thyroid hormones act in a permissive manner in BAT metabolism (Himms-Hagen, 1983). The hyperthyroid state induced by administration of exogenous thyroid hormones is associated with suppression of BAT thermogenic function (Sundin, 1981; Triandafillou et al., 1982), most probably because the extra heat generated in the muscle, kidney, liver, gut, and white adipose tissue (Rothwell and Stock, 1984b) results in a central suppression of BAT thermogenesis, mediated by suppression of sympathetic activity in BAT (Knehans and Romsos, 1984b).

Thyroid hormone is required at multiple sites involved in the β-adrenergic pathway in BAT (Sundin et al., 1984; Seydoux et al., 1982b). Hypothyroid BAT has a reduced sensitivity to noradrenaline (Arieli and Chinet, 1985) and a decreased β-adrenergic receptor density (Seydoux et al., 1982a).

The α_1-adrenergic pathway in BAT is less susceptible to lack of thyroid hormones than the β-adrenergic mediated thermogenic response (Garcia-Sainz and Fain, 1980).

The first reports of the Type II T5'D enzyme activity in BAT coincided with the onset of my research (Silva and Larsen, 1983 and Leonard et al., 1983). Silva and Larsen and co-workers demonstrated that the local production of T_3 from T_4 was increased in cold exposed and hypothyroid rats and the enzyme was activated by noradrenaline through α_1-adrenergic receptors. More recent work has shown that insulin and glucagon can also activate BAT T5'D activity (Silva and Larsen, 1986a; Mills et al., 1987). BAT T5'D activity is suppressed by thyroid hormone and by growth hormone (Silva and Larsen, 1986b). Thus, BAT T5'D is under complex hormonal control.

The function of BAT T5'D is two fold; one to provide T_3 for the circulation (Fernandez J.A. et al., 1987; Silva and Matthews, 1984; Silva et al., 1984; Silva and Larsen, 1985) and the second to provide intracellular T_3 to saturate T_3 receptors (Silva and Larsen, 1985; Bianco and Silva 1987a,c) for the optimal expression of the BAT UCP gene (Silva, 1988; Bianco and Silva 1987 a,b,c).

In BAT thyroid hormones are also involved in the activity of Na^+K^+-ATPase, which is increased after T_3 administration, and in the activity of a number of lipogenic enzymes (Knehans and Romsos, 1984; Bianco and Silva, 1987b). Malic enzyme, which is thyroid-dependent in the liver, is not altered in BAT by thyroid status (Bianco and Silva, 1987b).

Spot 14 mRNA, which is rapidly induced by T_3 in the liver, is present in BAT at high levels and is increased by both acclimation to

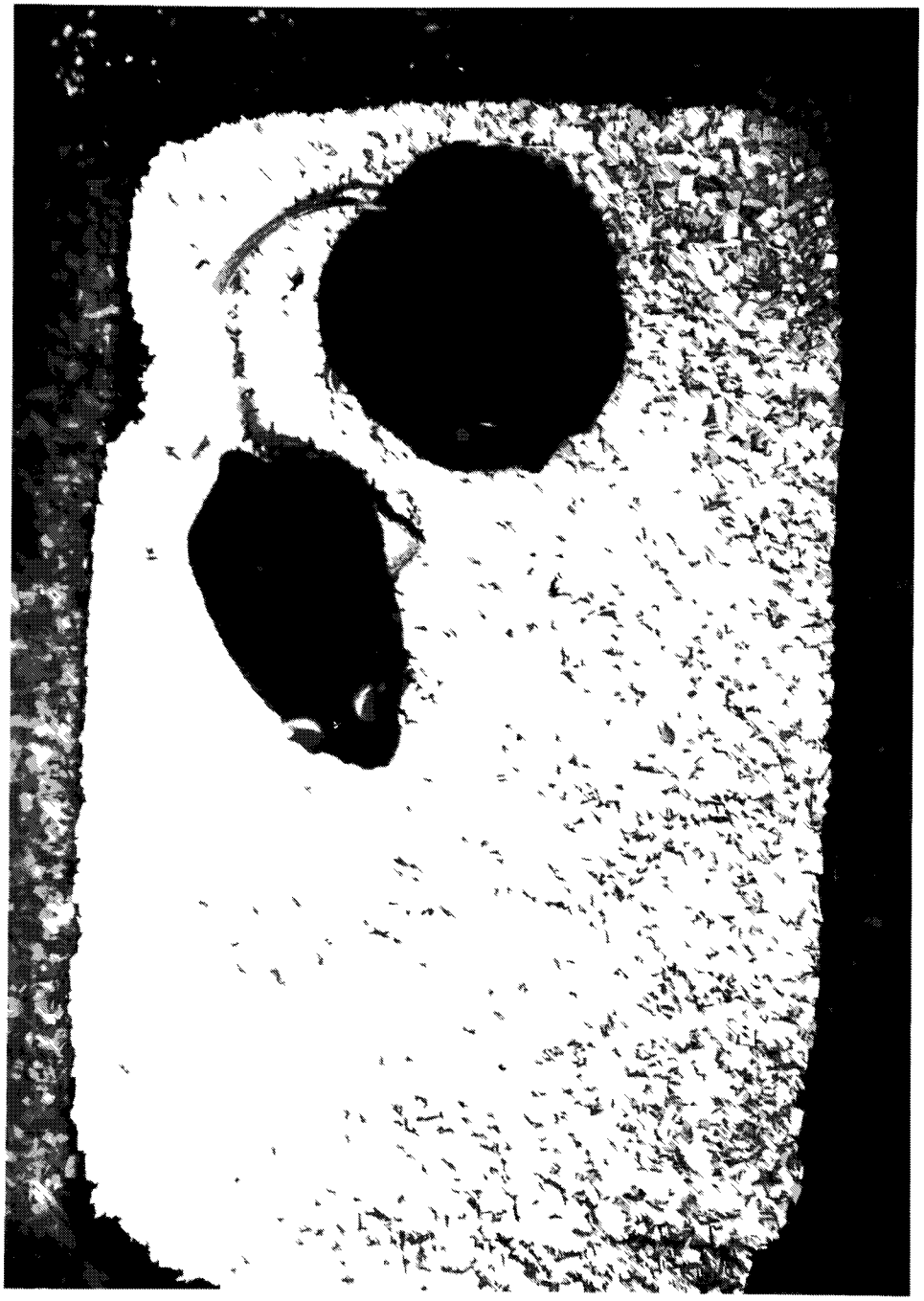
cold and by cafeteria feeding but is not dependent on the thyroid status in BAT (Jump and Oppenheimer, 1985; Freake and Oppenheimer, 1987). The identity of Spot 14 is not known but it has been suggested that Spot 14 may function in lipogenesis (Freake and Oppenheimer, 1987).

GENETICALLY OBESE (OB/OB) MICE

BACKGROUND AND OBJECTIVES

The genetically obese (ob/ob) mouse is a well studied model of obesity, similar in many respects to hyperplastic human obesity (Bray and York, 1971). The ob/ob mouse first appeared in a non-inbred stock at the Jackson Laboratories, Bar Harbor Maine (Ingalls et al., 1950). The obese-hyperglycemic syndrome which typifies this animal is inherited as an autosomal recessive gene (ob) located on chromosome 6 (Coleman, 1978). The gene can be maintained on several backgrounds, altering the severity of the syndrome but maintaining the phenotypic expression of the genome unchanged. In this thesis C57BL/6J ob/ob mice from Jackson Labs were studied. Another strain often studied in the U.K. is the Aston strain of ob/ob mouse. The ob/ob mouse is characterized by massive obesity (see Plate 1), hyperphagia, hyperglycemia, hyperinsulinemia, insulin resistance and an extreme sensitivity to cold (Bray and York, 1971, 1979). It has been postulated that obesity results from both a greater than normal efficiency in storing dietary energy associated with a subnormal metabolic rate as well as an elevated food intake since the obesity

PLATE 1: LEAN AND OB/OB C57BL/6J MICE.
Obese mice weigh more and have more body fat than lean mice. These mice are approximately 12 months old and weigh approximately 30 and 90 grams respectively.



will still occur even if food intake is limited to that of lean siblings (James and Trayhurn, 1976; Thurlby and Trayhurn, 1978, 1979). In fact, the preweanling ob/ob mouse is not hyperphagic (Rath and Thenen, 1979), thus the initiation and early development of obesity in the ob/ob mouse is due to a low level of energy expenditure and consequent high efficiency of energy utilization (Trayhurn, 1986).

The idea that thermogenesis might be abnormal in ob/ob mice resulted from the observation that cold (4°C) exposed ob/ob mice develop a rapid and profound hypothermia and usually die within 3 hours (David and Mayer, 1954). Defective nonshivering thermogenesis has been demonstrated in the ob/ob mouse by a lower metabolic rate at all temperatures below thermoneutrality compared to lean mice (Trayhurn and James, 1978). At thermoneutrality however, lean and ob/ob mice have similar energy expenditure expressed per whole animal (Trayhurn and James, 1978). Adult ob/ob mice fail to respond to acute cold exposure by the substantial increase in BAT mitochondrial GDP-binding that characterizes the lean mouse (Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980). The finding that ob/ob mice have a relatively normal sympathetic innervation of BAT (Ashwell and Dunnett, 1985) and noradrenaline content of BAT but decreased noradrenaline turnover at temperatures below thermoneutrality (Knehans and Romsos, 1982; Zaror-Behrens and Himms-Hagen, 1983) and a normal activation of these sympathetic nerves in response to cold (Knehans and Romsos, 1983; Young and Landsberg, 1983; Zaror-Behrens and Himms-Hagen, 1983), led to the concept that BAT of the ob/ob mouse is refractory to noradrenaline. This hypothesis was confirmed by the direct observation of a reduced

response of BAT of the ob/ob mouse to noradrenaline in blood flow studies (Thurlby and Trayhurn, 1980), and at the level of adenylate cyclase (Bégin-Heick and Heick, 1982) and lipolysis (Assimacopoulos-Jeannet et al., 1982).

The well known interaction between thyroid hormone and the adrenergic nervous system (Nilsson and Karlberg, 1983) and the refractoriness of ob/ob mice to noradrenaline led to the hypothesis that ob/ob mice may also exhibit defective thyroid hormone thermogenesis. The cold sensitivity, low metabolic rate and low body temperature are consistent with a hypothyroid condition as well as with an impairment in NST. However, there is no histological evidence for hypothyroidism in the thyroid gland of young ob/ob mice (Joosten and Vander Kroon, 1974). Blood T_3 levels are similar or slightly higher than those found in lean mice (Ohtake et al., 1977; Mobley and Dubuc, 1979; York et al., 1978a) and the thyroid-sensitive α-glycerophosphate dehydrogenase activity in liver is normal (York et al., 1978a). After an extensive review of the literature, Bray and York (1979) have concluded that the hypothalamic-pituitary-thyroid axis is normal. Thus, it appears that ob/ob mice are euthyroid. However, Kaplan et al. (1985) suggest that there have been many conflicting reports of the level of serum T_4 in ob/ob mice. Some researchers have found lower (Joosten and Van der Kroon, 1974, Ohtake et al., 1977) no different (Gambert and Garthwaite, 1981; Garthwaite et al., 1979) or higher (Mobley and Dubuc, 1979) levels. Kaplan et al. (1985) suggest that this variation in the literature could be due to abnormal thyroid hormone binding proteins in sera from ob/ob mice which suggest that

thyroid hormone levels are not easily predicted from serum measurements. For this reason, in most of my experiments, thyroid hormone free ob/ob mouse serum was used to produce standard T_3 and T_4 curves in order to more accurately estimate the thyroid hormone status of the ob/ob mouse.

A number of investigators have demonstrated that catecholamine-stimulated lipolysis (Bégin-Heick and Heick, 1977) is impaired in white adipose tissue of ob/ob mice but treatment with thyroid hormones improves the lipolytic response to adrenaline (Bégin-Heick and Heick, 1977). This indicates that a refractoriness to thyroid hormone exists in white adipose tissue of the ob/ob mouse.

Treatment with thyroxine is also able to improve the abnormally low binding of GDP to brown adipose tissue mitochondria and permit a cold induced increase in GDP-binding to occur in ob/ob mice (Hogan and Himms-Hagen, 1981). On the basis of these observations a hypothesis was proposed that the defect in the ob/ob mouse is a selective refractoriness of BAT to the permissive effect of T_3 on metabolic responsiveness to noradrenaline (Himms-Hagen, 1983). At the same time Silva and Larsen (1983) and Leonard *et al*. (1983) demonstrated the presence of Type II T5'D in BAT of rats. Thus, it was possible that the refractoriness of BAT of the ob/ob mouse to T_3 could be due to a defect in BAT T5'D which converts T_4 to the active thyroid hormone T_3, resulting in an intracellular hypothyroid state of BAT. Thus, the initial objective of this thesis was to study BAT T5'D activity in lean and ob/ob mice at 28 °C and during cold exposure, when BAT thermogenesis had been shown to be defective (Hogan and Himms-Hagen, 1980).

Initial results of this study disclosed a defective response of T5'D activity in BAT of the cold-exposed ob/ob mouse (Kates and Himms-Hagen, 1985; see Chapter 1 Part 1). Subsequent studies, therefore, had as their general objective the study of the relationship of BAT T5'D with thermogenic responsiveness of BAT to various stimuli in the ob/ob mouse.

Adrenalectomy is known to normalize many of the abnormalities of the ob/ob mouse; for example, hyperphagia, accelerated weight gain and obesity (Holt and York, 1984; Smith and Romsos, 1985). Adrenalectomy also improves hyperinsulinemia, hyperglycemia and insulin resistance (Smith and Romsos, 1985; Solomon et al., 1977; Solomon and Mayer, 1973). Adrenalectomy also improves BAT thermogenesis as measured by an increase in GDP-Binding (Holt and York, 1984) and adrenalectomy improves the reduced sympathetic nervous system activity in BAT (Vander Tuig et al., 1984). Since adrenalectomy was able to improve BAT thermogenesis in the ob/ob mouse, another objective of this thesis was to study the effect of adrenalectomy on BAT T5'D activity of lean and ob/ob mice at 28 °C and during cold exposure.

Gradual acclimation to mild cold has been shown to improve the poor cold-resistance of the ob/ob mouse and at the same time enhance the thermogenic functioning of its BAT as measured by an increase in GDP-binding and an increase in its capacity to respond to noradrenaline by an increase in metabolic rate (Bas et al., 1983; Hogan and Himms-Hagen, 1980). Thus, another objective of this thesis was to study the effect of gradual cold acclimation on BAT T5'D activity in lean and ob/ob mice to determine if BAT T5'D activity is related to the improvement of BAT

thermogenesis of the ob/ob mouse.

Feeding a palatable "cafeteria diet" is also known to improve BAT thermogenesis in ob/ob mice (Himms-Hagen et al., 1986; Trayhurn et al., 1982) as measured by an increase in GDP-binding. Another objective was therefore to measure BAT T5'D activity in lean and ob/ob mice fed a cafeteria diet to determine if the improved thermogenic function of the ob/ob mouse is related to BAT T5'D activity.

In the rat fasting and food restriction are known to reduce sympathetic-mediated thermogenesis in BAT (Landsberg and Young, 1983; Rothwell and Stock, 1982). Another objective of this thesis was to study the role of BAT T5'D activity in BAT when thermogenesis was switched off in order again to relate BAT T5'D activity to the thermogenic activity of BAT.

Silva and Larsen (1983) demonstrated that BAT T5'D activity was regulated by noradrenaline through α_1-adrenergic receptors in rats. The final objective of this thesis was to study the adrenergic regulation of BAT T5'D activity in lean and ob/ob mice in order to determine if the α_1-adrenergic receptor-mediated regulation of T5'D in BAT of ob/ob mice is defective.

SUMMARY OF OBJECTIVES

Defective BAT function in the ob/ob mouse, believed to be associated with refractoriness to the permissive effect of T_3 on thermogenic responsiveness to noradrenaline, might be due to an abnormality in the recently discovered enzyme, thyroxine 5'-deiodinase (T5'D) in BAT. This enzyme produces T_3 from T_4 within BAT and its activity is controlled by noradrenaline. Thus, the initial hypothesis was that BAT of the ob/ob mouse might be in a state of intracellular hypothyroidism secondary to a defective functioning of T5'D.

1. The initial objective was to study BAT T5'D activity in lean and ob/ob mice during exposure to cold, when BAT thermogenesis was known to be defective in the ob/ob mouse (see Chapter 1, Part 1).

2. When initial results disclosed a defective responsiveness of T5'D activity in BAT of the cold-exposed ob/ob mouse, subsequent studies had, as their general objective, the study of the relationship between BAT T5'D responsiveness with thermogenic responsiveness of BAT to various stimuli in the ob/ob mouse.

   These stimuli included:

   2a. Treatments which improve the defective response of the ob/ob mouse to acute cold-exposure, including adrenalectomy and gradual acclimation to cold (see Chapter 1 Part 2).

2b. Effects of diet to increase or to decrease thermogenic activity in BAT (see Chapter 2).

2c. Treatment with a variety of adrenergic agonists and antagonists (see Chapter 3).

3. When the opportunity arose to obtain BAT from a primate species, the cynomolgus monkey, a study was performed with the objective of finding out whether BAT of this monkey possessed T5'D, as did BAT of rodents. Since there was doubt about the presence of BAT in this species, it was also necessary to demonstrate that the tissue studied was authentic BAT (see Chapter 4).

MATERIALS AND METHODS

MATERIALS AND METHODS

1) ANIMALS

Female C57Bl/6J mice, genetically obese (ob/ob) and their lean littermates (+/?), were obtained from Jackson Laboratories, Bar Harbor, Maine. They arrived at the animal care facility at the Health Sciences Building in cages of littermates, usually 4-6 mice per cage. Upon arrival, animals were weighed and separated into individual 27 x 17 x 12 cm plastic cages with wood-chips as bedding or they were caged in groups of 3-4 lean or 3-4 obese mice. The animals were housed at 28 ± 1 ^{o}C with a Tempscribe temperature recorder (Model #14-9007 Bacharach, Inc.) recording room temperature 24 hours per day for 7 continuous days. The humidity was maintained between 45-55 %. The light cycle was 12 hours light / 12 hours dark with lights on at 06:00 or 09:00 depending on the experiment. The animals had access to tap water ad libitum and were fed ad libitum Purina Chow #5012. The metabolizable energy of the chow was 3.43 Kcal/g and was composed of 22.5 % protein, 53.2 % carbohydrate and 4.5 % fat by weight. This diet fulfilled all the nutrient requirements of mice for growth and reproduction (National Research Council, 1978). In some experiments animals were fed a cafeteria diet, 30% sucrose, or a restricted diet. The details of each of these protocols will be discussed before each experiment.

2) ADRENALECTOMY

Lean (+/?) and obese (ob/ob) mice were adrenalectomized at 4.5 weeks of age. A greater survival rate (80-90%) in ob/ob mice was found when the animals were young. This was probably due to the fact that young obese mice have considerably less body fat than older (6-8 week) mice making the surgical procedure easier to perform.

The surgery was performed under sterile conditions in the animal care facility. The mice were anesthetized with halothane and either bilateral adrenalectomy or sham adrenalectomy performed by the dorsal approach. Wound clips were used to close the incision and all mice were given a single injection of corticosterone (100µg in 100µl saline) at the end of the operation.

Animals were housed at 28°C in single cages with free access to chow (#5012) and tap water. A saline (0.9% NaCl) solution was also provided.

3) EXPERIMENTAL CONDITIONS

The animals were killed in the same room as they were housed. This was done to minimize the stress and sympathetic activation that occurs when the animals are moved. The animals were killed quickly by cervical dislocation and were bled immediately. Blood was collected in 1.5 ml microcentrifuge tubes with the aid of a small plastic funnel and kept on ice. At the same time, rectal temperatures were measured using a probe (Bailey Instruments-BAT-8 digital thermometer, probe RET-3) inserted to a depth of 1.8 cm. Final body weights were measured using a Mettler balance (PE 1600). Interscapular and subscapular BAT was

quickly removed and placed in ice cold isolation medium (0.25 M Sucrose, 1.0 mM HEPES, 0.2 mM EDTA, potassium salt, at pH 7.2). Gonadal white adipose tissue was removed and placed in a small plastic container until it was cleaned and weighed. This process took approximately 3-4 minutes per mouse and was performed as quietly as possible. All other procedures were performed in the laboratory.

4) PREPARATION OF SERUM

Fresh blood was kept on ice for approximately 30 minutes. The samples were centrifuged in an Eppendorf centrifuge (#5412) at maximum speed (14,000 x g) for 15 minutes in the 4°C cold room. Serum was removed as completely as possible and centrifuged again at maximum speed for 15 minutes at 4°C. Clear serum was then frozen in liquid nitrogen and stored at -70°C (Harris Manufacturing Co., Inc.)

5) PREPARATION OF BAT HOMOGENATES

BAT was cleaned of adhering muscle and white adipose tissue on ice. Gonadal fat was dissected free of uterus and ovaries. This procedure took approximately 5 minutes per mouse. The tissues were then blotted dry and weighed using a Mettler balance (AE 163).

BAT was finely minced with scissors and homogenized on ice in 3.5 ml of isolation medium (0.25 M sucrose, 1.0 mM HEPES, 0.2 mM EDTA, potassium salt, at pH 7.2) using a glass/teflon Potter - Elvehjem tissue grinder (Kontes #21) at 400 rpm. The final volume was measured and 500 µl of homogenate was rehomogenized manually on ice in a conical glass/glass Duall tissue grinder with 10 strokes of the pestle.

Samples of this second homogenate were frozen in screw cap microcentrifuge tubes (Sarstedt) in liquid nitrogen (100 µl for protein assay. 150 µl for deiodinase assay and 150 µl for UCP RIA). The remaining 3 ml of BAT homogenate was used to isolate mitochondria.

6) ISOLATION OF BAT MITOCHONDRIA

Mitochondria were isolated by differential centrifugation using the method of Slinde et al. (1975). BAT was homogenized as described above. Homogenates were centrifuged at 4°C for 10 minutes at (1,500 x g) 3,000 rpm in a Sorvall RC-5B or RC2-B centrifuge with brake off. using an HB-4 rotor and 42 ml Sorvall centrifuge tubes. The supernatant was filtered through 2 layers of cheese cloth and centrifuged at (16,000 x g) 10,000 rpm for 14 min at 4°C. For lean mice the pellet was resuspended in 42 ml of isolation medium using a glass pipette; for ob/ob mice the pellet and layer of fat that formed was rehomogenized in isolation medium using the glass teflon homogenizer at 400 rpm on ice. This was done to free any mitochondria that may have been trapped in the cake of fat. The homogenate was again centrifuged at 3,000 rpm for 10 minutes and the supernatant filtered through 2 layers of cheese cloth, the pellet and cake of fat were discarded. The supernatant was then centrifuged at 10,000 rpm for 14 minutes and the resulting mitochondrial pellet was combined with the first pellet with 42 ml of isolation medium and was centrifuged again at 10,000 rpm. Mitochondria were washed once more with 15 ml of isolation medium in smaller centrifuge tubes and centrifuged at 10,000 rpm. The final pellet was allowed to drain on Kleenex and was

resuspended in isolation medium to a final protein concentration of 10-30 mg/ml (lean: 180-300 µl and ob/ob: 180-200 µl). A sample (30-40 µl) was frozen in liquid nitrogen for protein determination. The remaining mitochondria were kept on ice for immediate use in the GDP-Binding assay.

7) PROTEIN ASSAY

BAT homogenate and BAT mitochondrial protein was estimated using the Lowry et al., method (1951) as modified by Schacterle and Pollack, (1973). Samples of BAT homogenate (20 µl) were precipitated in 5 ml 12.5 % TCA (trichloroacetic) acid at 4°C overnight to remove any fat that might interfere with the assay. Lean samples were precipitated in 15 ml polypropylene conical centrifuge tubes and ob/ob samples were precipitated in 15 ml COREX glass conical centrifuge tubes. The tubes were centrifuged at (1,500 x g) 3,000 rpm for 15 minutes at 4°C in a Beckman J6-B centrifuge. The supernatants were removed either by decanting (ob/ob) or by suction (lean). The pellets were dissolved in 1 ml of 0.5 N NaOH at 55°C for 15 minutes, vortexed, and allowed to cool to room temperature. Samples of BAT mitochondria (8 µl) were diluted with 1 ml of 0.5 N NaOH at room temperature. A series of standards (15-120 µg) were prepared fresh at each assay from a frozen stock solution of 10 mg/ml BSA. All samples, 1 ml, were incubated at room temperature for 10 minutes with an alkaline copper reagent, 5 minutes at 55°C with a phenol reagent and then cooled to room temperature in an ice water bath. The absorbance was read at 650 nM. A computer program was used to calculate the protein concentration

from the standard curve.

8) GDP-BINDING TO ISOLATED BAT MITOCHONDRIA

The binding of ^{3}H-GDP to isolated BAT mitochondria was performed essentially according to the method of Nicholls (1976) as modified by Desautels et al. (1978). The purine nucleotide GDP is usually used because it does not enter the mitochondria and it is not susceptible to hydrolysis. BAT mitochondria were isolated as described in section #6. Mitochondria,(20 µl), (200-500 µg protein), were incubated in microcentrifuge tubes for two minutes at room temperature in medium (280 µl) at pH 7.1 containing 100 mM Sucrose, 1 mM EDTA (disodium salt), 10 mM choline chloride, 20 mM TES (N-Tris(hydroxymethyl-2-aminoethane sulphonate), 5 µM rotenone, 100 µM potassium atractyloside, (all chemicals from Sigma) and [8-^{3}H] GDP (guanosine 5'-diphosphate, ammonium salt 11.3 Ci/mmol, Amersham), final concentration 10 µM. [U-^{14}C] sucrose was present to estimate the amount of water trapped in the pellet (0.15 µCi/ml of [^{14}C] Sucrose high (560 mCi/mmol) specific activity, Amersham). Non-specific binding was assessed in the presence of 100- fold excess (1.0 mM) ADP (Sigma). The incubation was started by the addition of mitochondria with vortexing. Binding was found to be in the linear range from 100-500 µg of protein for lean mice and 200-500 µg of protein for ob/ob mice. The incubation was stopped by a 2 minute centrifugation at maximum speed in an Eppendorf microcentrifuge (#3200) at room temperature. The supernatant was removed by suction and the pellet was dissolved by cutting off the tip of the microcentrifuge tube, placing it in a scintillation counting

vial, adding 1 ml of NCS tissue solubilizer (Amersham), and incubating at 55°C overnight. When the pellet was dissolved. 50 µl of 10 % ascorbic acid (Sigma) followed by 10 ml of toluene containing 0.7 % PPO (2,5-diphenyloxazole) (Sigma) was used for scintillation counting. The samples were left in the dark for at least 12 hours before they were counted to minimize quenching. The samples were counted with a Beckman LS6800 liquid scintillation counter The binding of ^{3}H GDP was calculated by subtracting non-specific binding from the total binding measured after correction for the amount of water trapped in the pellet which was estimated by the [^{14}C] sucrose. GDP-binding is expressed as pmoles GDP bound/mg mitochondrial protein. Total GDP-binding to BAT was not calculated because the recovery of mitochondria isolated from the homogenate was not measured. The calculations were performed using a computer program.

9) PURIFICATION OF MOUSE UNCOUPLING PROTEIN (UCP)

The purpose of this procedure was to obtain pure lean mouse UCP to prepare a standard curve in order to quantitate lean and obese (ob/ob) mouse UCP by solid phase radioimmunoassay (RIA). The method outlined follows the method of Lin and Klingenberg (1980, 1982) with some modifications.

Twenty-four homozygous lean (+/+) female C57Bl/6J mice were obtained from Charles River at 4-5 weeks old. They were housed in single cages at 28°C with free access to rodent chow (#5012) and tap water for 1 week, then they were acclimated to 4°C for 3 weeks. The animals were killed at 8-9 weeks of age. They had body weights ranging

from 20-24 g. Brown adipose tissue was removed from the intrascapular and subscapular regions, cleaned of muscle and connective tissue and weighed. BAT wet weights ranged from 0.3 - 0.4 g. BAT from 3 animals was pooled and homogenized in isolation medium (see method section #5). Mitochondria were isolated as previously described (section #6). The 8 final mitochondrial pellets were resuspended in 25 ml of isolation medium and samples were taken for the measurement of mitochondrial protein. The remaining mitochondrial suspension was aliquoted evenly into four centrifuge tubes, washed with isolation medium and centrifuged at 10,000 rpm for 14 minutes. The pellets were quickly frozen in liquid nitrogen and stored at -70 °C overnight.

The mitochondrial pellets were allowed to thaw on ice just enough to allow chipping them into small pieces. The mitochondria were resuspended in 2.5 ml (1mg/ml) standard medium (20 mM MOPS, 50 mM Na_2SO_4, 0.16 mM EDTA, Sodium salt at pH 6.7) in a glass-teflon homogenizer. The mitochondria were then quantitatively transferred into ultracentrifuge tubes with 9 ml of standard medium + 3.2 % lubrol. Soluble and peripheral proteins were extracted with lubrol for 30 min at 0 °C and then the suspension was centrifuged at 100,000 g (35K in a Beckman rotor 55.2 Ti) for 30 min at 4 °C in a Beckman L8-55M ultracentrifuge. The pellet was washed once by resuspending in 9.0 ml STE buffer (0.3 M sucrose, 10 mM Tris-base 2 mM EDTA, pH 7.2) and centrifuging again at 100,000 g for 30 min. The remaining lubrol was removed by swirling a small amount of STE buffer in the ultracentrifuge tube and decanting. The pellet was again frozen in liquid nitrogen and stored overnight at -70°C.

The pellet was thawed on ice, chipped into the glass-teflon homogenizer and resuspended in 8 ml of standard medium + 5 % triton X-100 (BDH Chemicals). Samples were taken for protein determination and to be run later on SDS PAGE (see method #10, LUBROL). These samples contained all the mitochondrial proteins present in the homogenate except soluble and peripheral proteins extracted by lubrol. The high level of triton and low ionic strength of the 5 % triton X-100 extraction selects for the extraction of UCP over the ADP/ATP carrier (Lin and Klingenberg, 1982). The mitochondria were extracted for 30 min at 0 °C and then centrifuged in the ultracentrifuge at 100.000 g for 30 min. The supernatant (8 ml) containing UCP was retained and samples were taken for protein determination and for SDS PAGE (see methods #10, TRITON). These samples contained proteins soluble in triton X-100.

The hydroxylapatite column was prepared by washing 15 g of hydroxylapatite (Bio-Rad) with 100 ml of standard medium 3 times. The column (26 mm x 12 cm, LKB #2137) was poured, packed and the void volume determined using the following formula:
(volume = π radius^2 x height). The supernatant (8 ml) was warmed to room temperature and loaded onto the column. The column was run at room temperature at 3.8 ml/min. After the void volume, 0.95 ml aliquots were collected every 15 seconds resulting in 50 fractions collected. Protein content was estimated by the modified method of Lowry (Peterson, 1977) in the presence of 5 % SDS (sodium dodecyl sulfate). Absorbance was read at 760 nm in a quartz cuvette and bovine serum albumin (Sigma) was used as the standard. The development of

serum albumin (Sigma) was used as the standard. The development of colour was time dependent, so only 20 samples + standard curve were measured at one time. Triton X-100 was determined by absorption at 276 nm using a standard curve of 0.001 to 0.1 % triton X-100. The samples were diluted 1:100 and their absorbance was measured in quartz cuvettes using a Gilford Instrument spectrophotometer (model # 2400-2).

Figure 1 shows the elution profile of the hydroxylapatite column. The sucrose density gradient step was not performed. Tubes # 8-23 were pooled and stored in 1 ml aliquots at - 70 °C. This protein (425 µg/ml, 1.76 % triton) was used as a standard in the solid phase RIA.

TABLE 1: PURIFICATION OF MOUSE BAT UNCOUPLING PROTEIN

	PROTEIN mg	TRITON X-100 %	YIELD %
MITOCHONDRIA	253.1	-	100.0
LUBROL	58.8	-	23.2
TRITON	31.7	5.0	12.5
HYDROXYLAPATITE COLUMN			
VOID VOLUME	0.0	-	-
#1-7	0.4	0.19	-
#8-23	6.5	1.76	2.6
#24-35	3.1	0.11	-
#35-50	1.4	0.27	-

LUBROL: BAT mitochondria after extraction with lubrol.
TRITON: extract of BAT mitochondria after extraction with lubrol.

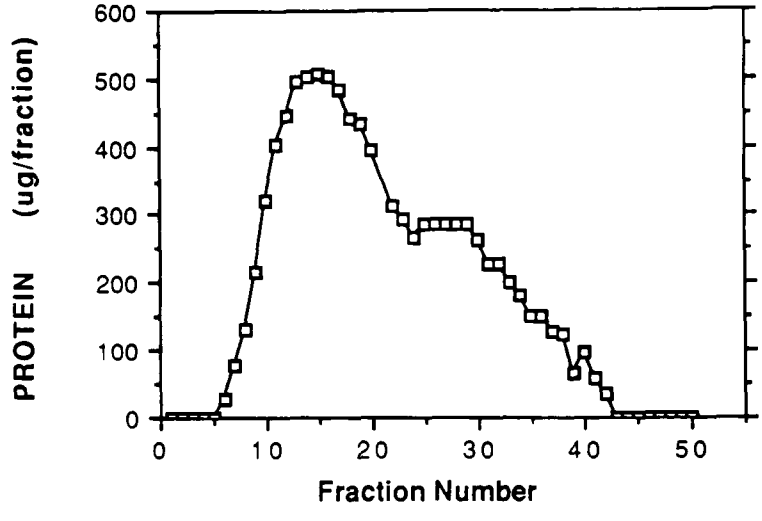


Figure 1: ELUTION PROFILE FROM THE HYDROXYLAPATITE COLUMN. Each fraction was 0.95 ml. The ADP/ATP translocase is degraded at room temperature and is adsorbed to the column whereas BAT UCP is stable at room temperature and is eluted from the column (see Chapter 4 for western blots). Fraction # 8-23 was collected and used as purified mouse BAT UCP standard curves in solid phase RIA for UCP (See Method Section # 12).

10) SDS-PAGE, ELECTROTRANSFER AND IMMUNOBLOT

SDS-PAGE:

The quality of the purified mouse UCP was examined and compared to samples obtained in the same manner as described in section #9 for rat and hamster. (The rat and hamster purifications were performed by Dr. Gloria Zaror-Behrens). Human and monkey BAT homogenates were also included in the SDS-PAGE and immuno-blot technique to compare purity and size. All chemicals and equipment were obtained from Bio-Rad (See Chapter 4 for results).

The discontinuous gel system of Laemmli, (1970), was used in a double vertical slab gel assembly. The stacking gel contained 10 % acrylamide (29 % acrylamide and 1 % N, N-methylene-bis-acrylamide diluted with 0.5 M TRIS, 0.4 % SDS, pH 6.8) and the gradient gel 14-20 % acrylamide (29 % acrylamide and 1 % N, N-methylene-bis-acrylamide diluted with 1.5 M TRIS, 0.4 % SDS, pH 8.8) Low molecular weight protein standards (Bio Rad.) (10,000-100,000 Daltons) consisted of lysozyme (14,400 M_r), soybean trypsin inhibitor (21,500 M_r), carbonic anhydrase (31,000 M_r), ovalbumin 45,000 M_r), bovine serum albumin (66,200 M_r), phosphorylase B (92,500 M_r), β-galactosidase (116,250 M_r), and myosin (200,000 M_r). Samples and standards were prepared by boiling aliquots in sample lysis buffer (7.4 % SDS, 30 % glycerol, 15 % mercaptoethanol, 0.5 M Tris-Cl and 2 mg bromophenol blue pH 6.8) for 3 minutes. Samples were loaded and the gel was run using the following buffer: 125 mM TRIS, 1M glycine, 0.5 % SDS, pH 8.3. The gel was run in the cold room with a circulating water bath for cooling at 50 V for 30 minutes (through the stacking gel) and then at 250 V for 4-

5 hours. Both gels were run at the same time, one was stained and the other was used for electrotransfer.

One gel was stained by incubating in fixing solution [(water: methanol:glacial acetic acid), 5:5:1] for 10 minutes, in staining solution (same as fixing solution + 0.05 % Coomassie Blue R250) for one hour and in several washes of destaining solution [(water:ethanol:glacial acetic acid) 8:3:1] for 2 hours. The gel was then photographed using a Polaroid Land Camera (Model #MP3) and Ilford FP4 film.

Electrotransfer:

The second gel was placed immediately in blotting buffer (150 mM glycine, 20 mM Tris-base, 0.02 % SDS, 20 % methanol, pH 8.3) on a shaking platform at room temperature for 30 minutes. Filter paper, (Whatman 3MM), sponge pads, and nitrocellulose membrane (NCM) were also presoaked in blotting buffer. A "sandwich" containing: plexiglass support, sponge pad, 2 filter papers, gel, NCM, 2 filter papers, sponge pad, and plexiglass support was inserted in the Trans Blot apparatus (Bio-Rad) with the NCM facing the anode. The transfer was run in the 4 °C cold room at 7 V/cm (60 volts with Bio-Rad power pack) for 3 hours with mixing using a magnetic stirer. Completeness of transfer was checked by staining the transferred gel.

Immunodetection:

The NCM was incubated in a physiological buffered saline (PBS) solution, containing 0.05 % Tween-20 pH 7.4 in order to block the membrane, on a shaking platform at room temperature for 30 minutes. The membrane was then transferred to 100 ml PBS-Tween containing a

final dilution of 1:400 of serum containing rabbit anti-hamster UCP antibody and incubated for 2 hours at room temperature on a shaking platform. After 4 x 30 min washes of 300 ml PBS-Tween the membrane was incubated in 100 ml PBS-Tween containing 7 x 10^6 cpm ^{125} I Protein A (ICN Radiochemicals 30-70 µCi/µg) for 1 hour in the 4 °C cold room. The membrane was washed with 300 ml PBS-Tween three times for 30 minutes and a fourth time overnight. The NCM was then blotted between 2 sheets of filter paper, air dried, covered with Saran Wrap and taped to an index card for support. The NCM was then placed together with X-ray film into a cassette equipped with intensifying screen (Kodak) in the - 70 °C freezer for 19 hours. The film was then developed for 1 minute.

11) PREPARATION OF UCP ANTIBODIES

Hamster UCP prepared using the same protocol described in section #9 was used to generate antibodies in rabbits. The method used was essentially that described by Fernandez et al. (1987). The hamster UCP was obtained in a greater yield (4.0 % vs 2.6 %) than the mouse UCP preparation and more UCP was therefore isolated from hamster (20 mg vs 6.5 mg).

Two female New Zealand White rabbits were each injected with 100 µg of purified uncoupling protein with Freund's complete adjuvant in 2 intramuscular sites and 2 subcutaneous sites. The following week 100 µg of UCP with Freund's incomplete adjuvant was injected subcutaneously at several sites in the back. A week later a booster was given by injecting 100 µg UCP in a total volume of 500 µl of saline,

injecting 100 µg UCP in a total volume of 500 µl of saline, intravenously via the ear vein. The following week the rabbits were bled into polycarbonate tubes and the blood was allowed to clot on ice in the 4 °C cold room overnight. The next day the blood was centrifuged at low speed 2,500 x g for 30 minutes. The pellet was discarded and the supernatant was centrifuged at the same speed for 15 minutes. The serum was then diluted 1:1 with glycerol by weight in 1 ml microcentrifuge tubes with screw caps and stored at - 70 °C. Preimmune serum was obtained by drawing 10 ml of blood from the rabbits (before the injection protocol began) following the same protocol.

12) UNCOUPLING PROTEIN SOLID PHASE RADIOIMMUNOASSAY (RIA)

A solid phase RIA was developed for the uncoupling protein (UCP) based on the method of Lean et al. (1983). Other references such as the book by Chard, (1982) and a paper by Darnule et al. (1982) were also of great help.

The plates:

Microtiter plates (96 flat bottom well, Falcon #3912 Becton Dickinson) were carefully washed in Pierce RBS Concentrate Surface Active Agent (a detergent from Fisher made by Pierce Chemical Company). The plates were soaked in detergent diluted with hot tap water for 30 minutes and then rinsed 6 times in cold tap water followed by 3 times with double distilled water The plates were "slapped" (forcibly) on paper towels, air dried and stored in cardboard boxes at room temperature. This washing procedure increased total binding of UCP by

Coating the plates:

The plates were coated with mouse UCP (see method #9) (425 µg/ml, 1.76 % triton). The mouse UCP was diluted to 10 µg/ml with PBS (phosphate buffered saline) (5 mM K_2HPO4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl pH 7.4) in a siliconized glass test tube (16 x 100mm). The plate was coated with 50 µl or 500 ng UCP per well. Non-specific binding was assessed with 50 µl of PBS + 0.1 % triton. The plate was placed in a ziplock plastic bag in a 37 °C incubator (Fisher Scientific Model 630D) for 2 hours. Temperatures from 27-37 °C work well but the total binding increases with increasing temperature. The protein reached maximum binding at 2 hours. After the coating procedure, the solution in the plate was removed by "slapping" (forcibly) the plate on 5 layers of Kleenex tissue followed by "tapping" (firmly but gently) the plate onto a stack of 5 Kleenex tissues, with clockwise rotation of the plate 3 to 4 times changing Kleenex stacks each time until the liquid was negligible. The plate was then filled with 200 µl PBS + 1 % Albumin + 0.1 % sodium azide (Albumin Bovine RIA Grade Sigma Chemical Co.) in each well using a multipipette (Titertek) and incubated for 10 minutes at 37 °C to block the uncoated portions of the well. Albumin was found to be a better blocking agent than 0.025 % w/v Tween-20 (Lean et al., 1986). The plate was emptied by dumping out the liquid into a waste container, then inverting the plate onto a stack of Kleenex followed by the tapping procedure. The plate was rinsed once again using the 200 µl multipipette and tapped again. A siphon was used to fill each well with PBS + 1 % Albumin, then the solution was dumped out into a waste container and the plate was tapped as described above.

The siphon was again used for the fourth wash followed by tapping. The plate was left inverted on 5 layers of Kleenex for 10 minutes.

Competition:

Each sample was incubated in 3 coated wells to measure total binding and also in 2 uncoated wells to measure non-specific binding for each sample. The plates were loaded in the following order: 40 µl PBS + 0.1 % triton in uncoated wells for non-specific binding (NSB for the plate) and in coated wells for total binding (B_o for the plate). 40 µl standards (+ NSB). 40 µl samples (+ NSB), and finally serum containing the UCP antibody. 10 µl per well. The antibody is competed for by either the bound UCP or the added UCP (standard or sample). The plate was then placed in a zip-lock plastic bag in the 4 °C cold room for 18 - 24 hours.

DIAGRAM OF A 96 WELL MICROTITER PLATE

	1	2	3	4	5	6	7	8	9	10	11	12
a	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
b	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
c	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
d	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
e	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
f	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
g	◘	•	◙	◙	o	o	o	◙	◙	o	o	o
h	◘	•	◙	◙	o	o	o	◙	◙	o	o	o

◘ Well coated with PBS + 0.1 % triton and competed with PBS + triton. This represents NSB of the plate.

• Well coated with UCP and competed with PBS + 0.1 % triton. This represents B_o of the plate.

◙ Well coated with PBS + triton and competed with standard or sample. This represents NSB of the standard or sample.

o Well coated with UCP and competed with standard or sample. This represents B_o for the standard or sample.

The next day the plate was inverted on a stack of 5 Kleenex tissues and the liquid was absorbed by the tissue. The plate was then tapped as previously described onto stacks of Kleenex. The plate was washed with PBS + 1 % albumin twice with the multipipetter and 3 times with the siphon. The last wash was left for 5 minutes before tapping. Finally the plate was left to drain upside down on a stack of Kleenex for 10 minutes.

Detection:

^{125}I Protein A (ICN Biomedicals Canada, specific activity 30-70 µCi/µg) was used to detect binding of the antibody to the UCP coated on the wells. A 1:1,000 dilution with PBS of protein A in a plastic scintillation vial resulted in a solution such that 50 ul contained 50-70,000 cpm. Total counts added were measured in triplicate: at the beginning, middle and end position of each plate. The plate was again placed in the zip-lock bag and left at room temperature for 90 minutes. The plate was then inverted on a stack of 10 Kleenex tissues to absorb the radioactive liquid. The plate was washed twice with PBS + 1 % albumin + 0.1 % sodium azide using the 200 µl multipipetter and twice with the siphon. Finally the plate was left inverted for 10 minutes before each well was cut out and placed in a plastic test tube to be counted along with the total counts (also in plastic tubes) in the Gamma counter (Beckman #5500).

Antibody titer:

The antibody titer was determined by measuring total binding (B_o) with antibody dilutions ranging from 1:100 to 1: 50,000 (in triplicate) and subtracting non-specific binding (in duplicate). Figure 2a and 2b

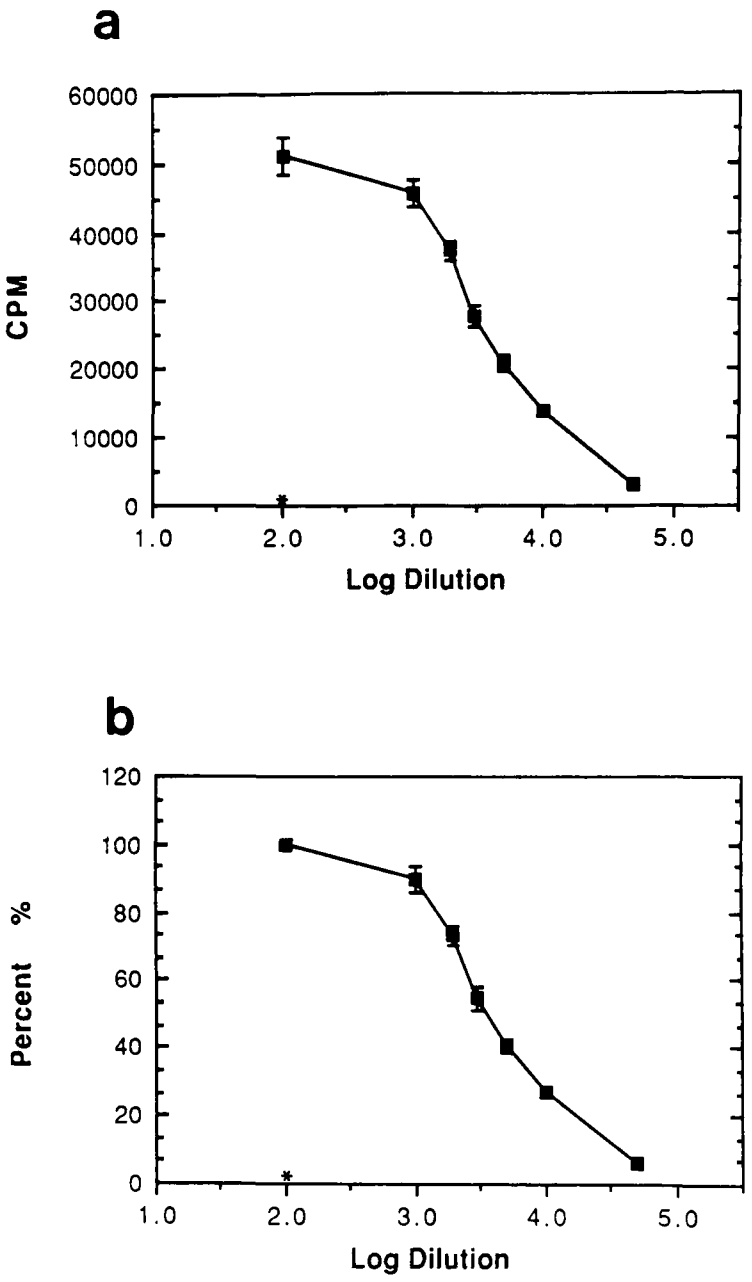


Figure 2: DILUTION CURVE OF SERUM CONTAINING HAMSTER UCP ANTIBODIES. Total binding of various dilutions of serum containing hamster BAT UCP antibody (squares) and preimmune serum (*) to purified mouse UCP coated plates. The data are expressed as (a) total cpm bound or (b) percent of total cpm bound. The values plotted are means ± sem of triplicate measurements. The optimum dilution which gave 50 % binding was determined to be 1:6,000.

shows that even a 1:100 dilution of pre-immune serum showed only background level counts. The titer showed the optimum range for the antibody dilution to be from 1:3,000 to 1:6,000.

Standard Curve:

The standard curve was prepared using mouse UCP purified from cold acclimated lean mice (see methods section #9). The UCP was diluted in siliconized glass test tubes first with PBS then with PBS + 0.1 % triton to obtain 6 dilutions such that 40 µl contained 2.2 to 70.8 ng UCP. The non-specific binding for each standard was assayed in duplicate (by adding 40 µl standard to an uncoated well) and each standard was assayed in triplicate (by adding 40 µl standard to a coated well). A standard curve was included on each microtiter plate. The total binding (B_0) of each plate was determined by adding 40 µl PBS + 0.1 % triton to 8 coated wells and NSB was determined by adding 40 µl to 8 uncoated wells. This was done to test the uniformity of binding down the plate. The standard curve was run with antibody dilutions from 1:3,000 to 1:9,000. The curves were analyzed using linear regression and it was found that an antibody dilution of 1:6,000 gave the best standard curve with slope and r^2 optimised.

Preparation of samples:

The solid phase RIA was used to determine UCP in BAT homogenates from lean and obese (ob/ob) mice and monkeys. The optimum concentration of the BAT homogenate to be extracted by 0.5% Triton was determined by incubating homogenates (0, 0.25 0.5 1.0 and 2.0 mg protein /ml) with 5 % triton (final concentration was 0.5 %) in a total volume of 200µl for 30 minutes at room temperature. The extraction was stopped by

diluting the extracts to 1.0 ml with PBS and centrifuging at maximum speed for two minutes in a benchtop Eppendorf microcentrifuge (#3200). The supernatant was decanted and stored at - 20 °C until assay. Figure 3a and 3b show that the best extraction of UCP was obtained from 0.5 mg protein/ml lean and ob/ob mice and monkey BAT homogenates. The optimum amount of extracted protein from the 0.5 mg/ml sample to give a value of UCP within the linear (5-60 ng) range of the standard curve was 4 µg or 40 µl of the extracted homogenate.

SUMMARY OF FINAL PROCEDURE:

Microtiter plates were coated with 500 ng mouse UCP for 2 hours at 37 °C. The plates were sealed with PBS + 1 % Albumin for 10 minutes at 37 °C. Standards from 2.2 to 70.8 ng mouse UCP (40 µl) were loaded along with samples (40 µl) of 0.5 mg/ml BAT homogenates extracted with 0.5 % triton diluted 1:5 or 4 µg of extracted BAT homogenate protein, then 10 µl of rabbit anti hamster UCP antisera final dilution 1:6,000 was added to all wells. The plates were incubated overnight at 4 °C. ^{125} I Protein A was used to detect binding (50,000 cpm/well) for 90 minutes at room temperature. The plates were then washed and counted in a Gamma counter A computer program using Lotus 1-2-3 was used to calculate the results.

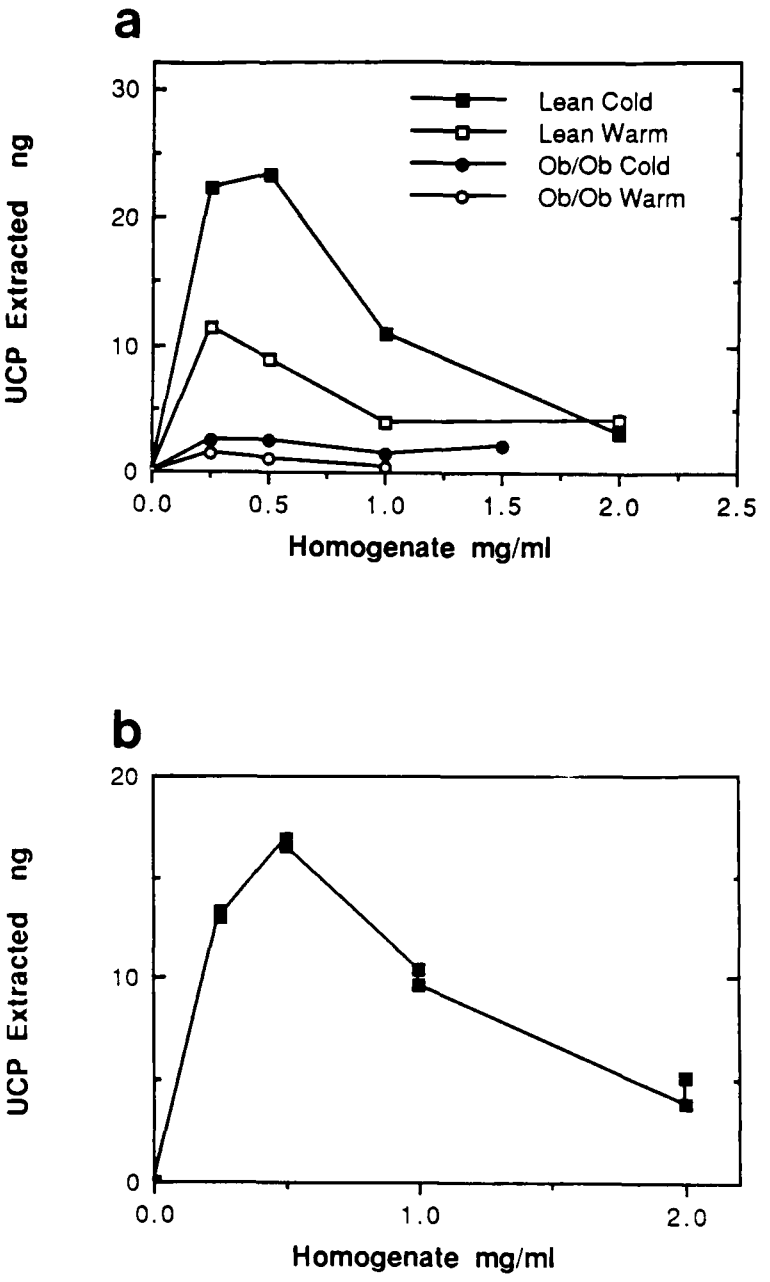


Figure 3: TRITON X-100 EXTRACTION OF BAT HOMOGENATES. Lean and ob/ob BAT homogenate (3a), values are means of triplicate measurements of 1 sample and monkey BAT homogenates (3b) values are means of triplicate measurements of 2 samples. Maximum extraction of UCP by triton X-100 was found to occur in 0.5 mg/ml homogenates.

SERUM RADIOIMMUNOASSAYS

13) CORTICOSTERONE RIA

A direct radioimmunoassay of total serum corticosterone was developed which requires only 5 µl of serum. This assay did not require a preparative extraction of chromatography step and had much greater sensitivity than protein binding methods. The method used was based on that of Al-Dujaili et al., 1981 and was modified to use the commercially available antisera and tritiated tracer by Deeks (1987).

A 1:1 dilution of rabbit anti-corticosterone-21-thyroglobulin serum (ICN ImmunoBiologicals #61-362-1), 5 µl sample or 100 µl standard (0-1000 pg/tube) were diluted to 350 µl with 50 mM NaH_2PO_4, 25 mM sodium citrate, 0.1 % BSA (RIA grade), pH 3.0. The low pH maximized the binding of corticosterone to corticosterone binding globulin. The antibody, sample or standard were incubated at room temperature for 30 minutes. A tracer amount, (12,000 cpm) of [1,2,6,7 ^3H]-corticosterone (Amersham 213 mCi/mg TRK.406) was added to all tubes which were then incubated for 60 minutes in a 37 °C shaking water bath after which the tubes were cooled in a ice water bath for 15 minutes. Separation of bound antibody bound from free corticosterone was accomplished by addition of an ice cold dextran coated charcoal suspension (0.05 % dextran T 70, Sigma, 0.5 % Norit A charcoal, Sigma) which adsorbs the free hormone, followed by centrifugation (3,200 rpm, 4 °C, 15 min, Beckman J6-B). A portion of the supernatant (400 µl) was counted in 10 ml Aquasol (NEN Research) in the Beckman LS6800 liquid scintillation counter.

The sensitivity of the assay was 30 pg/tube (0.15 µg/dl). The

average recovery was 101.5 % (n=3). The interassay coefficient of variation was 33.1 % for low corticosterone samples and 7.6 % for high corticosterone samples. Corticosterone was measured in µg/dl. A computer program written using Lotus 1-2-3 was used for the calculations.

14) THYROXINE (T_4) AND TRIIODOTHYRONINE (T_3) RIA

Total T_4 and T_3 were initially measured by RIA using commercial kits from Amersham (Amerlex T-3 RIA and Amerlex T-4 RIA). These kits use human serum standards and are only valid for measurements of human serum samples. The kits were very easy to use and the results were available in 24 hours but the assay was very expensive. Kaplan et al. (1985) noted large variations in values for serum T_3 and T_4 in ob/ob mouse serum reported in the literature. They discovered that ob/ob mice showed increased binding of tracer T_3 and T_4 to a postalbumin with mobility similar to that of human T_4-binding globulin. It was also shown that the non specific binding values in the serum RIAs for the ob/ob mice were abnormal compared to lean values. This paper prompted me to develop individual standard curves for lean and ob/ob mice for both the T_4 and the T_3 assay in order to prevent erroneous results due to the differences in thyroid hormone binding proteins.

The method used was essentially that of Larsen (1976). Dr. Larsen graciously provided antisera to T_3 and T_4 as a gift. This method was also easy to use and was inexpensive to run.

Preparation of Thyroid Hormone Free MOUSE Serum (THFS):

Serum was prepared from lean and obese (ob/ob) mice as described in section #4. Blood was collected from male and female C57Bl/6J mice over a period of 3-4 months. Researchers in the laboratory of Dr. N. Bégin-Heick donated mouse blood whenever animals were sacrificed. This was a generous gift as ob/ob mice are expensive. The collected serum (6 ml ob/ob and 6.5 ml lean) was thawed on ice and transferred to an ultracentrifuge tube. Tracer, ^{125} I T_3 (Amersham) was purified by paper electrophoresis (section #15), 50,000 cpm/ml was used to follow the stripping of thyroid hormones from the serum. An aliquot. 100 µl, was counted before stripping of hormones to measure total counts. The serum was stripped by incubating Norit A charcoal 0.023 g/ml with the serum and a magnetic stir bar overnight in the 4 °C cold room. The tube was centrifuged at 100,000 g for 30 minutes at 4 °C (35K in 55.2 Ti rotor) and 100 µl of serum was counted. This process continued until the supernatant contained $<$ 5 % thyroid hormones. The THFS was then frozen in liquid nitrogen and stored at - 70 °C until needed.

Preparation of Standards:

Standard solutions of thyroid hormones were prepared by dissolving 12 mg (free acid, Sigma) in 3 ml 0.05 N NaOH + 0.1 % BSA (RIA grade). a 1:100 dilution gave 40 µg/ml. The T_4 solution was standardized by reading the absorption at 325 nm and the concentration (mg/ml) was calculated knowing the molar extinction coefficient (6,250) and the molecular weight (776.93). C = (A/6205) x (776.93). Similarly the absorption of T_3 was read at 320 nm and C = (A/4658) x (651.01). Dilutions of each standard were prepared such that a 1:20 dilution in

THFS would give the following concentrations:

T_4 (µg/100ml): 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and

T_3 (ng/100ml): 10, 25, 50, 100, 200, 300.

The lean and ob/ob standards for T_3 and T_4 were aliquoted and stored at - 70 °C. THFS for lean and ob/ob mice was used to determine total binding and non specific binding. The antibody dilution curves were also determined using THFS.

Assay Method:

The T_4 assay buffer was 0.2 M glycine, 0.13 M acetate pH 8.6 containing 0.2 % BSA and 2 % sodium salicylate (Fisher). The T_3 assay buffer was the same except that it had 1.1 % sodium salicylate. The function of sodium salicylate was to inhibit binding of thyroid hormones to serum binding proteins (Larsen, 1976). Standards or samples (5 µl) were incubated with 1:10,000 dilution of T_4 antibody and 7-8,000 cpm/tube fresh ^{125} I T_4 (Amersham) in a total volume of 1.0 ml in the 4 °C cold room overnight. B_o was measured with 5 µl THFS + antibody and NSB was measured with 5 µl THFS without the antibody. The T_3 assay followed almost the same protocol except the volume of serum was 15 µl, the T_3 antibody dilution was 1:25,000 and the sample and antibody were incubated in the 4 °C cold room for 24 hours before the addition of 3-4,000 cpm ^{125} I T_3 (Amersham). The tubes were then incubated for a further 24 hours in the 4 °C cold room. To separate bound from free thyroid hormone, an ice cold suspension of 0.04 % Norit A charcoal and 0.004 % dextran T 70 (1.0 ml) was added to each tube except the total counts tube and the tubes were incubated in the 4 °C cold room for 45 minutes. The tubes were centrifuged at 3,200 rpm in

the Beckman J6-B centrifuge for 15 minutes at 4 °C. The supernatant was decanted into a new tube and was counted in the Beckman Gamma 5500. A computer program using Lotus 1-2-3 was used to calculate the values. It was found that it was important to have the appropriate standard curve for each animal type to properly interpret results.

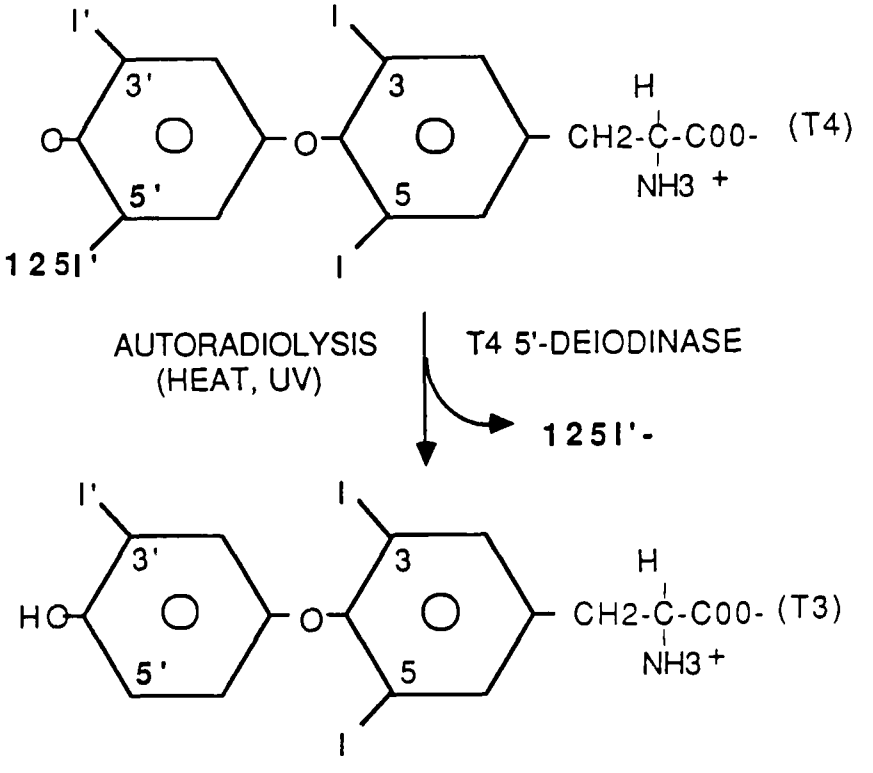


Figure 4: BAT THYROXINE 5'-DEIODINASE ASSAY. The 5'-deiodination of ^{125}I-thyroxine (T_4) resulting in triiodothyronine (T_3) and $^{125}I^-$ can be accomplished enzymatically (BAT homogenates) or by autoradiolysis caused by heat or UV radiation. The purification of substrate tracer by paper electrophoresis is necessary to remove $^{125}I^-$ produced by autoradiolysis.

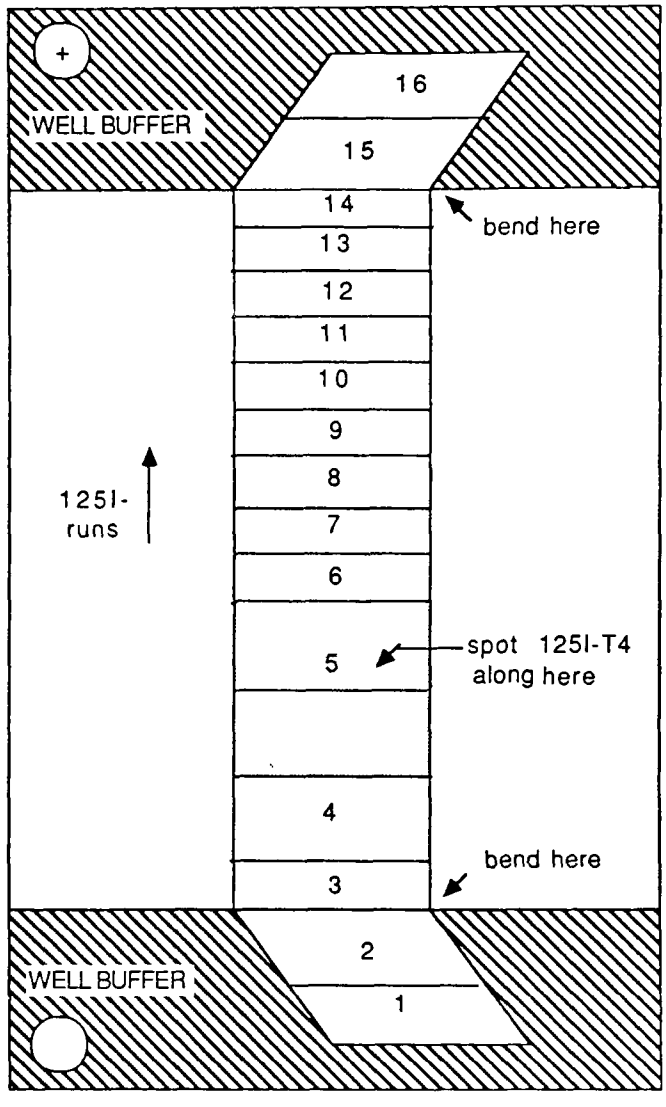


Figure 5: DIAGRAM OF PAPER ELECTROPHORESIS USED TO PURIFY TRACER ^{125}I T_4 OF $^{125}I^-$ RELEASED BY AUTORADIOLYSIS. Radioactivity was spotted at line 5 and dried under N_2. Electrophoresis was run at 500 volts for 10 minutes with a 50 mM ammonium acetate buffer (pH 7.1).

15) THYROXINE 5'-DEIODINASE (T5'D) ACTIVITY IN BAT HOMOGENATES.

BAT homogenates were prepared as described in section # 5 and aliquots were frozen at - 70 °C for at least 1-2 weeks before assay. The enzyme was stable for at least 4 months under these conditions. Many researchers include dithiothreitol (DTT) in the homogenate to stabilize the enzyme (Kaplan and Young, 1987; Silva et al., 1987)). we were unable to do so since most of the homogenate was used to isolate mitochondria.

T5'D activity was assayed by measuring ^{125} I- release from T_4 by the ion exchange method of Leonard and Rosenberg, 1980. [3'-5'-^{125} I] Thyroxine (Amersham IM.141, specific activity > 1200µCi/µg) was used as the tracer Autoradiolysis occurs spontaneously and the rate of this reaction is increased by light and heat. The tracer was therefore purified by paper electrophoresis immediately before use.

Paper Electrophoresis:

No more than 250 µl of ^{125} I T_4 was spotted with a Hamilton syringe onto a strip of Whatman 3 MM paper and dried under nitrogen. The strip was placed in an electrophoresis chamber with 50 mM ammonium acetate (pH 7.1) buffer The electrophoresis was run at 500 volts for 10 minutes. The product of the autoradiolysis, free iodide (^{125} I-), is charged and will move towards the anode. The tracer [^{125} I]- T_4 is neutral at pH 7.1 and will not migrate. The [^{125} I]- T_4 was extracted from the filter paper with 2.0 ml of methanol:ammonium hydroxide (99:1) and concentrated to the desired volume under nitrogen. This method gave a free iodide concentration of < 1 % .

Assay Method:

The method used was essentially that of Silva et al., 1987. The incubation included 10 mM DTT, 1 mM 6-propyl-2 thiouracil (PTU) (used to inhibit Type I T5'D), 2.57 nM unlabelled T_4 (Sigma) and about 0.15 nM ^{125} I T_4 tracer in 150 µl of 0.1 M potassium phospnate buffer (pH 7.0), 1.0 mM EDTA. The reaction was started by the addition of 50 µl of BAT homogenate containing 20 - 60 µg protein. The tubes were flushed with nitrogen and incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 50 µl ice cold thyroid hormone free normal human serum (AMF Biological Diagnostics Co.) which caused the substrate T_4 and the product T_3 to bind to the thyroid hormone binding proteins in the serum. This complex and all other proteins were precipitated with 350 µl of 12.5 % TCA. The tubes were centrifuged at maximum speed for 2 minutes in a bench top Eppendorf centrifuge #3200. Aliquots (500 µl) were loaded onto 2.0 ml columns of Dowex AG 50 W-X_2 100-200 mesh cation exchange resin (Bio-Rad) equilibrated with acetic acid: H_2O (1:10). The cation exchange column bound any excess ^{125}I T_4 that may not have been bound by the serum and precipitated by TCA. The columns were washed 3 times with 1.0 ml aliquots of acetic acid:water and the wash (containing free iodide) was collected in plastic tubes (16 x 100 mm) and counted in the Beckman Gamma 5500 counter. Autoradiolysis that occurred during the assay was measured using tubes where 50 µl of buffer or boiled tissue instead of homogenate were incubated along with the samples. All samples were measured in triplicate.

A computer program was used to calculate enzyme activity. Activity

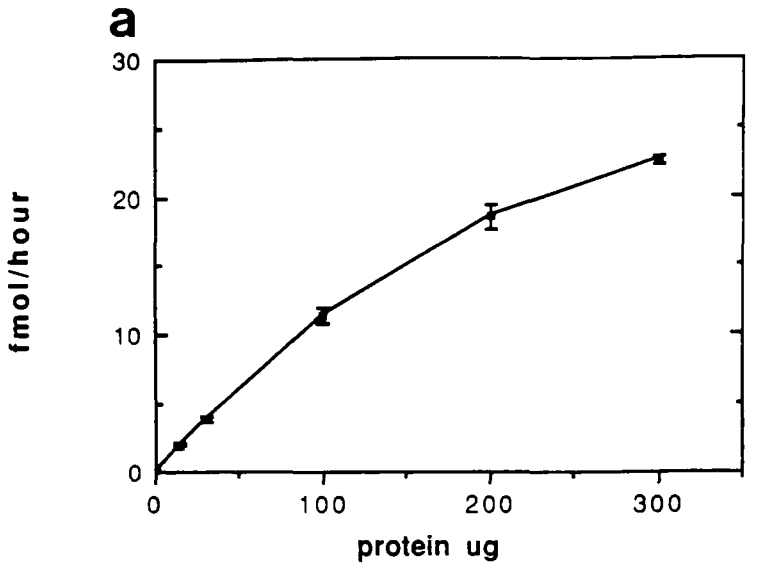
was measured as specific activity (pmol/h/mg protein) and total activity (pmol/h). Corrections were made for the decay of ^{125}I (half life = 60 days), background counts, autoradiolysis and counting efficiency of the Gamma counter

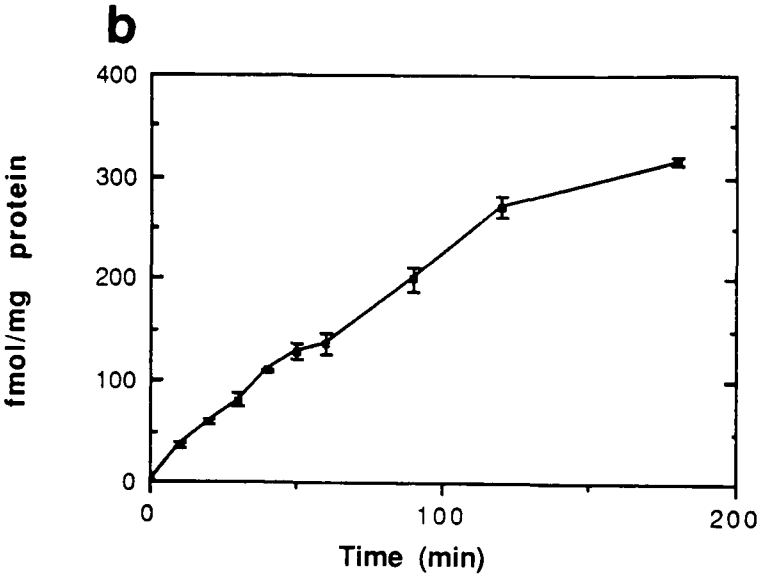
Preliminary studies using this method showed that protein concentrations from 15-100 µg were within the linear range of the assay (Figure 6a). The time course study showed the assay was linear up to 50 minutes (Figure 6b). The optimum pH was 7.0 (Figure 6c), DTT enhanced enzyme activity (Figure 7a) and PTU did not inhibit the enzyme up to a concentration of 1 mM (Figure 7b). These preliminary results confirmed that the enzyme studied in this assay was type II thyroxine 5'-deiodinase (T5'D).

Figure 6a: BAT T5'D ASSAY PROTEIN CURVE. Protein concentrations (mean ± SEM, triplicates) from lean 12 hour cold exposed (14 °C) mice were varied from 15 to 300 µg. The linear range of this assay was found to be 15-100 µg. Method Section #15 provides more details on assay conditions.

Figure 6b: BAT T5'D ASSAY TIME CURVE. The time course of enzyme activity was measured using homogenates (40 µg protein) incubated from 10 to 180 minutes. The assay was measured using cold exposed lean homogenates (means ± SEM, triplicates) and was found to be linear up to 50 minutes.

Figure 6c: pH CURVE OF BAT T5'D ACTIVITY. Lean cold exposed homogenates (40 ug protein) were incubated in triplicate for 30 minutes in 0.1M potassium phosphate buffers from pH 6.0 to 8.0. The maximum activity was found to occur at pH 7.0.


a
30
20
10
0
fmol/hour
0
100
200
300
protein ug


b
400
300
200
100
0
fmol/mg protein
0
100
200
Time (min)

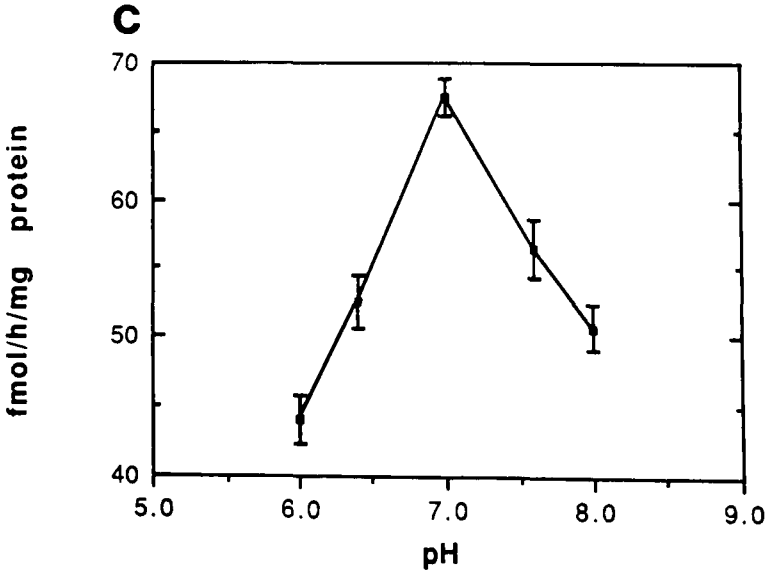
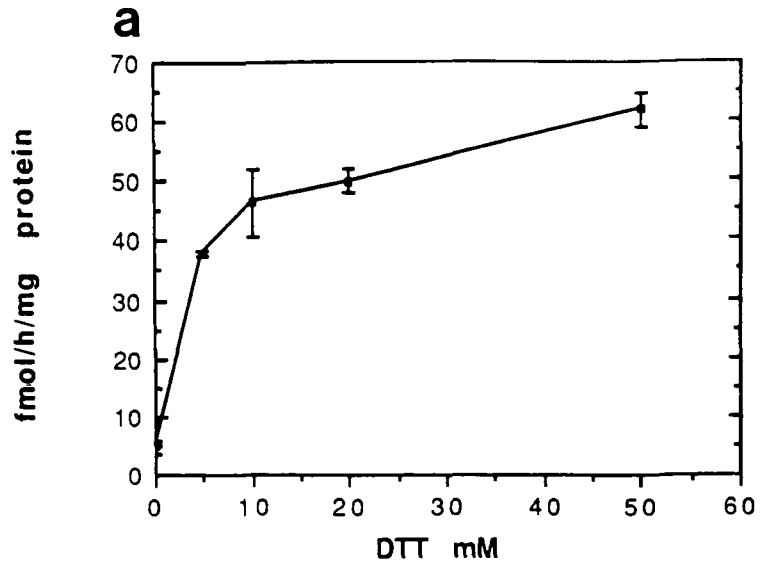

c
70
60
50
40
fmol/h/mg protein
5.0
6.0
7.0
8.0
9.0
pH

Figure 7a: EFFECT OF DTT ON BAT T5'D ACTIVITY. Cold exposed lean BAT homogenates were incubated (in triplicate) with various concentrations of DTT (0 - 10 mM). DTT was found to stimulate BAT T 5' D activity almost 10 fold at 10 mM.

Figure 7b: EFFECT OF PTU ON BAT T5'D ACTIVITY. Cold exposed lean BAT homogenates were incubated (in triplicate) with varying concentrations of PTU (0 - 10 mM). No inhibition of BAT T5'D activity was found at 1 mM but at 10 mM a 50 % inhibition was observed.


a
fmol/h/mg protein
70
60
50
40
30
20
10
0
0
10
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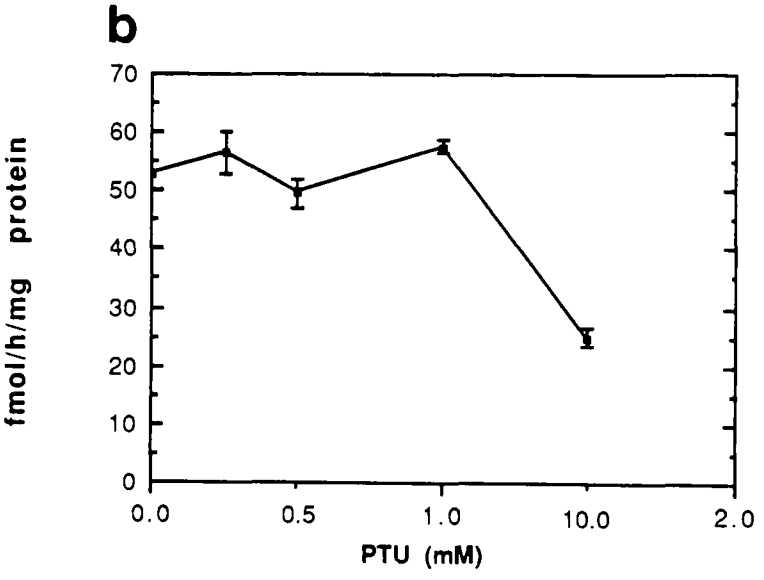
30
40
50
60
DTT mM


b
fmol/h/mg protein
70
60
50
40
30
20
10
0
0.0
0.5
1.0
10.0
2.0
PTU (mM)

16) STATISTICS

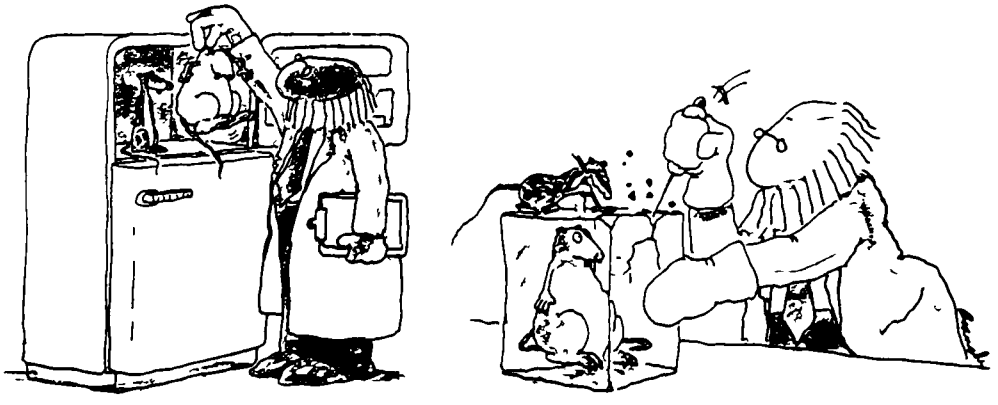
Statistical analysis was done using a main frame computer (VM/370) at the University of Ottawa Computing center at the Health Sciences center. The statistics package used was SAS (SAS Institute, inc. Statistical Analysis System, Box 8,000, Cary, North Carolina 27511). The procedure used was GLM (general linear models) for unbalanced ANOVA (analysis of variance) in which the number of animals (n) per group are not the same. The post-hoc tests used were Scheffe's test when $n < 5$ and Tukey's studentized HSD (honestly significant difference) test when $n > 5$. In some cases repeated measures ANOVA was performed for repeated measurements made on the same animal, GLM was also used for this variation. The level of significance was $p < 0.05$ unless otherwise stated. Dr. R. Nair, Dept. of Epidemiology at the University of Ottawa was consulted to confirm the validity of the tests used and to interpret results.

CHAPTER 1:

EFFECT OF COLD ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 1: CHARACTERIZATION OF THE DEFECTIVE RESPONSE OF THE OB/OB MOUSE TO ACUTE COLD EXPOSURE.

PART 2: TREATMENTS WHICH IMPROVE THE DEFECTIVE RESPONSE OF THE OB/OB MOUSE TO ACUTE COLD EXPOSURE.



CHAPTER 1:

THE EFFECT OF COLD ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 1: CHARACTERIZATION OF THE DEFECTIVE RESPONSE OF THE OB/OB MOUSE TO ACUTE COLD EXPOSURE.

EXPERIMENT # 1: EFFECT OF 12 HOUR EXPOSURE TO 14 °C ON BAT OF LEAN AND OB/OB MICE.

BACKGROUND

Previous work from this laboratory demonstrated that the genetically obese (ob/ob) mouse did not activate brown adipose tissue thermogenesis in a normal way when the mouse was exposed to cold (Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980). Studies with rats indicated that thyroid hormones participated in cold-induced activation of BAT thermogenesis only in a permissive manner (Triandafillou et al., 1982; Himms-Hagen, 1983). Other workers had demonstrated that the adult ob/ob mouse had normal or even elevated serum thyroid hormone levels (Mobley and Dubuc, 1979; Gambert and Garthwaite, 1981), so it seemed that the thermogenic defect in BAT of the ob/ob mouse was not due to a lack of serum thyroid hormone.

The onset of this research project coincided with the demonstration by Silva, Larsen and colleagues that rat brown adipose tissue was able to produce T_3 from T_4 by the enzyme thyroxine 5'-deiodinase and that

the enzyme activity was regulated by noradrenaline and was markedly increased by acute cold-exposure in rats (Silva and Larsen, 1983; Leonard et al., 1983). This work suggested that brown adipose tissue may not depend upon serum T_3 but rather upon generation of its own T_3 from serum T_4 when required for stimulated thermogenesis.

OBJECTIVE:

The objective of the experiment was to find out whether ob/ob mouse BAT was able to respond to acute cold exposure by an increase in BAT T5'D activity and thus generate the T_3 needed by the tissue for a normal thermogenic response to noradrenaline.

METHOD:

Female C57Bl/6J mice, obese (ob/ob) and lean (+/?) littermates arrived at 4-5 weeks of age and were housed in pairs (one lean, one ob/ob) at 28 °C with a 12 hour light cycle (lights on at 06:00) with free access to food and water Six pairs of mice were exposed at 4 months of age to 14 °C or to 28 °C for 12 hours starting at 21:30.

Rectal temperatures were measured, then the mice were killed by cervical dislocation and blood collected. White gonadal adipose tissue was removed and weighed. Interscapular and subscapular BAT was cleaned, weighed, and homogenized in an all-glass homogenizer. Samples of homogenates were frozen immediately in liquid nitrogen and stored at -70 °C. Protein content was estimated, and T5'D activity was measured as described in Methods section #7 and # 15.

The kinetic study shown in Figure 9 was performed on homogenates of

lean and ob/ob mouse BAT exposed to 14 °C for 12 hours. The method used was exactly as described in Methods #15 except the substrate concentration was varied from 2.6 to 10.3 nM. Each substrate concentration was performed in triplicate. The data from this experiment was analyzed by linear regression. The rest of the data was analyzed using a 2-way ANOVA followed by Tukey's HSD post-hoc test.

RESULTS:

Obese mice of this age weighed considerably more than their lean littermates, had more white adipose tissue and a lower rectal temperature (Table 2). Exposure to cold induced a slight hypothermia in the lean mice and a marked hypothermia in ob/ob mice. Obese mice have more BAT with greater total protein than lean mice (Table 3). Obese mice also have slightly higher total and specific T5'D levels at 28 °C than lean mice. However, upon cold exposure lean mice demonstrate a dramatic 26 fold increase in both specific and total T5'D activity while the ob/ob mouse shows little more than a doubling of enzyme activity (Table 3 and Figure 8).

The Lineweaver-Burke plot of the kinetic study data is shown in Figure 9. The regression line for lean 12 hour cold exposed mice gave a K_m of 1.8 ± 1.2 nM and a V_{MAX} of 1.7 ± 0.6 pmol/h/mg protein. Similarily the regression line of ob/ob mice exposed to cold for 12 hours gave a higher K_m (9.7 ± 3.0 nM and a slightly lower V_{MAX} (1.0 ± 0.4 pmol/h/mg protein). Lean K_m values were within the range found by others (Silva et al., 1987; Leonard et al., 1983).

TABLE 2

EFFECT OF 12 HOUR COLD EXPOSURE ON BODY WEIGHT, BODY TEMPERATURE, AND WHITE ADIPOSE TISSUE WET WEIGHT OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CONTROL 26°C n=6	COLD EXPOSED 12 h at 14°C n=6	CONTROL 26°C n=6	COLD EXPOSED 12 h at 14°C n=6
Body Wt. g	22.4 ± 0.8	21.3 ± 0.7	54.1§ ± 1.8	55.2§ ± 2.4
Body Temp. °C	37.6 ± 0.2	36.3* ± 0.4	35.8§ ± 0.7	21.8§* ± 0.9
WAT Wet Weight g	0.45 ± 0.06	0.39 ± 0.06	3.79§ ± 0.23	4.04§ ± 0.34

Values are means ± SEM for the number (n) of animals per group. The symbols are as follows: § indicates a significant effect of obesity, * indicates a significant effect of cold exposure, ($p < 0.05$). The statistics were measured using a two-way ANOVA followed by Tukey's post-hoc test.

TABLE 3

EFFECT OF 12 HOUR COLD EXPOSURE ON BROWN ADIPOSE TISSUE OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CONTROL 26°C n=6	COLD EXPOSED 12 h at 14°C n=6	CONTROL 26°C n=6	COLD EXPOSED 12 h at 14°C n=6
BAT Wet Weight g	0.14 ± 0.01	0.12 ± 0.01	0.89§ ± 0.07	1.17§ ± 0.16
BAT Protein mg	7.4 ± 0.5	7.7 ± 0.8	4.0§ ± 1.3	14.6§ ± 1.6
T_4 5' D pmol/h/mg prot.	0.06 ± 0.01	1.57* ± 0.22	0.12§ ± 0.02	0.33§* ± 0.08
T_4 5' D pmol/h	0.46 ± 0.07	11.89* ± 1.68	1.64§ ± 0.24	4.50§* ± 1.14

Values are means ± SEM for the number (n) of animals per group. The symbols are as follows: § indicates a significant effect of obesity, * indicates a significant effect of cold exposure, ($p < 0.05$). The statistics were measured using a two-way ANOVA followed by Tukey's post-hoc test.

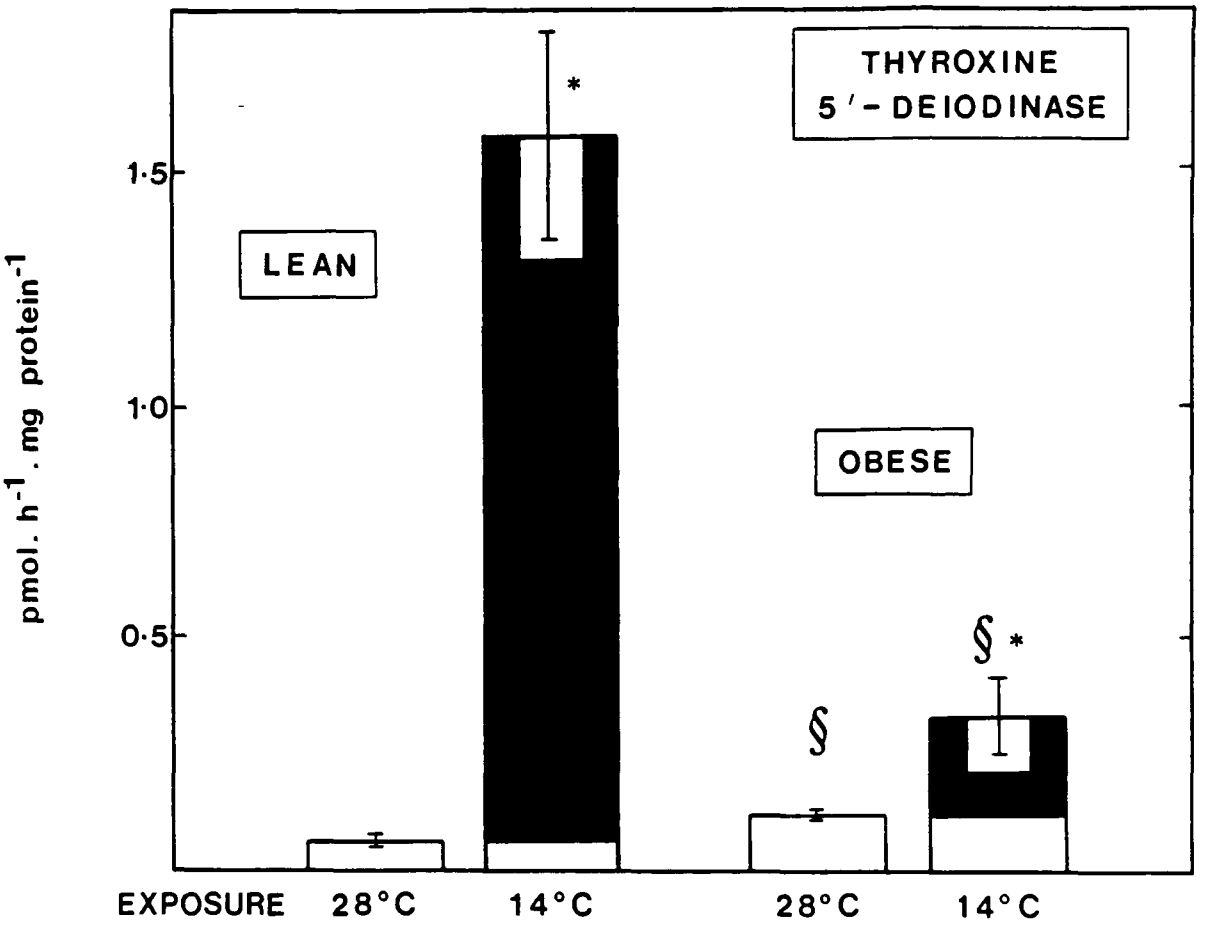


Figure 8: EFFECT OF COLD EXPOSURE (12 HOURS AT 14 °C) ON THYROXINE 5'-DEIODINASE SPECIFIC ACTIVITY IN BROWN ADIPOSE TISSUE HOMOGENATES OF LEAN AND OB/OB MICE. Values shown are means ± SEM for 6 animals in each group. The symbol *, represents a significant effect of cold exposure and §, represents a significant effect of obesity. The statistics were measured using two-way ANOVA followed by Tukey's HSD post-hoc test.

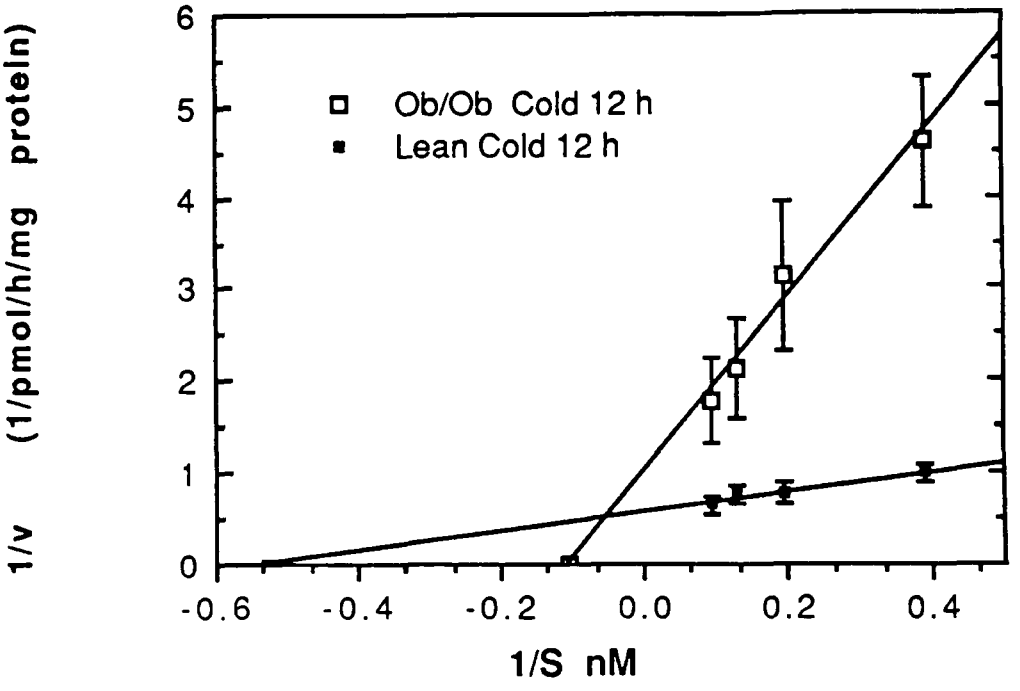


Figure 9: KINETIC STUDY OF BAT T'5D ACTIVITY IN LEAN AND OB/OB MICE. Lean (solid squares) and ob/ob (open squares) 12 hour cold exposed BAT homogenates were incubated with substrate concentrations varying from 2.6 to 10.3 nM. The values are means ± SEM from 3 different homogenates assayed in triplicate (n = 3). See results for more detail.

DISCUSSION:

The principal finding in this experiment is that obese (ob/ob) mice fail to stimulate T5'D activity in response to acute cold exposure. This finding has since been confirmed by Kaplan and Young (1987). The stimulated increase in BAT T5'D activity in rats is known to require transcription and protein synthesis (Jones et al., 1986). Thus, the ob/ob mouse fails to activate transcription and protein synthesis. This defective response of the T5'D resembles the defective cold-induced stimulation of brown adipose tissue mitochondrial GDP-Binding observed in this animal (Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980). Both of these events are believed to be regulated by the sympathetic nervous system via noradrenaline.

Obese mice have a higher basal activity of T5'D in BAT than lean mice. The reason for this higher basal activity in ob/ob mice living close to thermoneutrality is not clear. Since insulin is known to stimulate BAT T5'D activity (Silva and Larsen, 1986a; Mills et al., 1987) it is possible that the hyperphagic and hyperinsulinemic conditon of the ob/ob mouse could lead to a long-term adaptation in which basal levels of T5'D are more stimulated than in lean mice. Kaplan and Young, (1987), also report the same phenomena. They note that the increase may be related to intracellular hypothyroidism in BAT since they also noted a 33 % increase in cerebrocortical type II 5'-deiodinase activity in ob/ob mice.

The results of the kinetic study show that the K_m of BAT T5'D in lean and ob/ob mice is within the range described by others for the Type II T5'D in BAT (Silva et al., 1987; Leonard et al., 1983). The

variability of the measurement of BAT T5'D activity in the cold exposed ob/ob mouse makes it difficult to compare the kinetic parameters of lean and ob/ob mice. Leonard and Visser (1986) propose that the low K_m Type II T5'D is regulated by the availability of substrate since the K_m is near physiological levels of T_4. Calculations based on studies by van Doorn et al. (1985) in rats suggest the physiological level of T_4 in BAT is approximately 3 nM.

Thyroid hormone is essential for the thermogenic action of noradrenaline on BAT at many levels including activation of adenylate cyclase, and hormone sensitive lipase, and the fatty acid-induced activation of mitochondrial respiration (Sundin et al., 1984). The actions of noradrenaline to stimulate adenylate cyclase (Bégin-Heick and Heick 1982,1984) and to promote thermogenesis (Thurlby and Trayhurn, 1980) are both reduced in ob/ob mice. We know that sympathetic nervous system activity is increased in BAT of the cold exposed ob/ob mouse (Zaror-Behrens and Himms-Hagen, 1983; Knehans and Romsos, 1983). A refractoriness of BAT of the ob/ob mouse to the normal level of T_3 in the blood has previously been postulated to be the explanation for its poor thermogenic response to noradrenaline (Himms-Hagen, 1983). The results of this experiment suggest that we must now consider the contribution of the reduced endogenous production of T_3 from T_4 secondary to a failure to increase T5'D activity when the sympathetic nerve supply to brown adipose tissue is stimulated, as during acute exposure to cold, in the failure of this tissue to respond adequately to the action of noradrenaline to increase thermogenesis.

BAT contains nuclear T_3 receptors (Bürgi and Bürgi-Saville, 1986; Bianco and Silva, 1987c) which are highly occupied with T_3 produced locally by T5'D. T_3 is required for the response of UCP, α-glycerophosphate dehydrogenase and lipogenic enzymes to adrenergic stimulation (Bianco and Silva, 1987a,b). In the case of UCP, two factors seem to be important for gene expression. The first is adrenergic activation of T5'D and the second is a high nuclear T_3 receptor saturation. Bianco and Silva have shown that T_3 was necessary for cold to increase UCP mRNA levels in rats (Bianco and Silva, 1987a).

In a recent collaborative study with Dr K. B. Freeman's group at M^c Master University we showed that ob/ob mice exposed to cold (12 hours at 14 °C) had reduced levels of UCP mRNA (Reichling et al., 1988). It is likely that the defective increase in BAT T5'D in the ob/ob mouse after 12 hours of cold exposure could lead to a shortage of T_3 with which to saturate nuclear T_3 receptors. This lack of saturation of T_3 receptors would then lead to reduced levels of UCP mRNA and a defective trophic response.

With this in mind, the next experiment was designed to further characterize the acute response of lean and obese (ob/ob) mice to cold. The response of BAT T5'D activity was compared to the thermogenic response of the tissue by measuring GDP-binding and the amount of UCP in order to test the hypothesis that a normal activation of BAT T5'D activity is necessary for a normal thermogenic and trophic response to acute cold exposure.

CHAPTER 1:

THE EFFECT OF COLD ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 1: CHARACTERIZATION OF THE DEFECTIVE RESPONSE OF THE OB/OB MOUSE TO ACUTE COLD EXPOSURE.

EXPERIMENT #2: TIME COURSE OF EXPOSURE TO 14 °C.

BACKGROUND AND OBJECTIVES:

Obese (ob/ob) mice have a defective response of BAT T5'D activity to 12 hours a 14 °C (see Part 1, Experiment #1). The objective of this experiment was to further characterize the acute response to cold with a time course including points before and after the 12 hour time point. The time course was limited to 24 hours because the ob/ob mouse becomes hypothermic when exposed to cold, thus the maximum time the ob/ob mouse can survive 14 °C is 24 hours. The control animals used for this experiment were maintained at 28 °C. Thus by killing animals at various times of the day I was also able to study rhythms in body temperature, serum hormones, and BAT thermogenic activity in lean and ob/ob mice.

METHODS:

Female C57BL/6J lean (+/?) and genetically obese (ob/ob) mice arrived from Jackson Labs at 4 weeks of age. They were placed in individual cages at 28 °C with a 12:12 lighting schedule with lights on at 09:00. The animals were fed Purina rodent chow #5012 ad libitum and

were allowed free access to tap water. These experiments were performed from December 1986 to February 1987.

At 8 weeks of age the animals were either exposed to 14 °C at 08:00 for 1, 3, 6, 12, 16, or 24 hours or they remained at 28 °C but were moved to another shelf within the same room in order to act as a same time control animals. Food intake was measured starting at 08:00 on both cold exposed and warm acclimated 28 °C mice. The lighting schedule was 12:12 with lights on at 09:00.

After the cold exposure, animals were killed by cervical dislocation, blood collected and rectal temperatures were measured. Gonadal white adipose tissue was removed, cleaned and weighed. Interscapular and subscapular BAT was removed and placed in isolation medium. These procedures were performed in the 14 °C cold room and 4 mice were killed in approximately 15 minutes. The control animals were killed in the 28 °C room following the same procedure. Each time point consisted of 2 experimental days in which 2 lean and 2 ob/ob mice were killed at each temperature per experimental day, no more than 30 minutes separated the first and last animal at each time point.

In the laboratory BAT was cleaned, weighed and homogenates were prepared as described in method section #5. Mitochondria were prepared as described in section #6. Protein was estimated as described in section #7, and GDP-binding to isolated BAT mitochondria was measured as described in section #8. BAT T5'D activity was measured as described in section #15. Serum corticosterone, T_3 and T_4 (Larsen, 1976) were measured by RIA as described in sections # 4, 13 and 14. BAT UCP was measured by solid phase RIA as described in methods section

#12. Statistical analysis was by three-way ANOVA followed by Scheffe's post-hoc test.

RESULTS:

BODY WEIGHT, WAT WET WEIGHT, AND FOOD INTAKE:

Obese (ob/ob) mice have greater body weight and more white adipose tissue than lean mice under all conditions (Table 4 and 5). There was no effect of acute cold exposure on body weight or the amount of gonadal white adipose tissue of lean or obese mice. At 28 °C obese mice consume more metabolizable food energy than lean mice at all times studied. Both lean and obese mice consumed more energy during the dark phase of the lighting schedule (55 %) than during the day (45 %). When lean animals are exposed to cold they increase their energy intake by 55 % with no change in body weight or amount of gonadal WAT. In contrast, when obese mice are exposed to cold they decrease their energy intake to one third that of 28 °C control ob/ob mice, with no change in body weight or gonadal white adipose tissue weight (Table 4 and 5).

BODY TEMPERATURE:

Both lean and obese mice have lower body temperatures during the day than during the night. Obese mice however, regulate their body temperature at least 1-2 °C lower than lean mice at most times of the day (Figure 10). When lean mice are exposed to cold they are able to maintain their body temperature, however, the diurnal rhythm in body temperature was not maintained. The increase in body temperature found during the dark phase does not occur in lean mice exposed to 14 °C,

TABLE 4

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON BODY WEIGHT, WAT WET WEIGHT, FOOD INTAKE AND THYROID HORMONES OF LEAN MICE.

	CONTROL 28° C						COLD EXPOSED 14° C from 08:00					
	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=3	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=4	24:00 n=4	08:00 n=5
Body Weight g	17.9 ± 0.4	18.5 ± 0.4	17.9 ± 1.2	19.6 ± 0.4	18.0 ± 1.2	18.9 ± 0.7	17.6 ± 0.2	18.2 ± 0.8	18.4 ± 0.3	19.3 ± 0.2	18.9 ± 0.6	19.2 ± 0.6
WAT Wet Wt. g	0.26 ± 0.08	0.39 ± 0.09	0.27 ± 0.04	0.31 ± 0.03	0.18 ± 0.06	0.28 ± 0.03	0.24 ± 0.03	0.31 ± 0.05	0.24 ± 0.07	0.22 ± 0.03	0.30 ± 0.05	0.19 ± 0.04
Food Intake Kcal from 08:00	2.30 ± 0.35	3.18 ± 0.26	2.79 ± 0.14	6.65¶ ± 0.69	8.51¶ ± 0.22	14.50¶ ± 1.62	1.99 ± 0.13	2.57 ± 0.29	4.39 ± 0.54	9.14 ± 1.95	12.83* ± 2.01	22.47* ± 1.51
Ratio T_3/T_4 %	1.85 ± 0.16	2.30 ± 0.13	2.38 ± 0.04	3.18 ± 0.28	2.13 ± 0.10	1.83 ± 0.18	2.01 ± 0.25	2.52 ± 0.49	2.52 ± 0.19	5.16* ± 0.71	6.18* ± 0.35	8.47* ± 0.57

Values are means ± SEM for the number (n) of animals per group. The animals were housed in single cages. Symbols are as follows: Significant effect of obesity §, significant effect of cold *, significant effect of time ¶, ($p < 0.05$). Statistics were measured using ANOVA followed by Scheffe's post-hoc test.

TABLE 5

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON BODY WEIGHT, WAT WET WEIGHT, FOOD INTAKE AND THYROID HORMONES OF OB/OB MICE

	CONTROL 28° C						COLD EXPOSED 14° C from 08:00					
	09:00 n=3	11:00 n=4	14:00 n=4	20:00 n=4	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=5	24:00 n=4	08:00 n=4
§Body Weight g	38 2 ± 1.4	42 9 ± 1 6	40 5 ± 0 3	45 9 ± 0 9	37 3 ± 3.0	42 3 ± 0 8	35 9 ± 2 3	40 2 ± 1 2	42 2 ± 1 4	43 6 ± 1 3	39 9 ± 2 7	36 6 ± 2 1
§WAT Wet Wt. g	2 2 ± 0 1	2 6 ± 0 2	2 7 ± 0 1	3 0 ± 0 2	2 3 ± 0 2	2 2 ± 0 1	1 9 ± 0 2	2 4 ± 0 1	2 9 ± 0 1	3 0 ± 0 2	2 5 ± 0 3	2 0 ± 0 2
Food Intake Kcal from 08:00	4 7§ ± 0 2	4 5§ ± 0 6	5 6§ ± 0 7	9 0§¶ ± 0 5	12 7§¶ ± 0 1	20 5§¶ ± 4 1	1 7* ± 0 3	1 6* ± 0 4	1 1*§ ± 0 8	3 0*§ ± 0 9	4 2*§ ± 2 3	7 5*§ ± 2 5
Ratio T_3/T_4 %	2 1 ± 0 1	2 2 ± 0 1	2 8 ± 0 3	3 2 ± 0 3	2 3 ± 0 2	1 9 ± 0 1	1 9 ± 0 1	2 7 ± 0 3	2 9 ± 0 2	3 2 ± 0 2	3 3*§ ± 0 4	4 4*§ ± 0 1

Values are means ± SEM for the number (n) of animals per group. Animals were housed in single cages. Symbols are as follows: Significant effect of obesity §, (at the begining of the data this signifies that all means have a significant effect of obesity), significant effect of cold *, and significant effect of time ¶, ($p < 0.05$). Statistics were measured using ANOVA and Scheffe's post-hoc test.

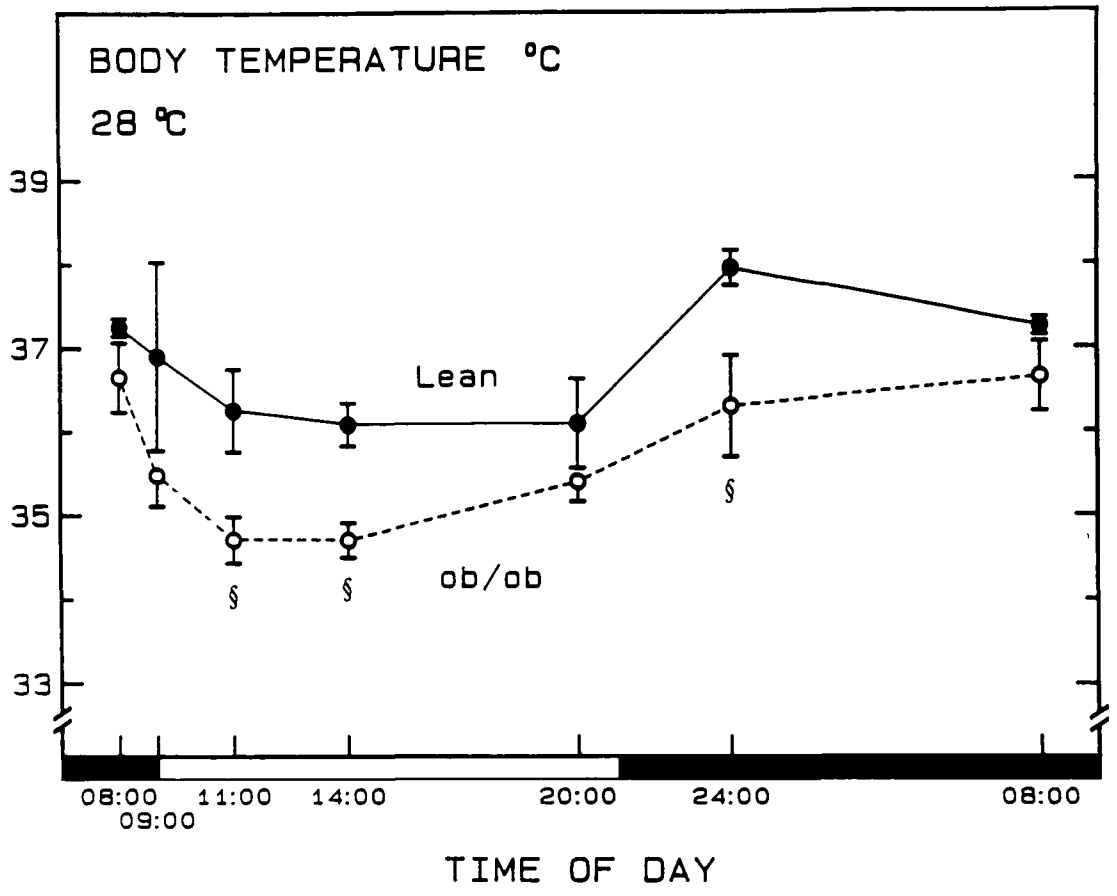


Figure 10: DIURNAL RHYTHM OF BODY TEMPERATURE IN LEAN AND OB/OB MICE AT 28°C. Values are means ± SEM for the number (n) of animals noted in Tables 8 and 9. Body temperatures between 24:00 and 08:00 were higher in both lean and ob/ob mice than those during daylight. A significant effect of obesity is symbolized by §.

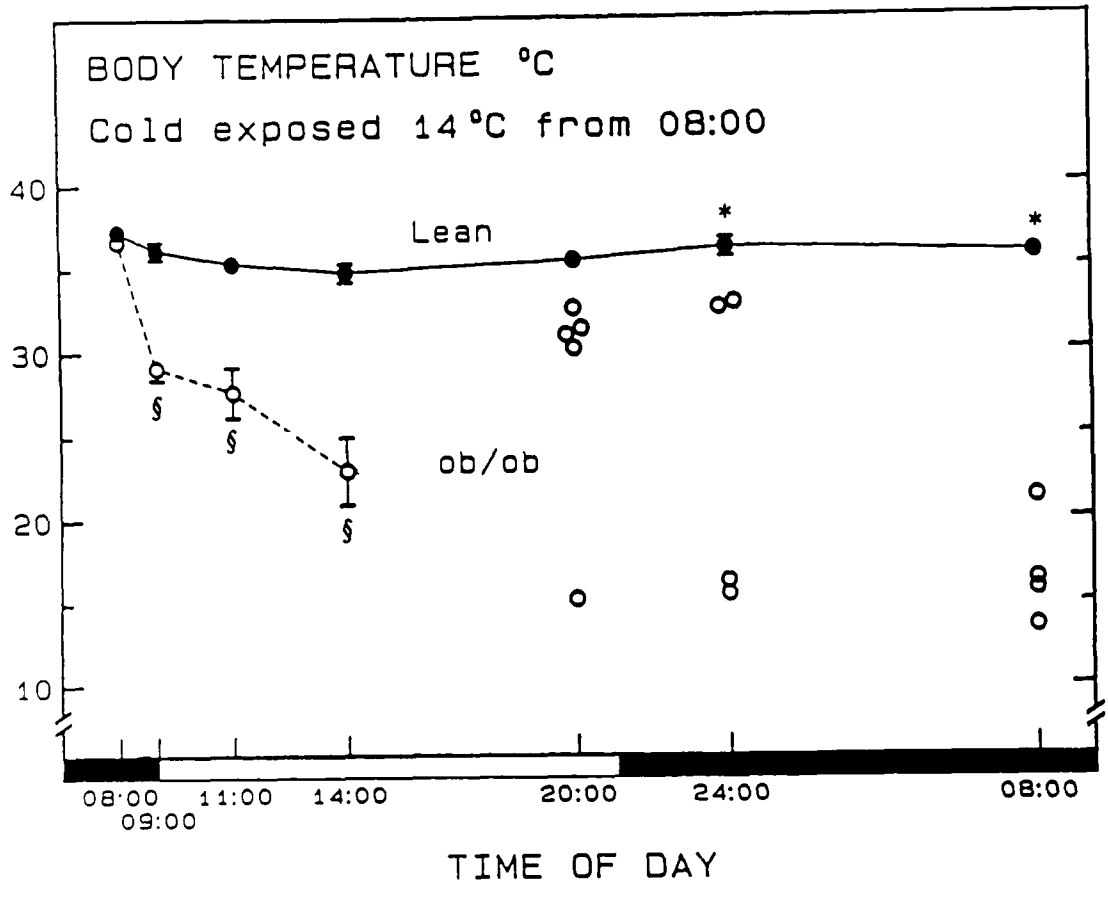


Figure 11: BODY TEMPERATURE OF LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals noted in Table 8 and 9. Individual values for ob/ob mice (open circles) are given at 12, 16 and 24 hours of cold exposure. A significant effect of cold is symbolized by *. All ob/ob measurements are significantly different from 28 °C ob/ob values and lean cold exposed values.

thus cold exposure abolished the increase in body temperature seen between 24:00 and 08:00 (Figure 10 and 11). In contrast, obese mice are unable to maintain their body temperatures when exposed to cold (Figure 11). After 12 hours of cold exposure some mice were able to thermoregulate better than others (see individual data values in Figure 11). In spite of this improvement in thermoregulation in some ob/ob mice, after 24 hours of cold exposure 3/4 ob/ob mice had dropped their body temperatures to room temperature (14 °C) and were near death.

SERUM THYROID HORMONES AND CORTICOSTERONE:

Obese mice are euthyroid (Figure 12). Both lean and obese mice follow the same circadian rhythm, in serum T_4 and serum T_3. There was a significant increase in serum T_3 just before the dark phase in both lean and obese mice. There was no significant change in the ratio of T_3/T_4 with time in lean or ob/ob mouse serum (Table 4 and 5). When lean mice were exposed to cold there was no change in serum T_3 for 6 hours after which there was a dramatic 3-4 fold increase. The ob/ob mouse however, was only able to increase serum T_3 levels 45 % after 24 hours at 14 °C. Serum T_4 levels were not affected by cold exposure in lean mouse serum, obese mice had a tendency to decrease serum T_4 levels but the decrease was not significant (Figure 13).

Obese mice had significantly higher levels of serum corticosterone at all times of the day studied. Both lean and obese mice followed a similar circadian rhythm in that they both had lower levels during the day than at night (Figure 14). When lean mice are placed in the cold they significantly increase serum corticosterone levels to the high

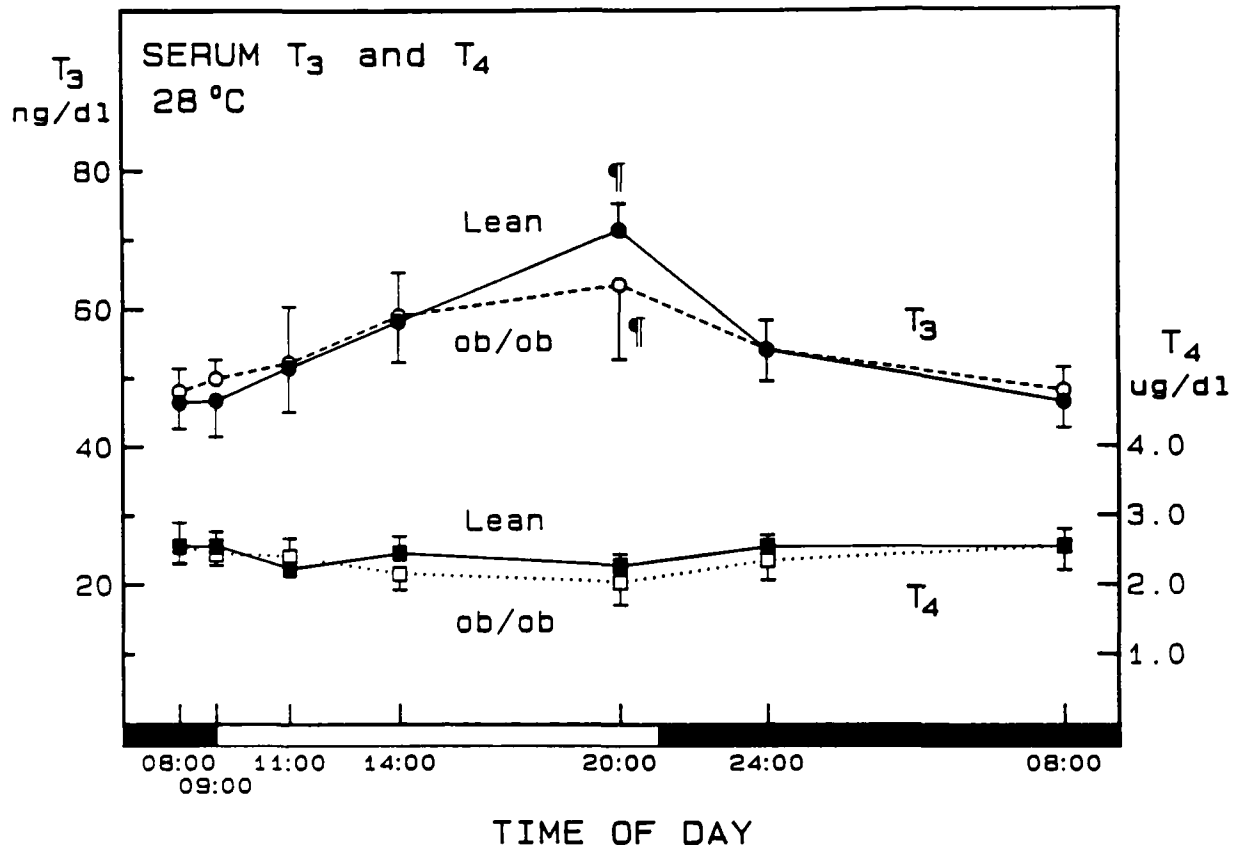


Figure 12: DIURNAL RHYTHM OF SERUM THYROID HORMONES IN LEAN AND OB/OB MICE AT 28 °C. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. A significant effect of time is represented by ¶.

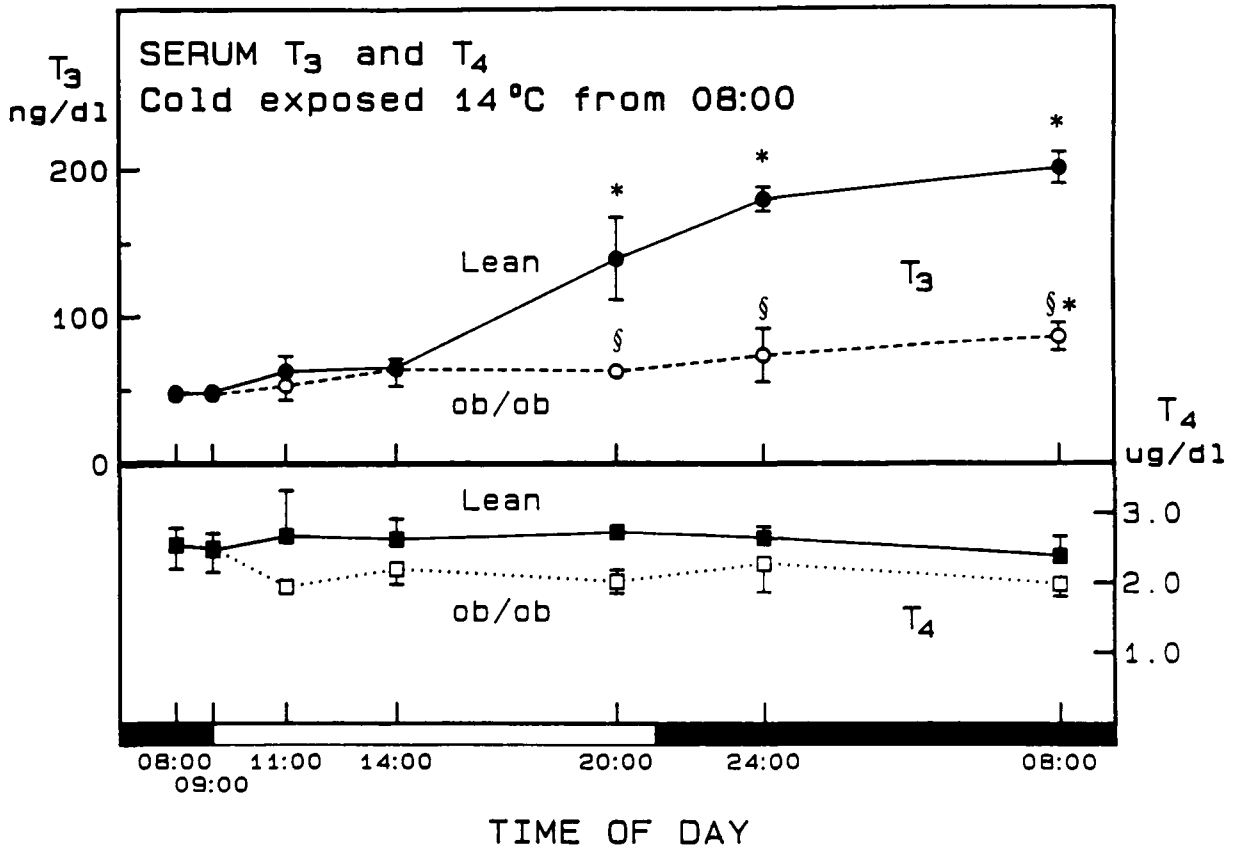


Figure 13: SERUM THYROID HORMONE LEVELS OF LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. A significant effect of cold exposure is denoted by * and § denotes a significant effect of obesity.

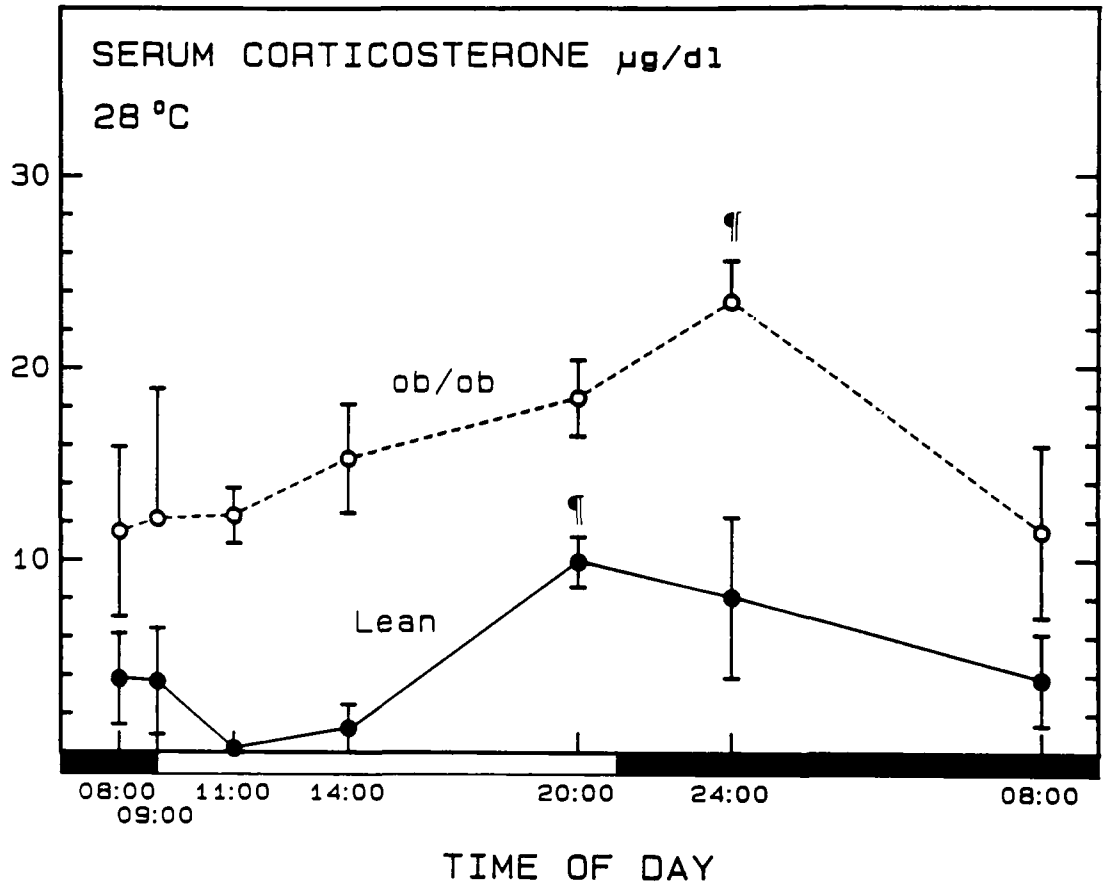


Figure 14: DIURNAL RHYTHM OF SERUM CORTICOSTERONE IN LEAN AND OB/OB MICE AT 28 °C. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. Obese mice had significantly higher values at all times of the day than lean mice. A significant effect of time was denoted by ¶.

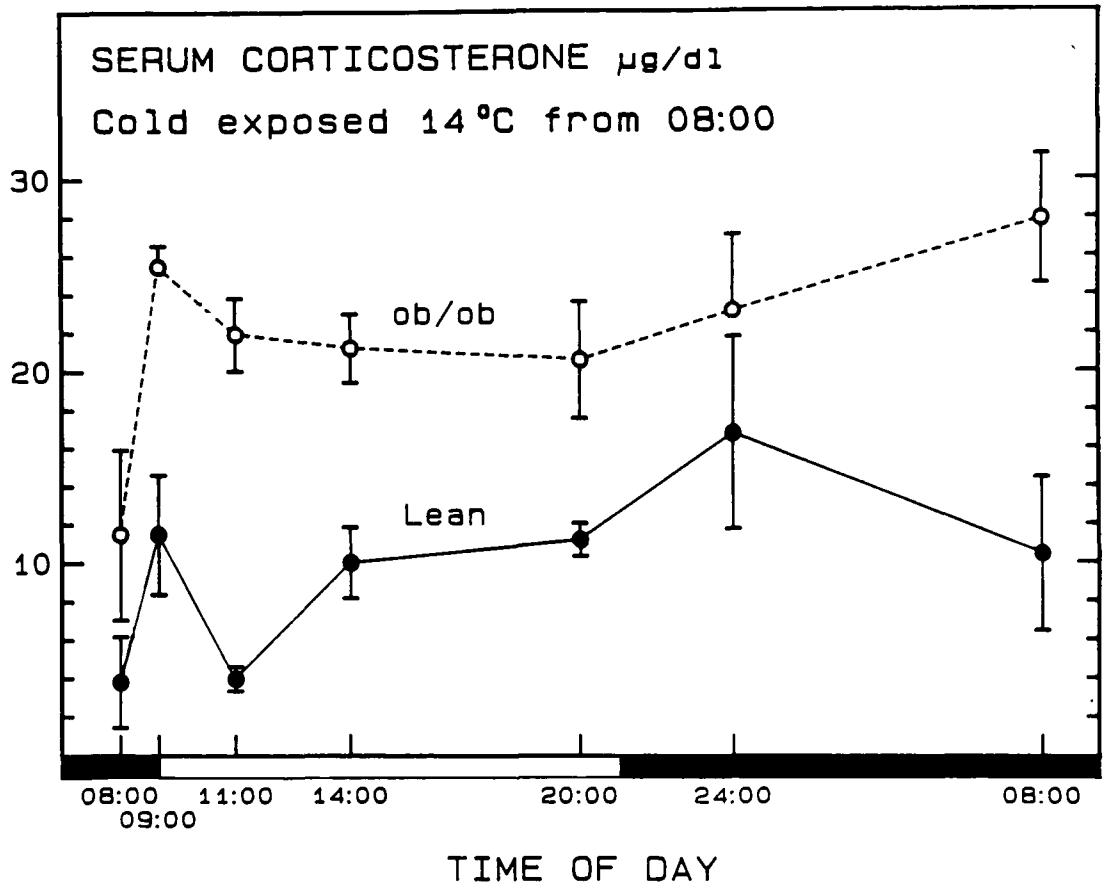


Figure 15: SERUM CORTICOSTERONE LEVELS IN LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. Obese mice had significantly higher values at all times of the cold exposure than lean mice (except at 24:00) Cold stimulated a significant increase in corticosterone levels in both lean and ob/ob mice at most times of the day (See Table 8 and 9).

levels observed during the dark phase. Obese mice also significantly increase serum corticosterone levels when exposed to cold (Figure 15).

EFFECT ON BROWN ADIPOSE TISSUE:

Obese mice have more BAT than lean mice at all times studied (Table 6 and 7). There was no effect of cold on the amount of BAT in either lean or ob/ob mice. Lean and obese mice had the same amount of BAT protein (in this experiment). Upon cold exposure both lean and obese mice were able to increase BAT protein. Obese mice were able to increase BAT protein 45 % whereas lean mice were capable of only a 20 % increase.

GDP-BINDING:

Lean mice have significantly greater GDP-binding to isolated BAT mitochondria than obese mice at all times studied (Figure 16). Lean mice have a pronounced circadian rhythm in GDP-binding with almost a 3 fold difference between the low binding levels at night and early morning and the highest levels recorded just before lights off in the evening. Obese mice follow the same but greatly blunted rhythm with much smaller differences between high and low values (Figure 16).

When lean animals are exposed to cold they are able to double their GDP-binding values within one hour After 12 hours in the cold they have increased GDP-binding values 3 fold. In contrast obese mice are able to increase GDP-binding values only after 6 hours of cold exposure. The maximum increase representing a 4 fold stimulation also occurs after 12 hours, however, the maximum binding found in the ob/ob mouse only approaches values of lean mice at 28 °C. After 24 hours of

TABLE 6

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON BAT OF LEAN MICE

	CONTROL 28° C						COLD EXPOSED 14° C from 08:00					
	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=3	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=4	24:00 n=4	08:00 n=5
BAT Wet Wt. g	0.17 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.19 ± 0.01	0.15 ± 0.01	0.20 ± 0.02	0.17 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
BAT Protein mg	9.5 ± 1.0	11.7 ± 0.9	10.8 ± 0.9	10.9 ± 0.7	11.5 ± 0.2	11.2 ± 0.5	10.9 ± 0.5	11.2 ± 0.6	11.2 ± 0.8	13.9* ± 0.4	13.2* ± 0.7	13.0* ± 0.6
UCP µg/mg protein	4.87 ± 0.72	3.35 ± 0.47	4.64 ± 0.27	4.48 ± 0.57	2.90 ± 0.96	3.44 ± 1.20	7.44* ± 0.47	4.80* ± 0.53	6.37 ± 1.67	5.64 ± 0.76	7.00* ± 1.23	7.68* ± 1.11
T_4 5' D pmol/mg P/h	0.11 ± 0.02	0.07 ±0.01	0.09 ±0.01	0.14 ±0.01	0.10 ±0.01	0.12 ±0.02	0.24 ± 0.03	0.77* ±0.08	1.54* ±0.08	3.22* ±0.21	3.79* ±0.40	1.08* ±0.19

Values are means ± SEM for the number (n) of animals per group. Symbols are as follows: Significant effect of obesity §, significant effect of cold compared to controls at the same time of day *, ($p < 0.05$). Statistics were measured using ANOVA followed by Scheffe's post-hoc test.

TABLE 7

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON BAT OF OB/OB MICE

	CONTROL 28° C						COLD EXPOSED 14° C from 08:00					
	09:00 n=3	11:00 n=4	14:00 n=4	20:00 n=4	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=5	24:00 n=4	08:00 n=4
§BAT Wet Wt. g	1.01 ± 0.12	1.24 ± 0.07	1.21 ± 0.04	1.44 ± 0.06	0.73 ± 0.08	1.10 ± 0.06	1 05 ± 0.10	1.26 ± 0.07	1.36 ± 0.08	1.32 ± 0.13	0.77 ± 0.05	0.88 ± 0.06
BAT Protein mg	10.0 ± 0.9	7.5 ± 1.2	8.8 ± 0.3	8.9 ± 0.5	10.8 ± 0.2	11.8 ± 1.4	11.4 ± 0 4	11.0* ± 1.0	14.5* ± 1.8	15.3* ± 1.0	15.9* ± 0.9	16.7*§ ± 0.9
UCP µg/mg Prot.	2.69§ ± 0.11	1.96 ± 0.38	1.53§ ± 0.23	2.33§ ± 0.16	3.79 ± 1.10	2.39 ± 0.23	1.81§ ± 0.22	1.99§ ± 0.21	1.39§ ± 0.33	1.32§ ± 0.16	3.08§ ± 0.48	3.13§ ± 0.53
T_4 5'D pmol/mg P/h	0 10 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.08§ ±0.01	0.08 ± 0.01	0.08 ±0.01	0.06§ ± 0.01	0.14§ ±0.04	0.36§ ±0.27	1.25*§ ±0.51	1.42*§ ±0.59	1.98* ±0.67

Values are means ± SEM for the number (n) of animals per group. Symbols are as follows: Significant effect of obesity §, (at the begining of the data this signifies that all means have a significant effect of obesity), significant effect of cold *, ($p < 0.05$). Statistics were measured using ANOVA followed by Scheffe's post-hoc test.

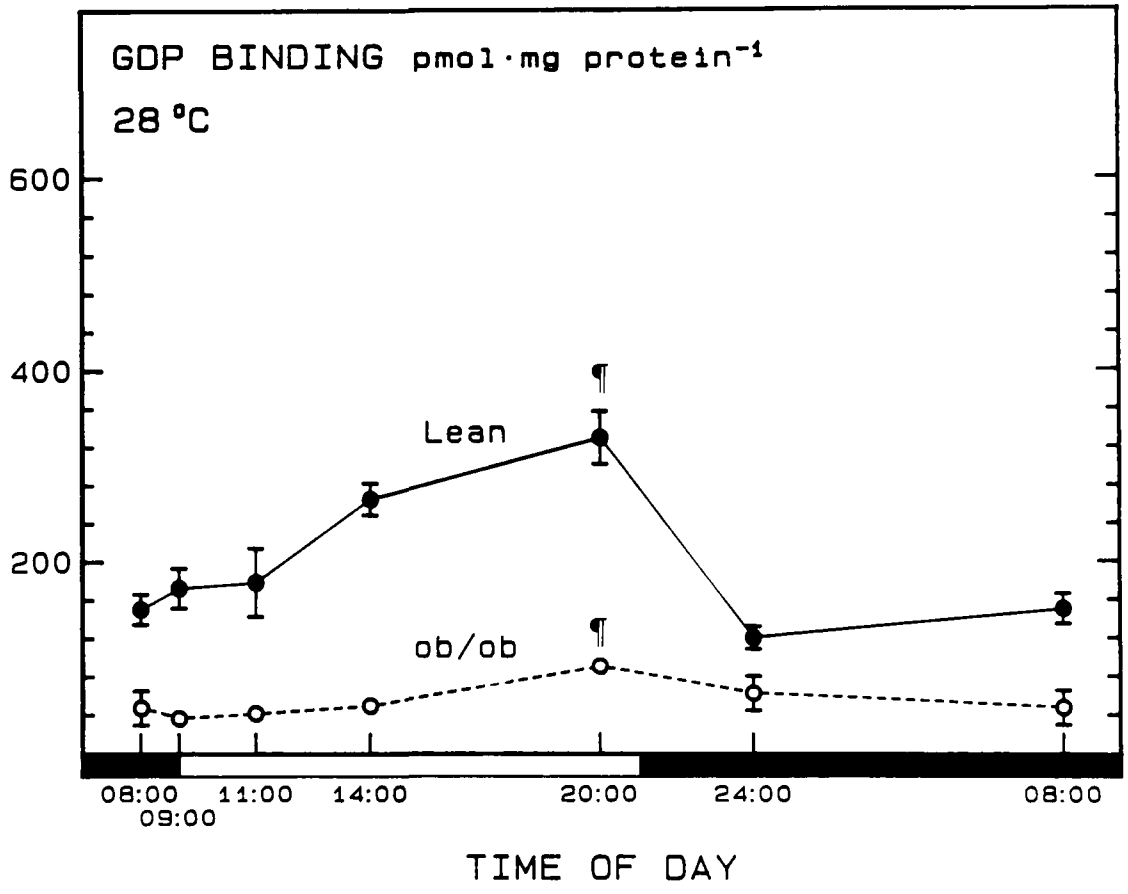


Figure 16: DIURNAL RHYTHM OF GDP-BINDING TO ISOLATED BAT MITOCHONDRIA OF LEAN AND OB/OB MICE AT 28 °C. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. Obese mice have significantly lower binding values at all times measured. A significant effect of time was denoted by ¶.

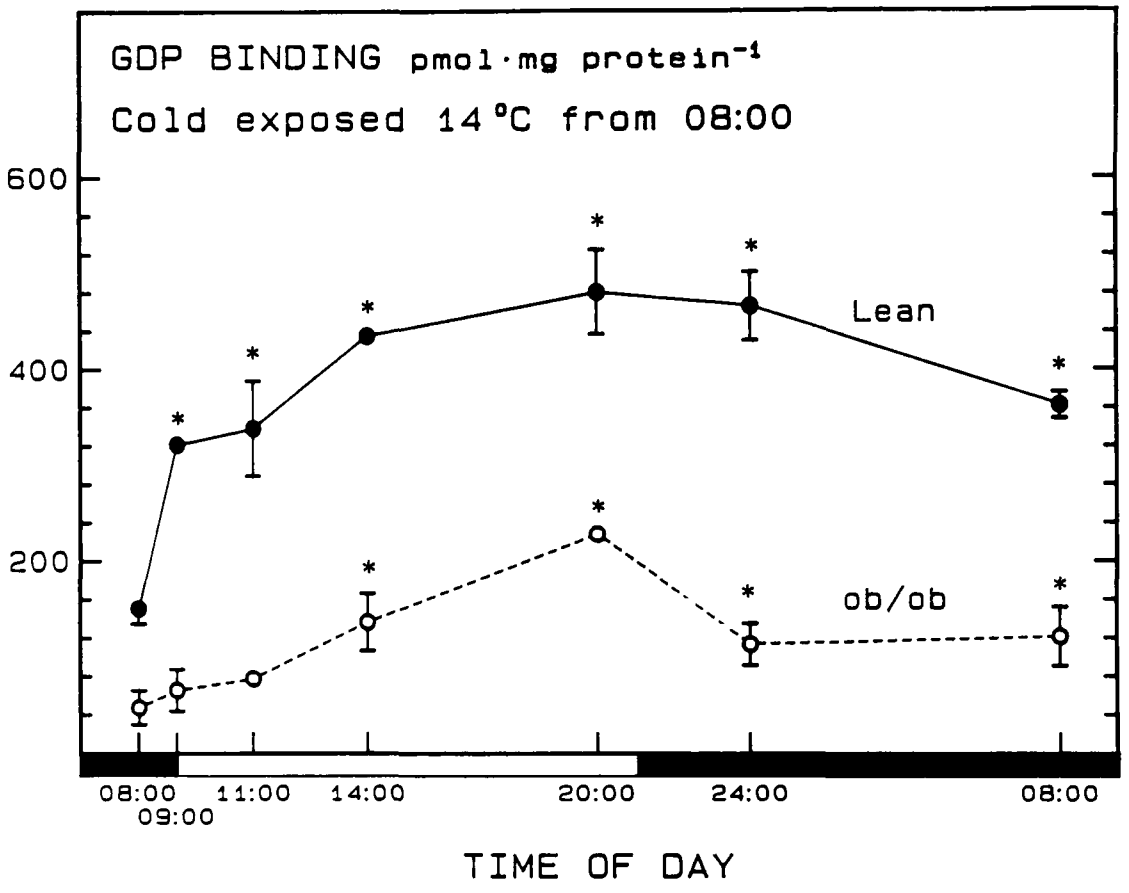


Figure 17: GDP-BINDING TO ISOLATED BAT MITOCHONDRIA OF LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. Obese mice have significantly lower binding values at all times measured. A significant effect of cold was denoted by *.

cold exposure both lean and obese mice have a 2 fold stimulation of GDP-binding values (Figure 17).

UNCOUPLING PROTEIN:

Obese mice have lower UCP levels expressed as µg UCP/mg homogenate protein (Table 6 and 7) and as µg UCP/total BAT homogenate (Figure 18) than lean mice at most times of the day studied. The obese mouse has a curious circadian rhythm, with high levels at night and low levels during the day (see Figure 18 and Table 9). In contrast lean animals do not have a significant rhythm in uncoupling protein. This indicates that in lean mice the rhythm found in GDP-binding was accomplished by unmasking of binding sites rather than synthesis of new protein. The changes in UCP found in the ob/ob mouse do not relate to changes found in GDP-binding. The higher level of binding found at 20:00 corresponds to a lower level of UCP (Figure 16 and 18).

When lean mice were exposed to cold (Figure 19) there was a tendency for UCP values to increase over the 24 hour cold exposure but the increase was not statistically significant when expressed as µg UCP/total BAT homogenate. When expressed as µg UCP/mg protein, there was a significant doubling of UCP over the 24 hour cold exposure in lean mice (Table 6). Obese mice continue to have significantly lower UCP levels than lean mice exposed to 14 °C. Obese mice also show a trend towards higher UCP levels but the increase was not significant after a 24 hour cold exposure regardless of how the level was expressed (Figure 19 and Table 7). Thus lean mice were specifically able to

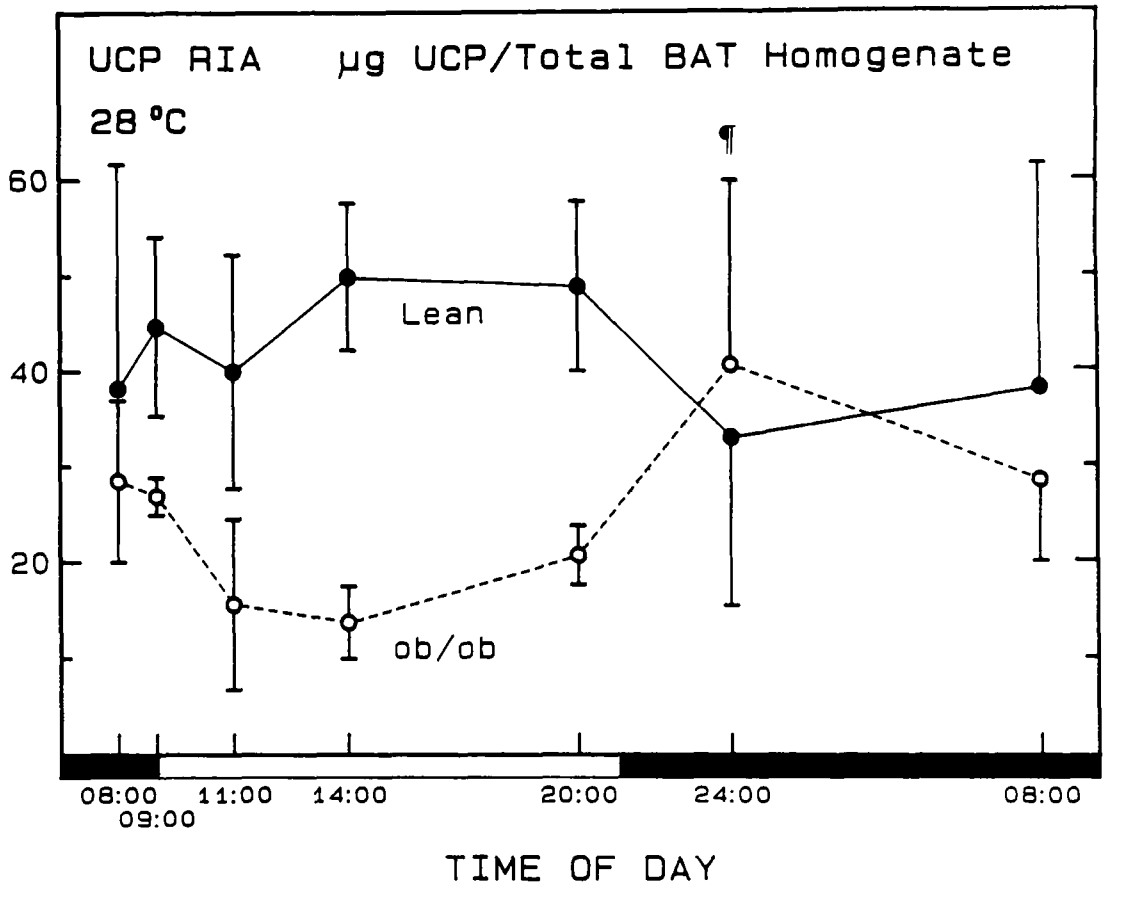


Figure 18: DIURNAL RHYTHM OF UCP MEASURED BY SOLID PHASE RIA IN BAT HOMOGENATES OF LEAN AND OB/OB MICE AT 28 °C. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. Obese mice had significantly lower values than lean mice at all times studied (except 24:00 and 08:00). A significant effect of time was denoted by ¶.

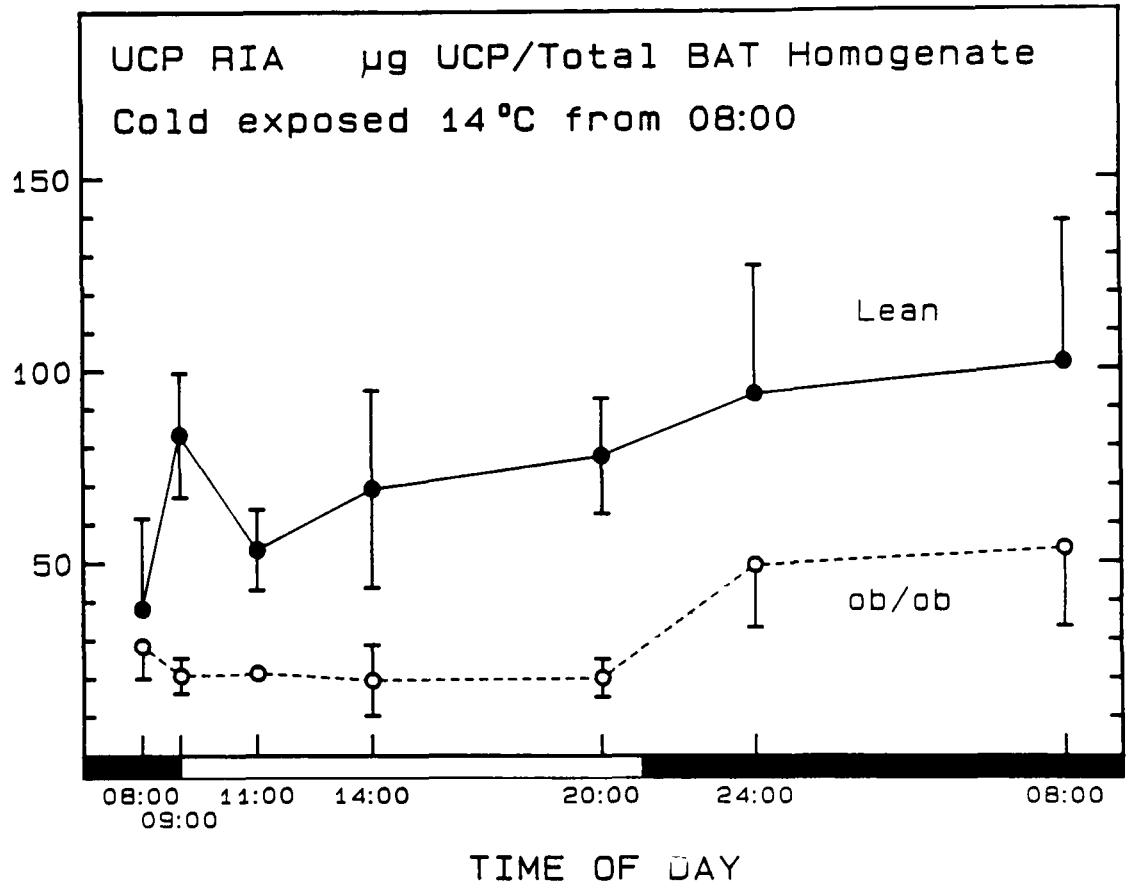


Figure 19: UCP MEASURED BY SOLID PHASE RIA IN BAT HOMOGENATES OF LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. There was no significant effect of cold exposure on lean or ob/ob mice. Obese mice had lower UCP values than lean mice at all times studied except at 08:00.

increase the level of BAT UCP after 24 hours of cold exposure whereas ob/ob mice were unable to increase UCP levels.

BROWN ADIPOSE TISSUE THYROXINE 5' DEIODINASE ACTIVITY:

Lean mice have lower total T5'D activity during the day and higher values just before dark and during the night (Figure 20). The specific T5'D activity however showed no significant effect of time (Table 6). Obese mice showed no rhythm in T5'D specific or total activity (Figure 20 and Table 7). At all times except 20:00 there was no significant effect of obesity on T5'D activity.

When lean mice are exposed to cold there was a lag phase of about 1 hour after which there was a dramatic increase in both total (Figure 21) and specific (Table 6) T5'D activity. The maximum total and specific activity was reached after 12-16 hours and represented a 50 and 40 fold increase respectively (Figure 21 and Table 6). After 24 hours of cold exposure both the total and specific activity decreased to a 14 and 10 fold increase above basal levels. In contrast obese mice are unable to increase total (Figure 21) or specific (Table 7) T5'D activity for at least 6 hours when exposed to cold. Figure 21 is similar to Figure 11 which shows the varying degrees of hypothermia found in ob/ob mice exposed to cold: Figure 21 shows the variation in the response of obese BAT T5'D activity to cold exposure. There was a relationship between the animals that were able to maintain body temperature at 20:00 and 24:00 (Figure 11) and the level of total BAT T5'D activity. However, after 24 hours at 14 °C most of the ob/ob mice were hypothermic and near death and 3/4 mice had managed to elevate

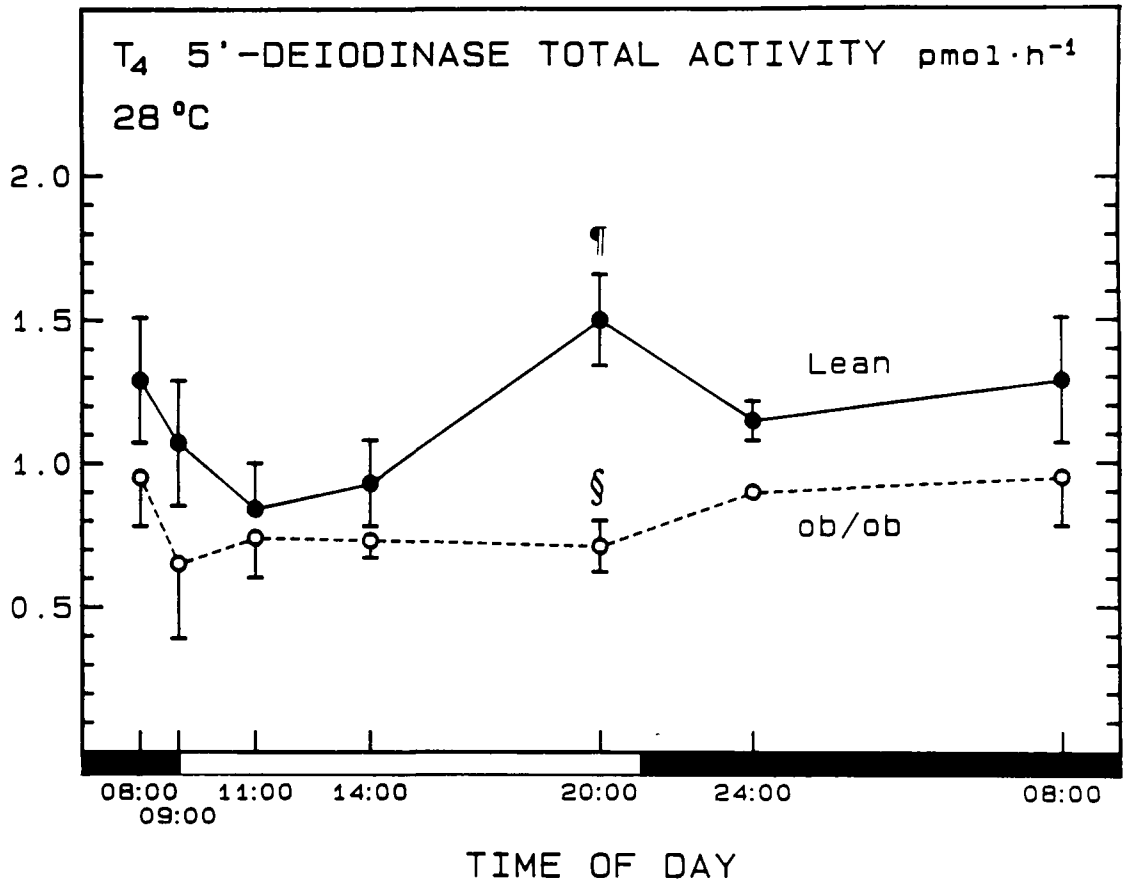


Figure 20: DIURNAL RHYTHM OF BAT T5'D TOTAL ACTIVITY IN BAT OF LEAN AND OB/OB MICE AT 28 °C. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. There was no significant difference between lean and ob/ob value (except at 20:00). A significant effect of time was denoted by ¶.

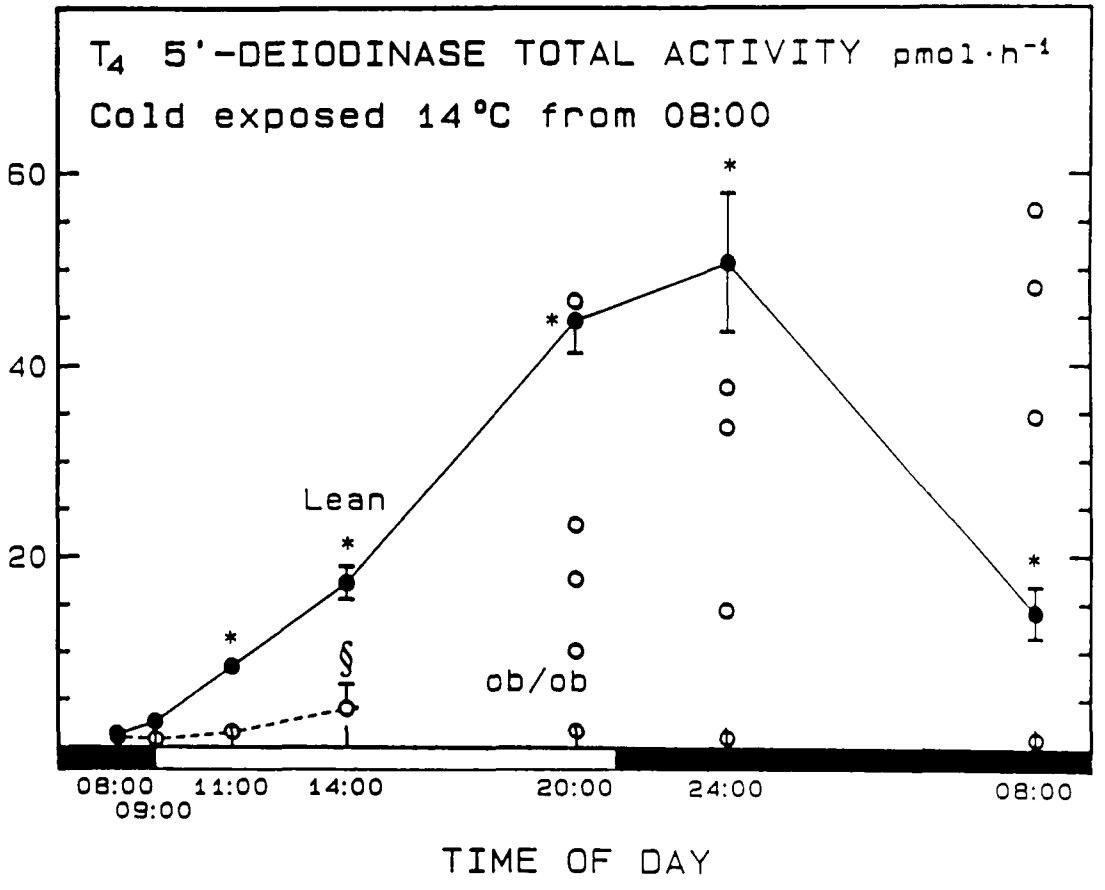


Figure 21: BAT T5'D TOTAL ACTIVITY IN BAT OF LEAN AND OB/OB MICE EXPOSED TO 14 °C FROM 08:00. Values are means ± SEM for the number (n) of animals in Tables 8 and 9. A significant effect of cold was denoted by * for lean mice. Obese mice had significantly lower values than lean mice at all times studied. Individual values for obese mice are denoted by the open circles. There was a significant effect of cold exposure on the average of these individual values (see Table 9).

TABLE 8

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON LEAN MICE:

GRAPH DATA

	CONTROL 28° C						COLD EXPOSED 14° C from 08 00					
	09:00 n=4	11 00 n=4	14:00 n=4	20:00 n=3	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=4	24·00 n=4	08 00 n=5
Body Temp °C	36 9 ± 0 7	36 3 ± 0 5	36 1 ± 0 3	36 1 ± 0 5	38 0¶ ± 0 1	37 3 ± 0 1	36 1 ± 0 3	35 3 ± 0 3	34 7 ± 0 6	35 4 ± 0 1	36 2* ± 0 3	35 7* ± 0 2
Serum T_3 ng/dl	46 7 ± 3 1	51 5 ± 3 7	58 2 ± 3 5	71 4¶ ± 2 7	54 1 ± 2 5	46 4 ± 2 2	48 6 ± 2 7	62 8 ± 6 1	65 3 ± 3 3	139 5* ± 16 3	179 8* ± 4 9	200 6* ± 5 4
Serum T_4 µg/dl	2 6 ± 0 1	2 2 ± 0 1	2 5 ± 0 1	2 3 ± 0 1	2 6 ± 0 1	2 6 ± 0 1	2 5 ± 0 2	2 7 ± 0 4	2 6 ± 0 2	2 7 ± 0 1	2 7 ± 0 1	2 4 ± 0 1
Serum Cort µg/dl	3 66 ± 1 61	0 01 ± 0 00	1 22 ± 0 73	9 90¶ ± 0 94	8 07 ± 2 42	3 77 ± 1 37	.1 5* ± 1 80	2 97* ± 1 04	9 98* ± 1 10	11 18 ± 0 51	16 78* ± 2 90	10 43* ± 2 01
GDP Binding pmol/mg prot	172 3 ± 20 7	178 3 ± 35 6	265 3 ± 17 2	329 3¶ ± 27 8	119 8 ± 12 1	150 0 ± 16 2	321 3* ± 10 7	338 0* ± 50 4	434 5* ± 9 4	480 5* ± 44 3	466 0* ± 36 0	362 0* ± 14 4
UCP Total µg/BAT homog	44 6 ± 5 5	39 9 ± 7 1	49 8 ± 4 4	48 8 ± 6 3	32 9 ± 10 1	38 1 ± 13 6	81 8 ± 8 3	53 3 ± 6 1	69 1 ± 14 9	77 6 ± 8 ~	93 8 ± 19 4	101 ~ ± .8 ~
T_4 5' D pmol/h	1 07 ± 0 22	0 84 ± 0 16	0 93 ± 0 15	1 50¶ ± 0 16	1 15 ± 0 07	1 29 ± 0 22	2 59 ± 0 42	8 44* ± 0 63	17 28* ± 1 73	44 72* ± 3 42	50 ~4* ± ~ 22	14 ,4* ± 2 ~0

Values are means ± SEM for the number (n) of animals per group. Symbols are as follows: Significant effect of obesity §, significant ef2fect of cold *, Significant effect of time ¶ ($p < 0.05$). Statistics were measured using ANOVA followed by Scheffe's post-hoc test.

TABLE 9

EFFECT OF UP TO 24 HOUR COLD EXPOSURE ON OB/OB MICE:

GRAPH DATA

	CONTROL 28° C						COLD EXPOSED 14° C from 08:00					
	09:00 n=3	11:00 n=4	14:00 n=4	20:00 n=4	24:00 n=4	08:00 n=4	09:00 n=4	11:00 n=4	14:00 n=4	20:00 n=5	24:00 n=4	08:00 n=4
Body Temp °C	35.5 ± 0.3	34.7§ ± 0.3	34.7§ ± 0.2	35.4 ± 0.2	36.3§¶ ± 0.4	36.7 ± 0.2	29.1§* ± 0.4	27.6§* ± 1.5	23.0§* ± 2.0	28.1§* ± 3.2	24.3§* ± 4.9	16.7§* ± 1.6
Serum T_3 ng/dl	49.9 ± 2.0	52.2 ± 4.8	59.0 ± 3.7	63.4¶ ± 6.2	54.1 ± 2.6	48.1 ± 2.0	46.8 ± 2.9	53.0 ± 5.8	64.1 ± 6.7	63.0§ ± 2.4	74.1§ ± 10.6	86.4§* ± 5.5
Serum T_4 µg/dl	2.4 ± 0.1	2.4 ± 0.2	2.2 ± 0.1	2.0 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	2.5 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.0§ ± 0.1	2.3 ± 0.2	2.0 ± 0.1
GDP Binding pmol/mg prot	35.7§ ± 20.7	41.0§ ± 35.6	48.8§ ± 17.2	90.3§¶ ± 27.8	62.3§ ± 12.1	47.0§ ± 16.2	65.3§ ± 10.7	77.0§ ± 50.4	135.5§* ± 9.4	206.0§* ± 44.3	112.5§* ± 36.0	121.8§* ± 14.4
Serum Cort µg/dl	12.1§ ± 4.7	9.2§ ± 3.2	15.5§ ± 1.5	18.4§ ± 1.1	23.5§¶ ± 1.2	11.5§ ± 2.6	25.4§* ± 0.6	21.97§* ± 1.1	21.2§* ± 1.0	20.6§ ± 1.5	23.2 ± 2.3	27.9§* ± 2.4
UCP Total µg/BAT homog	26.8§ ± 4.4	15.5§ ± 5.1	13.6§ ± 2.2	20.6§ ± 1.8	40.6¶ ± 11.1	28.4 ± 4.9	20.7§ ± 3.3	21.3§ ± 1.0	19.4§ ± 5.4	20.0§ ± 2.5	49.3 ± 9.4	53.5§ ± 11.6
T_4 5' D pmol/h	0.05 ± 0.15	0.75 ± 0.14	0.73 ± 0.06	0.71§ ± 0.09	0.90 ± 0.10	0.95 ± 0.17	0.72§ ± 0.15	1.50§ ± 0.34	3.99§ ± 2.57	19.90§* ± 7.64	21.69§* ± 8.59	34.91§* ±12.19

Values are means ± SEM for the number (n) of animals per group. Symbols are as follows: Significant effect of obesity §, significant effect of cold *, Significant effect of time ¶ ($p < 0.05$). Statistics were measured using ANOVA followed by Scheffe's post-hoc test.

total BAT T5'D activity (Figure 21 and 11). Thus ob/ob mice were unable to properly activate BAT T5'D activity in response to acute cold exposure.

DISCUSSION:

Lean mice acclimated to 28 °C have a distinct rhythm in BAT T5'D activity (Figure 20 and Eley and Himms-Hagen, 1989b). Low levels were seen during the day with higher levels observed at night. This rhythm was very similar to the diurnal rhythm observed in serum corticosterone in lean mice (Saito and Bray, 1983 and Figure 5) and rats (Oster et al., 1988). In rats, the rhythm of corticosterone can be explained by complementary rhythms in ACTH and CRF (Moldow and Fischman, 1984). The interaction of corticosterone and BAT T5'D activity will be studied further in the next section of this chapter. Petersen (1978) reported a 5 fold increase at dusk in the hourly rate of feeding of mice which was closely associated with a fall in blood glucose and a rise in insulin. The unusual rise in insulin at this time was speculated to be due either to a pulsatile secretion from the pancreas at that time or due to other hormones or factors present due to the great increase in food intake. I also found an increase in food intake at dark onset (20:00, Table 4). This time point (20:00) also coincides with the peak in T5'D activity (Figure 20). Silva and Larsen, (1986a) and Mills et al., (1987) have shown that insulin has a stimulatory effect on BAT T5'D activity in rats. Thus it is possible that an increase in insulin in mice (Petersen, 1978) could cause an increase in T5'D activity and since food intake is higher at night, insulin levels would be higher at

night. This is one possible explanation as to why T5'D activity is higher at night. The rhythm in BAT T5'D activity was also related to the rhythm found in serum T_3 with the highest values for both variables measured at 20:00. A diurnal rhythm in BAT lipogenesis of rats in which high levels of lipogenesis are observed at night and low levels during the day has been reported by Thompson and Grigor, (1987). Thyroid hormones are known to regulate lipogenic enzymes in the liver (for review see Hoch, 1988). Since BAT contains high amounts of the regulatory enzymes of fatty acid synthesis (McCormack and Denton, 1977; Bianco and Silva, 1987b) it is possible to speculate that the higher production of T_3 by BAT T5'D activity at night (Figure 20) and/or the higher level of serum T_3 (Figure 12) could be involved in the diurnal variation of BAT lipogenesis.

The rhythm in BAT T5'D activity seemed to lag 10-12 hours behind the rhythm found in GDP-binding to isolated BAT mitochondria. The GDP-binding rhythm (Figure 16) had high levels during the day and low levels at night. This pattern has also been reported by Eley and Himms-Hagen (1988b). The low levels of GDP-binding found at night could be related to the extremely high physical activity of mice during the early dark phase (Connoly and Becker Lynch, 1981). The high physical activity could cause the increase in body temperature seen in Figure 10. This increase in body temperature could be sufficient to suppress BAT thermogenesis. Such a phenomena has been known to occur in exercised mice (Richard and Trayhurn, 1984).

The rhythm observed in GDP-binding in lean mice was very similar to the rhythm in BAT sympathetic activity of mice reported by Yen *et al*.

(1988). Thus there seems to be a direct relationship between the stimulation by noradrenaline and the increase in GDP-binding but a lag between the stimulation by noradrenaline and an increase in BAT T5'D activity. In fact, since there was no rhythm in UCP found in lean mice (Figure 18) the changes in GDP-binding seen in Figure 16 were probably accomplished by unmasking and remasking of binding sites on BAT UCP in response to the rhythm of noradrenaline secretion. In contrast there was no rhythm found in BAT T5'D activity in ob/ob mice at 28 °C (Figure 20). This could be due to the known resistance or refractoriness of ob/ob mice to noradrenaline and insulin. At most times of the day there was no difference between lean and ob/ob BAT T5'D activity. This is in conflict with the higher basal activity observed in Experiment #1. Seasonal variations in T5'D activity in which lean mice have higher basal activities of BAT T5'D in the winter and lower basal values in the spring have been reported by Kaplan and Young (1987). The time course Experiment #2 was performed in the winter and the 12 hour cold exposure Experiment #1 was done in the spring, thus I also observed similar seasonal variations in my data.

The ob/ob mouse had higher levels of serum corticosterone than lean mice and the ob/ob mouse was able to maintain a similar rhythm in serum corticosterone compared to lean mice. These results agree closely with the work of Saito and Bray (1983). In spite of the lack of rhythm found in ob/ob BAT T5'D activity at 28 °C, ob/ob mice had euthyroid levels of serum T_3 and demonstrated a similar rhythm in T_3 levels compared to lean mice. Thus it seems that the lack of rhythm in BAT T5'D activity is not reflected in serum T_3 levels in the ob/ob mouse.

The obese mouse had lower levels of GDP-binding and UCP at all times of the day studied. The ob/ob mouse had a similar but blunted rhythm in GDP-binding compared to lean mice. Since there was no rhythm observed in UCP the increase in GDP-binding observed at 20:00 could have been accomplished by unmasking of GDP-binding sites. Thus ob/ob mice are capable of the unmasking phenomena. Ashwell et al., (1985) also report lower levels of UCP and GDP-binding in ob/ob mouse BAT compared to lean littermates. Thus ob/ob mice have both a lower thermogenic activity and capacity compared to lean mice. This is consistent with low levels of noradrenaline turnover in ob/ob mice of this age and at this temperature (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983).

Lean mice were able to maintain body temperature when exposed to cold (Figure 11). The exposure of lean mice to 14 °C caused a dramatic rise in BAT T5'D activity (Figure 21). There was a lag phase of approximately 1 hour before the rapid increase (Kaplan and Young, 1987 and Figure 21). When exposed to cold, lean mice are able to activate the sympathetic nervous system within 1 hour (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983). The cold stimulated increase in rat BAT T5'D activity is known to also have a 1-2 hour lag phase and require transcription and protein synthesis (Jones et al., 1986; Kopecky et al., 1986). Thus it is possible that the mechanism of activation of mouse BAT T5'D activity could be similar to that of rat and also require transcription and protein synthesis. Lean mice reached a 50 fold stimulation of BAT T5'D activity 12-16 hours after cold exposure, however by 24 hours, BAT T5'D activity had decreased to

a 14 fold stimulation. This increase in total BAT T5'D activity was due to a 40 fold increase in specific activity and a 20 % increase in BAT protein (Table 6). Kaplan and Young (1987) exposed lean mice to 4 °C and found a 14 fold increase after 4 hours. The pattern of cold stimulation of mouse BAT T5'D activity is similar to that of rats in that there was a lag phase followed by a 210 fold stimulation 12 hours after exposure to 4 °C followed by a decrease to a 50 fold stimulation at 24 hours and was different from that of hamsters which have a lag phase followed by a much greater maximum response which is delayed to 24 hours, also this high level of total BAT T5'D activity is maintained for 30-40 days (Kopecky et al., 1986). Thus rats and mice are similar in their BAT T5'D activity to cold whereas hamsters seem to have a different strategy. The dramatic rise in BAT T5'D activity in lean mice was related to a similar increase in serum T_3 (Figure 13). However, although serum corticosterone levels were significantly increased in lean mice by acute cold exposure there seemed to be no relationship to the pattern seen in BAT T5'D activity.

Lean mice exposed to 14 °C demonstrate a 2 fold increase in GDP-binding within one hour with no significant change in BAT UCP levels. Thus the rapid doubling of GDP-binding represents unmasking of GDP-binding sites on BAT UCP. This phenomena has been reported by many workers (Desautels et al., 1978; Trayhurn et al., 1987) and is consistent with the increase in sympathetic nervous system activity in response to cold exposure (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983). GDP-binding to lean BAT mitochondria reached a maximum 12-16 hours after cold exposure and then decreased by 24 hours

to a doubling of activity (Figure 17). This pattern is similar to the pattern of T5'D activity. Ashwell et al., (1983) reported a doubling of both GDP-binding and UCP levels in lean mice acclimated to 13 °C compared with lean mice acclimated to 22 °C.

In contrast to the lean mouse, the obese ob/ob mouse was unable to activate BAT T5'D activity in response to 14 °C exposure for at least 6 hours (Figure 21). During those 6 hours, the ob/ob mouse was also unable to maintain it's body temperature and became hypothermic. After 12-16 hours of cold exposure however, some ob/ob mice were able to increase BAT T5'D activity and improve their hypothermia (Figure 11). However, this increase in BAT T5'D activity was unable to prevent the hypothermia found in all mice after 24 hours of cold exposure (Figure 11). The variability of the onset of hypothermia in ob/ob mice has been reported before (Himms-Hagen et al., 1986; see Figure 22) and it has been suggested that this phenomena is a result of an inappropriate entry into torpor The delay in activation of BAT T5'D activity was accompanied by a failure to increase serum T_3 in response to cold (Figure 13), this relatively hypothyroid state also contributed to the extreme hypothermia observed in the ob/ob mouse 24 hours after cold exposure.

Obese mice were able to increase BAT protein synthesis within 3-6 hours of acute cold exposure (Table 7). The 45 % increase in protein synthesis was not reflected in an improvement in hypothermia. Obese mice did not increase GDP-binding for at least 6 hours of cold exposure (Figure 17). There was no significant change in BAT UCP in response to acute cold exposure in ob/ob mouse BAT although there was a tendency

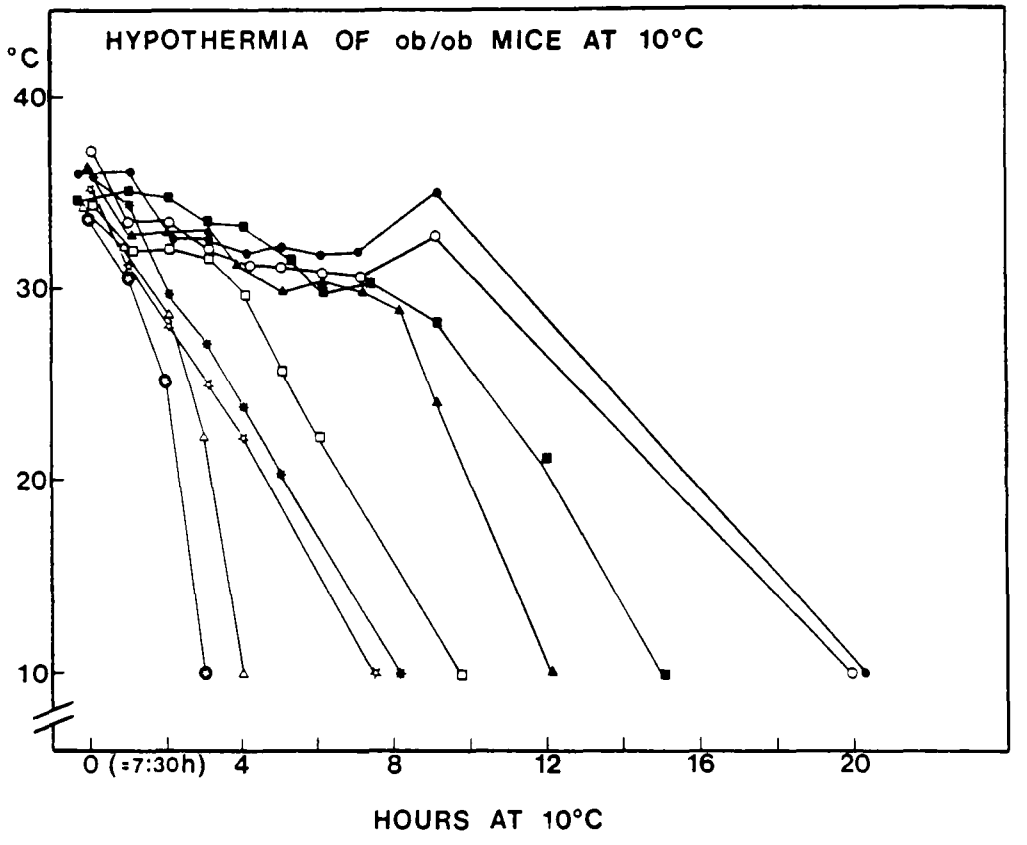


Figure 22: EFFECT OF COLD EXPOSURE (10°C) ON BODY TEMPERATURE OF OBESE (OB/OB) MICE. Individual values are shown for 9 ob/ob mice placed at 10 °C at 07:30. This figure shows the individual variation in body temperature of the ob/ob mouse in response to cold exposure. This figure was reproduced from Himms-Hagen et al. (1986) with the permission of Dr. J. Himms-Hagen.

towards higher levels (Table 7 and Figure 19). Thus ob/ob mice were initially unable to unmask GDP-binding sites on UCP but between 6-24 hours of cold exposure they were able to modestly increase GDP-binding to isolated BAT mitochondria by unmasking. These results are consistent with the attenuated increase in UCP mRNA found in 12 hour exposed ob/ob mice (Reichling et al., 1988) and with the hypothesis that T_3 produced by BAT T5'D is an important factor for the complete expression of the UCP gene (Bianco and Silva 1987a; Silva, 1988; Bianco et al., 1988). Obese mice are able to increase sympathetic activity in response to acute cold exposure relatively normally (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983). Thus it seems that BAT of ob/ob mice is refractory to the normal amount of noradrenaline since there was no effect of cold on BAT T5'D activity or GDP-binding for at least 6 hours.

SUMMARY:

Obese (ob/ob) mice exhibit a defective response to acute cold exposure. The following table summarizes the results of this experiment:

COMPARED TO LEAN MICE, OB/OB MICE HAVE:

28°C	EXPOSURE TO 14 °C
↑ BODY WEIGHT	
↑ WAT	
↑ BAT WET WEIGHT	
↑ FOOD INTAKE	NO ↑ FOOD INTAKE
↓ BODY TEMPERATURE	↓ BODY TEMPERATURE (NEAR DEATH)
↑ CORTICOSTERONE	↑ CORTICOSTERONE
NORMAL T_3	↓ T_3
↓ GDP-BINDING TO MITOCHONDRIA	↓ GDP-BINDING TO MITO
↓ UCP (TOTAL)	↓ UCP (TOTAL)
NO RHYTHM IN T5'D ACTIVITY	NO ↑ T5'D FOR 6 HOURS

The ob/ob mouse exhibits many defects compared to lean mice even at 28 °C and upon exposure to 14 °C many of the defects are exaggerated. To my knowledge a systematic study of all the variables studied over 24 hours in this experiment has not been previously reported. The

characterization of the response of lean and ob/ob mouse BAT T5'D activity to acute cold exposure adds supportive evidence to the hypothesis that T_3 produced by BAT T5'D activity is important for the expression of the UCP gene and the increase in UCP found in cold exposed mice.

I have clearly shown a defective response of ob/ob mouse BAT T5'D activity to exposure to 14 °C for 6 hours. The next section will examine the effect of two treatments known to improve many of the defects of the ob/ob mouse.

CHAPTER 1:

THE EFFECT OF COLD ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 2: TREATMENTS WHICH IMPROVE THE DEFECTIVE RESPONSE OF THE OB/OB MOUSE TO ACUTE COLD EXPOSURE.

EXPERIMENT #1: ADRENALECTOMY

BACKGROUND AND OBJECTIVES:

In the previous section the defective thermogenic response of ob/ob mice to cold exposure was defined and discussed. In this section I will discuss two treatments, adrenalectomy (ADX) and gradual cold acclimation, which were able to improve many of the defects of the ob/ob mouse.

Surgical removal of the adrenal gland is able to wholly or partially normalize most abnormalities of the ob/ob mouse. For example, adrenalectomy decreases hyperphagia, excessive weight gain, hyperglycemia and insulin resistance of skeletal muscle and is able to increase GDP-binding to isolated BAT mitochondria (Solomon et al., 1977; Yukimura and Bray, 1978; Ohshima et al., 1984; Saito and Bray, 1984; and Holt and York, 1984).

The objective of this experiment was to study the effect of adrenalectomy on the thermogenic response of BAT of lean and ob/ob mice to acute cold exposure (6 hours at 14 °C). The 6 hour time point was chosen because the defective response of the ob/ob mouse to cold discussed in the previous section was most evident at this time.

METHODS:

Female C57Bl/6J lean (+/?) and genetically obese (ob/ob) mice arrived from Jackson Labs at 4 weeks of age. They were placed in individual cages at 28 °C with a 12:12 lighting schedule with lights on at 09:00. The animals were fed Purina rodent chow #5012 ad libitum and were allowed free access to tap water This experiment was performed during March and April, 1987.

At 4.5 weeks of age the animals were surgically adrenalectomized (ADX) or sham-operated (Sham) as described in method section #2. Body weights were measured weekly for 4 weeks and the identity of ob/ob ADX animals was confirmed on the basis of lower body weight before death and low serum corticosterone values after death.

At 8.5 weeks of age groups of lean and obese sham and ADX animals were exposed to 14 °C for 6 hours starting at 08:00 as described in the previous experiment (Chapter 1, Part 1, Experiment 2). Animals were killed in the 14 °C cold room and 28 °C room as described previously. The same protocol described for the time course experiment was followed.

RESULTS:

<u>BODY WEIGHT, 4 WEEK BODY WEIGHT GAIN, NOSE-TAIL LENGTH, AND WHITE ADIPOSE TISSUE WET WEIGHT:</u>

Sham-operated obese (ob/ob) mice weigh more, have greater 4 week body weight gain, and gonadal WAT and are stunted compared to lean sham operated control animals (Table 10). There was no signficant effect of ADX on these parameters in lean mice at either temperature studied.

Adrenalectomy however, was able to significantly reduce body weight, 4 week body weight gain and the amount of gonadal WAT of ob/ob mice at both temperatures studied. Adrenalectomized ob/ob mice increased their nose-tail length and were no longer stunted. There was no significant effect of exposure to 14 °C for 6 hours on these parameters in ob/ob mice.

BODY TEMPERATURE:

Sham operated obese (ob/ob) mice have significantly lower body temperatures than lean sham-operated mice. Adrenalectomy had no effect on body temperature of lean mice at either temperature studied. Adrenalectomy was, however, able to significantly increase the body temperature of ob/ob mice at 28 °C and at 14 °C for 6 hours and was able to reduce the drop in body temperature (black bars, Figure 23) in cold exposed ob/ob mice and thus improve the hypothermia of cold-exposed obese mice.

SERUM CORTICOSTERONE:

Obese (ob/ob) mice have greater levels of serum corticosterone than lean 28 °C mice. Cold exposure significantly increased serum corticosterone levels in both lean and ob/ob sham operated animals. Adrenalectomy, as expected, caused a significant reduction of serum corticosterone levels in both lean and ob/ob ADX mice. Cold exposure did not stimulate serum corticosterone values in ADX lean or ADX ob/ob mice (Table 10).

SERUM THYROID HORMONES:

Obese mice are euthyroid compared to lean sham 28 °C mice (Figure 24 and 25). Adrenalectomy had no effect on serum T_4 or T_3 of lean or

TABLE 10

EFFECT OF ADRENALECTOMY ON LEAN AND OB/OB MICE

	LEAN				OB/OB			
	28° C		14° C for 6 h		28° C		14° C for 6 h	
	SHAM	ADX	SHAM	ADX	SHAM	ADX	SHAM	ADX
(N)	(7)	(6)	(16)	(7)	(5)	(5)	(17)	(7)
Body Weight g	18.0 ± 0 2	17 8 ± 0 6	17.1 ± 0 3	19.4 ± 0 6	36.2§ ± 0 7	31 2¶§ ± 1 3	34 4§ ± 0 8	25 0¶§ ± 2 1
4 WK Body Weight Gain g	2 1 ± 0 3	2 5 ± 0 5	1.3 ± 0 2	2.3 ± 0 4	15 1§ ± 0 2	8 2¶§ ± 2 2	14 0§ ± 0 2	2 3¶ ± 0 7
WAT Wet Weight g	0 29 ± 0 04	0 22 ± 0 03	0 29 ± 0 03	0.25 ± 0 04	2.07§ ± 0 15	1 50¶§ ± 0 19	1 79§ ± 0 10	1 00¶§ ± 0 15
Nose-Tail Length cm	15 2 ± 0 1	15 3 ± 0.1	15 0 ± 0 1	15.6 ± 0 2	14.4§ ± 0 3	16 2¶§ ± 0 1	14 3§ ± 0 1	15 5¶ ± 0 1
Corticosterone µg/dl	6 43 ± 1 26	0 41¶ ± 0 57	15 81* ± 1 97	0 61¶ ± 0 31	20 79§ ± 4 04	0 93¶ ± 0 68	32 86*§ ± 0 58	0 18¶ ± 0 08
RATIO T_3/T_4 %	2 55 ± 0 11	2 82 ± 0 36	3 27* ± 0 13	3 95* ± 0 24	2 49 ± 0 10	2 46 ± 0 24	3 06 ± 0 19	4 10*¶ ± 0 19

Values are means ± SEM. Symbols are as follows: significant effect of obesity §, significant effect of adrenalectomy ¶, significant effect of cold exposure *, ($p<0.05$). Statistics were measured using three-way ANOVA followed by Scheffe's post-hoc test.

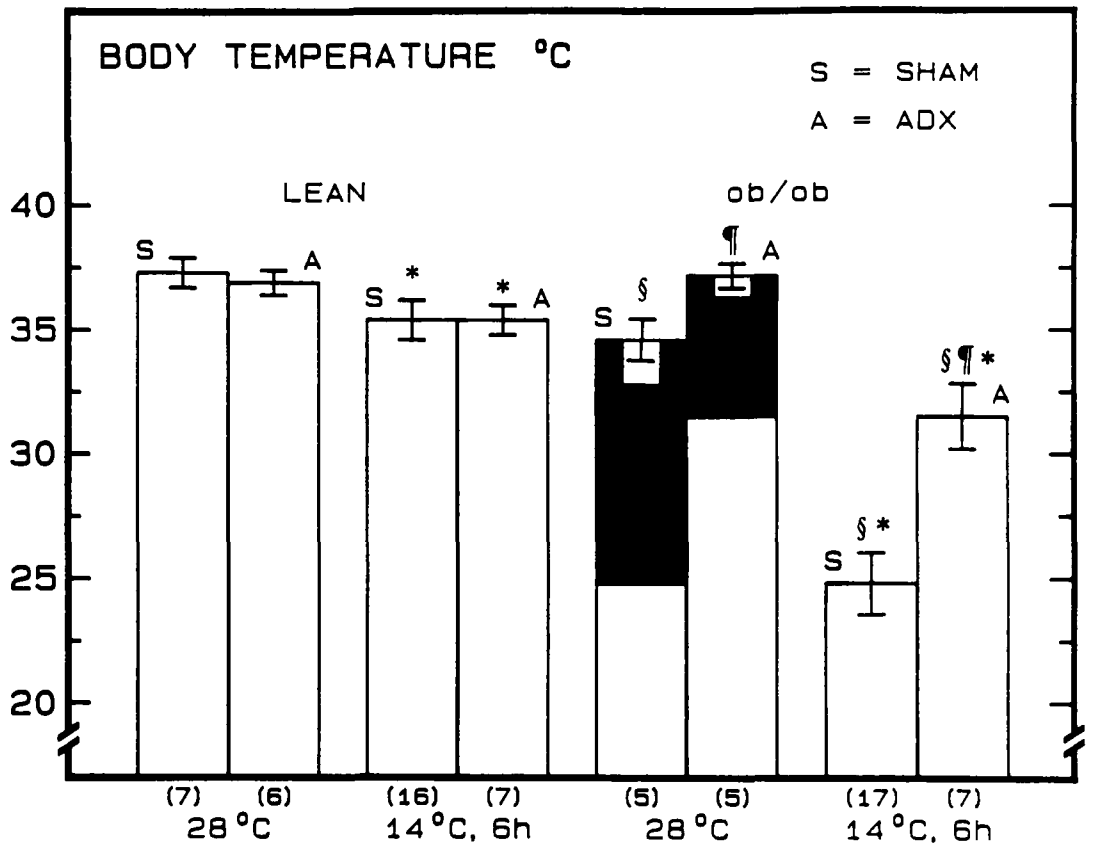


Figure 23: EFFECT OF ADX AND COLD EXPOSURE ON BODY TEMPERATURES OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 12. A significant effect of ADX is symbolized by ¶. A significant effect of cold exposure is symbolized by * and the dark portions of the bars signify the improvement in hypothermia due to ADX. A significant effect of obesity is denoted by §. ADX improved the response of ob/ob mice to cold exposure.

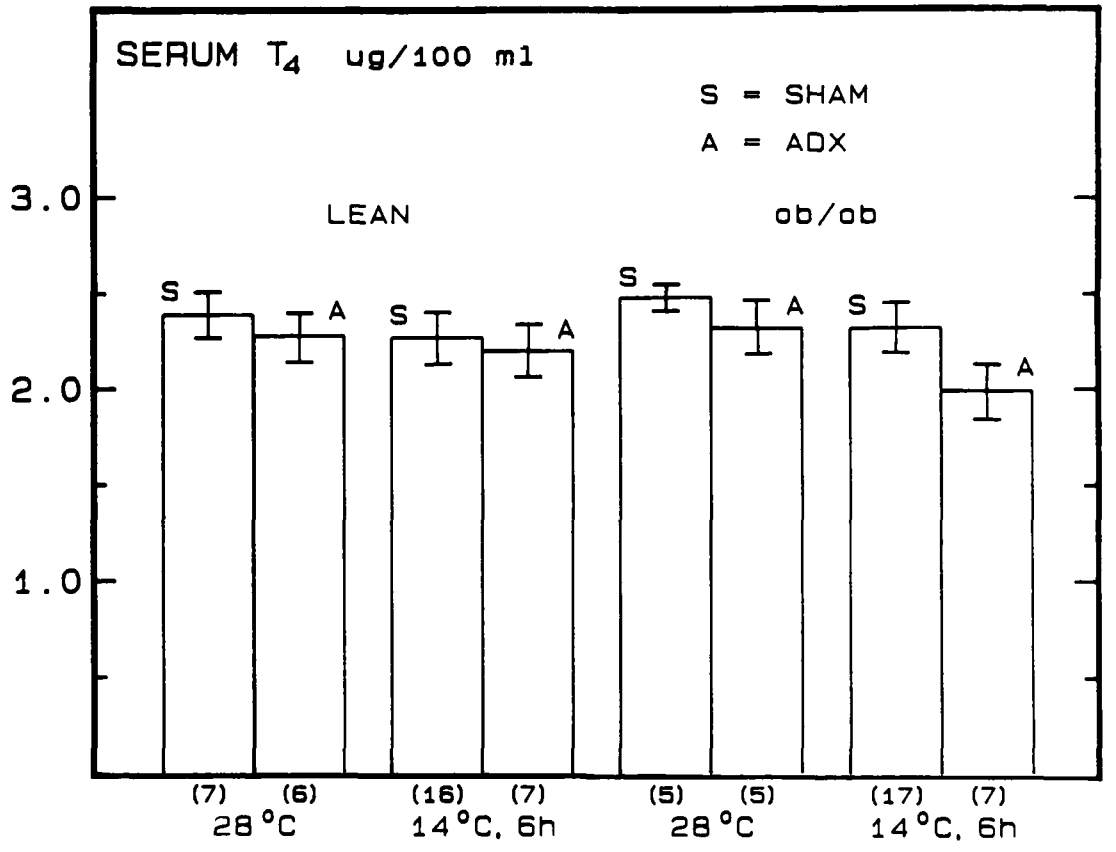


Figure 24: EFFECT OF ADX AND COLD EXPOSURE ON SERUM T_4 LEVELS IN LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 12. There was no significant effect of ADX, obesity or cold exposure on serum T_4.

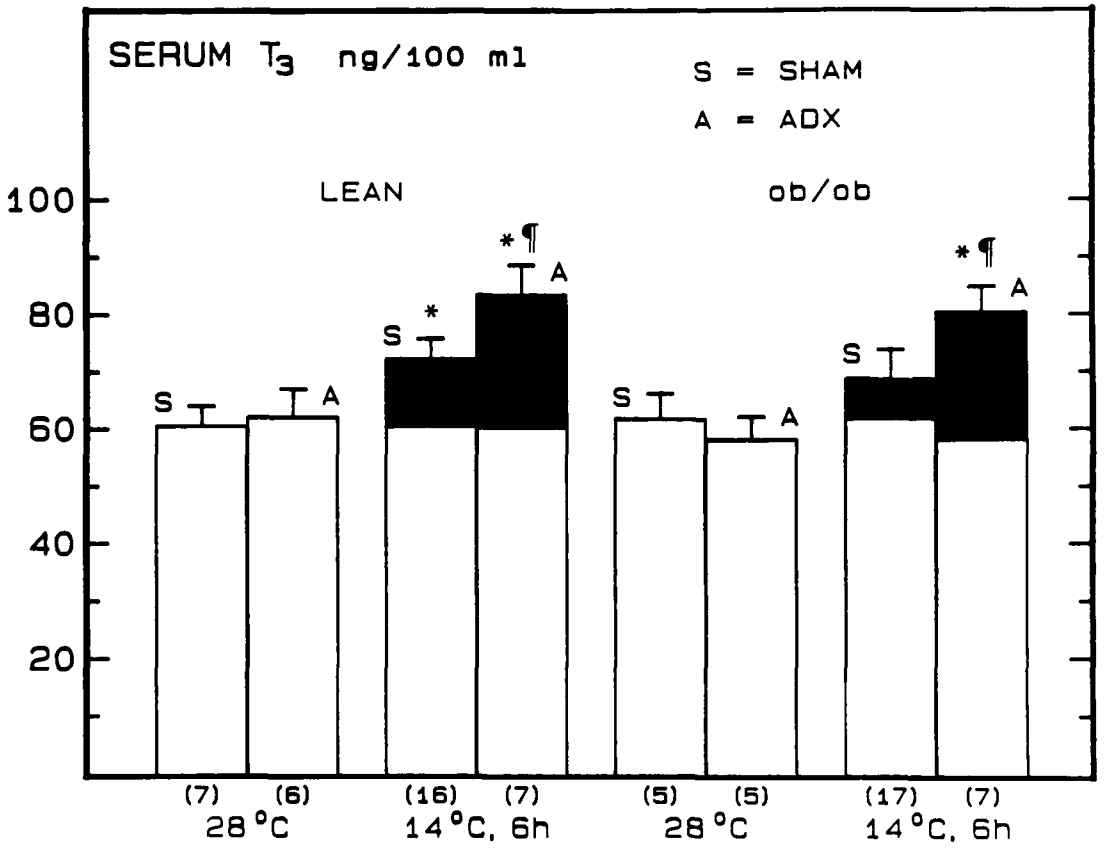


Figure 25: EFFECT OF ADX AND COLD EXPOSURE ON SERUM T_3 LEVELS IN LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 12. The symbols are as follows: * significant effect of cold, ¶ significant effect of ADX. The dark portions of the bars represent the increase due to cold exposure compared to 28 °C control animals. ADX normalized the response of ob/ob mice to cold exposure.

ob/ob mice at 28 °C. Cold exposure however, was able to significantly increase serum T_3 (Figure 25) and T_3/T_4 ratio (Table 10) in lean sham mice but not in ob/ob sham mice. Adrenalectomy was able to cause a small increase in serum T_3 and T_3/T_4 ratio in cold exposed lean and ob/ob mice and was also able to significantly increase T_3/T_4 ratio in ob/ob mice exposed to cold. Serum T_4 levels were not significantly altered by ADX or cold exposure in lean or ob/ob mice.

EFFECT OF ADX AND COLD EXPOSURE ON BAT:

Obese mice have more BAT wet weight than lean mice under all conditions studied (Table 11). The amount of BAT in lean mice was not significantly effected by ADX or cold exposure. Cold exposure does not affect the amount of BAT in obese mice but ADX caused a significant 15 percent reduction at 28 °C and a 50 % reduction in cold exposed ob/ob mice.

Obese mice have the same amount of BAT protein as lean mice at 28 °C. Adrenalectomy of lean 28 °C mice had no effect on BAT protein, whereas ADX was able to significantly increase BAT protein in 28 °C ob/ob mice by more than 50 %. Cold exposure had no significant effect on BAT protein of lean sham or ADX mice. However, cold exposure caused a 30 % increase in BAT protein of sham ob/ob mice but cold exposure was unable to further stimulate tha already high level of BAT protein in ob/ob ADX mice.

GDP-BINDING:

Obese mice at 28 °C have significantly lower GDP-binding values that lean mice. Adrenalectomy had no significant effect on GDP-binding of lean or ob/ob mice housed at 28°C. Cold exposure significantly

TABLE 11

EFFECT OF ADRENALECTOMY ON BAT OF LEAN AND OB/OB MICE

	LEAN				OB/OB			
	28° C		14° C for 6 h		28° C		14° C for 6 h	
(N)	SHAM (7)	ADX (6)	SHAM (16)	ADX (7)	SHAM (5)	ADX (5)	SHAM (17)	ADX (7)
BAT Wet Weight g	0 19 ± 0 01	0 17 ± 0 02	0 16 ± 0 01	0 16 ± 0 01	0 84§ ± 0 04	0 71¶§ ± 0 07	0 81§ ± 0 03	0 39¶§ ± 0 06
BAT PROTEIN mg	12 0 ± 0 4	11 1 ± 0 6	12 6 ± 0 5	12 5 ± 0 9	12 4 ± 0 8	18 9¶§ ± 0 8	16 1*§ ± 0 4	21 0¶§ ± 1 2
UCP µg/mg protein	6 33 ± 0 68	8 34 ± 1 45	5 44 ± 0 34	12 21*¶ ± 1 43	2 73§ ± 1 36	7 73¶ ± 1 86	3 69§ ± 0 44	16 87*¶ ± 2 45
T_4 5' D pmol/mg P/h	0 082 ± 0 008	0 131 ± 0 028	1 120* ± 0 111	0 784* ± 0 069	0 127§ ± 0 012	0 087 ± 0 007	0 316*§ ± 0 069	0 585*§ ± 0 066

Values are means ± SEM. Symbols are as follows: Significant effect of obesity §, significant effect of adrenalectomy ¶, significant effect of cold exposure *, ($p<0.05$). Statistics were measured using three-way ANOVA followed by Scheffe's post-hoc test.

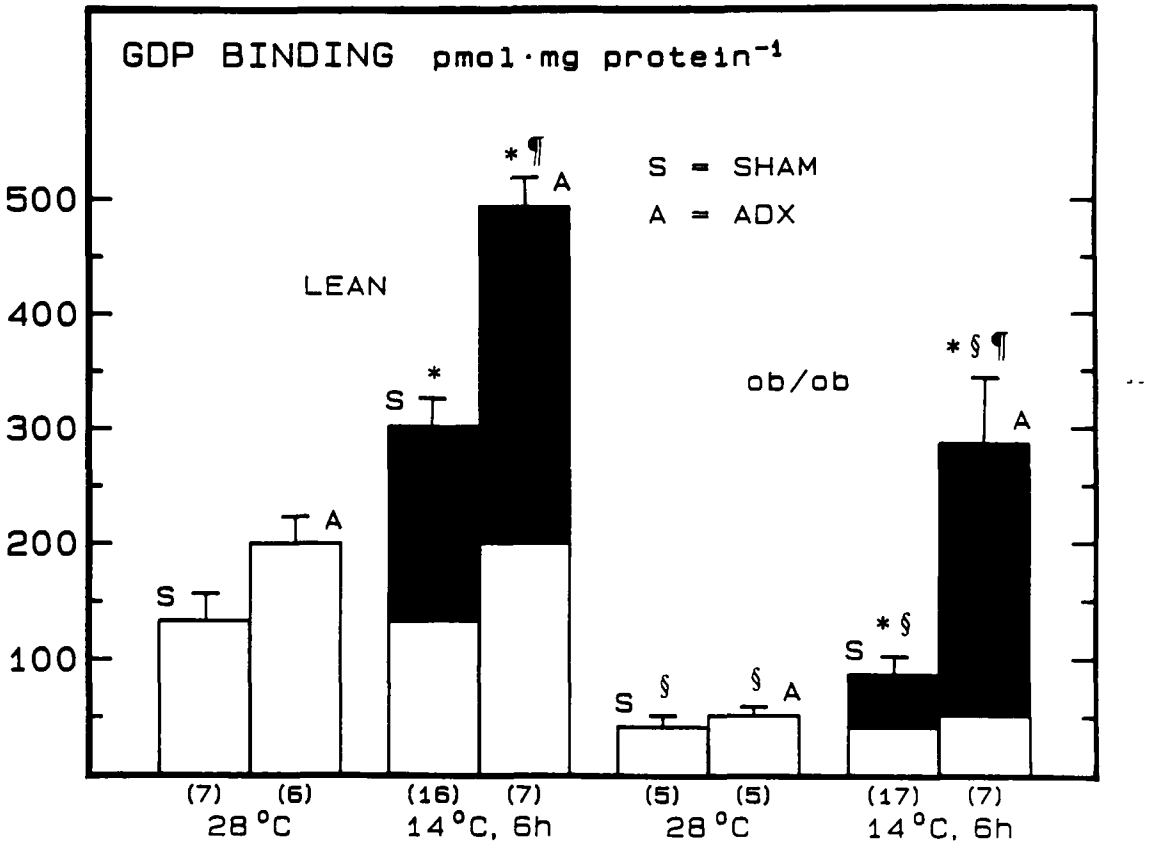


Figure 26: EFFECT OF ADX AND COLD EXPOSURE ON GDP-BINDING TO ISOLATED BAT MITOCHONDRIA OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 12. The symbols are as follows: * and the dark portions of the bars represent a significant effect of cold exposure, § a significant effect of obesity and ¶ a significant effect of adrenalectomy. ADX normalized the response of the ob/ob mouse to cold exposure.

increased GDP-binding in lean mice. Adrenalectomy caused a further stimulation of the cold stimulated increase in GDP-binding of lean mice (black bars, Figure 26). Cold exposure caused a small but significant increase in GDP-binding values of ob/ob mice but this increase was much smaller than that seen in lean mice (black bars. Figure 26). Adrenalectomy of obese mice enabled an increase in GDP-binding in response to cold exposure that was not significantly different from lean sham mice but was still less than the response of lean ADX mice.

UNCOUPLING PROTEIN (UCP):

Obese mice have 50 % less UCP than lean mice. Adrenalectomy had no significant effect on UCP of lean mice at 28 °C but was able to normalize UCP levels by a 3 fold increase in BAT UCP in ob/ob mice maintained at 28°C (Table 11 and Figure 27). Cold exposure of lean sham animals had no effect on BAT UCP. Adrenalectomy however, caused a doubling of BAT UCP levels in lean mice exposed to 14 °C for 6 hours. Cold exposure of ob/ob sham mice caused a small but significant increase in total BAT UCP but had no effect on the specific amount of BAT UCP. Adrenalectomy of cold exposed ob/ob mice caused a dramatic 10 fold increase in BAT UCP from basal 28 °C ob/ob sham levels. This increase was almost double that observed in lean ADX mice exposed to 14 °C for 6 hours (Figure 27 and Table 11).

THYROXINE 5'- DEIODINASE ACTIVITY:

Obese mice have significantly higher basal T5'D activity than lean mice (Figure 28 and Table 11). Adrenalectomy of lean and ob/ob mice at 28 °C does not significantly effect T5'D activity but the increase in basal activity observed in sham ob/ob mice was no longer significant.

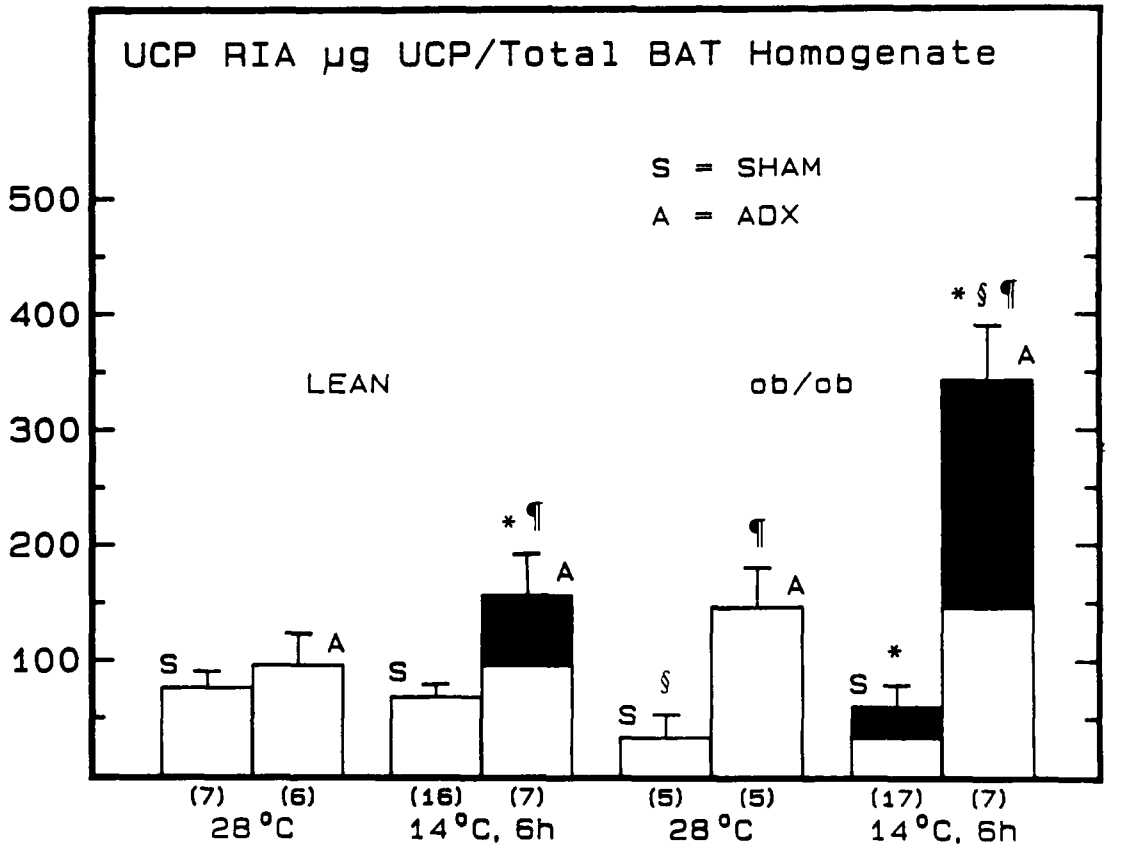


Figure 27: EFFECT OF ADX AND COLD EXPOSURE ON THE LEVEL OF UCP IN BAT HOMOGENATES OF LEAN AND OB/OB MICE. values are means ± SEM for the number (n) of animals in Table 12. The symbols are as follows: § a significant effect of obesity, ¶ significant effect of ADX and * and the dark portions of the bars represent a significant effect of cold exposure. ADX normalized the response of ob/ob mice to cold exposure.

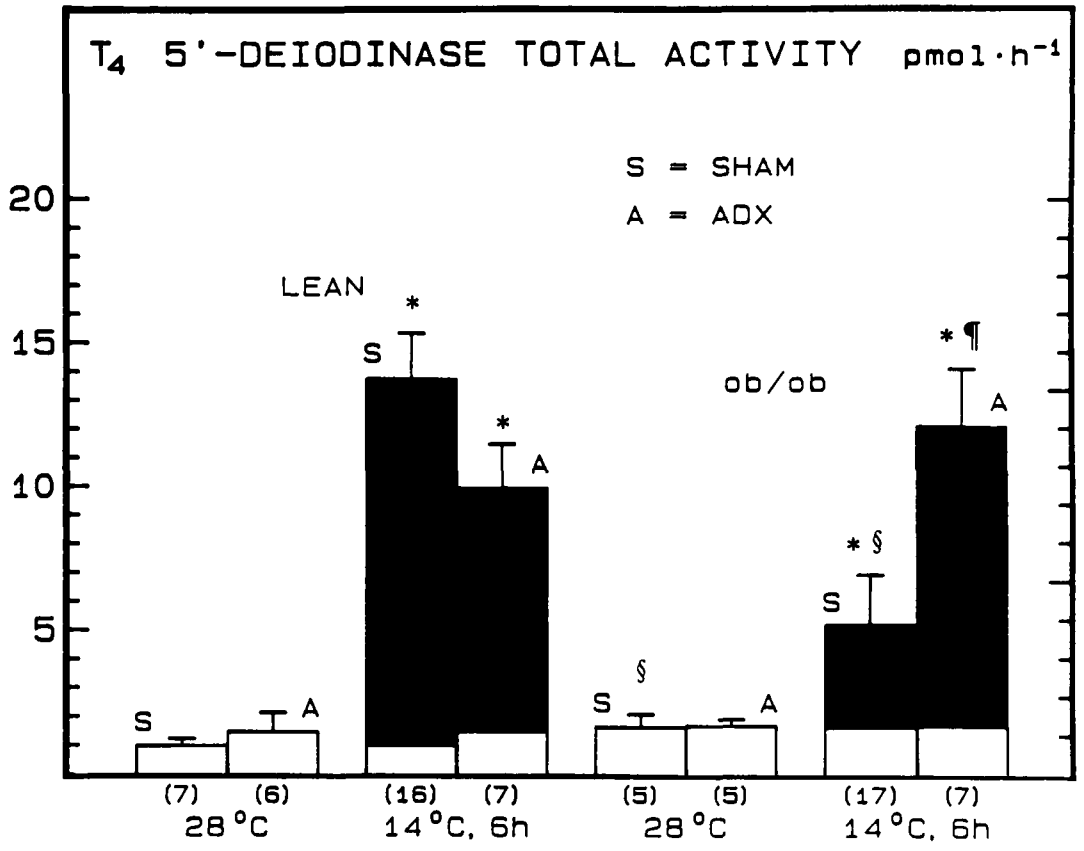


Figure 28: EFFECT OF ADX AND COLD EXPOSURE ON THE LEVEL OF BAT T5'D TOTAL ACTIVITY IN LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 12. The symbols are as follows: § a significant effect of obesity, ¶ a significant effect of ADX and * and the dark portions of the bars represent a significant effect of cold exposure. ADX normalized the response of ob/ob mice to cold exposure.

TABLE 12

EFFECT OF ADRENALECTOMY ON LEAN AND OB/OB MICE:

GRAPH DATA

	LEAN				OB/OB			
	28° C		14° C for 6 h		28° C		14° C for 6 h	
(N)	SHAM (7)	ADX (6)	SHAM (16)	ADX (7)	SHAM (5)	ADX (5)	SHAM (17)	ADX (7)
Body Temp °C	37 4 ± 0 2	36 9 ± 0 2	35 4* ± 0 2	35 4* ± 0 2	34 5§ ± 0 7	37 2¶ ± 0 2	24 8§* ± 1 1	31 4¶§* ± 1 1
Serum T_4 µg/dl	2 4 ± 0 1	2 3 ± 0 1	2 3 ± 0 1	2 2 ± 0 1	2 5 ± 0 1	2 3 ± 0 1	2 3 ± 0 1	2 0 ± 0 1
Serum T_3 ng/dl	60 5 ± 1 4	62 0 ± 4 0	72 7* ± 1 4	85 3*¶ ± 2 0	61 7 ± 2 2	56 1 ± 2 5	68 8 ± 2 6	80 4*¶ ± 2 6
GDP Binding pmol/mg Protein	133 1 ± 19 6	199 7 ± 15 3	302 0* ± 15 8	494 0*¶ ± 20 3	40 6§ ± 5 1	51 2§ ± 3 9	87 4§* ± 7 6	286 6§¶* ± 50 4
UCP RIA µg UCP/total homogenate	76 1 ± 9 0	95 9 ± 21 5	68 2 ± 5 4	156 4*¶ ± 28 6	32 9§ ± 15 6	145 7¶ ± 34 1	60 2* ± 7 6	343 3§¶* ± 39 5
T_4 5' D pmol/h	0 97 ± 0 10	1 45 ± 0 31	13 74* ± 1 22	9 94* ± 1 45	1 61§ ± 0 23	1 65 ± 0 12	5 18§* ± 1 22	12 08*¶ ± 1 34

Values are means ± SEM. Symbols are as follows: Significant effect of obesity §, significant effect of adrenalectomy ¶, significant effect of cold exposure *, ($p<0.05$). Statistics were measured using three-way ANOVA followed by Scheffe's post-hoc test.

Lean mice increase BAT T5'D total and specific activity 14 fold when exposed to 14 °C for 6 hours, while obese mice were capable of only a 3 fold increase in both total and specific activity. Adrenalectomy had no significant effect on the cold stimulated increase in BAT T5'D activity in lean mice but was able to significantly stimulate ob/ob BAT total T5'D activity 7 fold from basal values such that the absolute values of total and specific BAT T5'D activity were not significantly different from lean mice.

DISCUSSION:

Adrenalectomy significantly reduced but did not normalize the excessive body weight gain and obesity of ob/ob mice. This result is consistent with previous results from our laboratory (Tokuyama and Himms-Hagen, 1987) and those of others (Saito and Bray, 1984; Dubuc and Wilden, 1986 and Vander Tuig et al., 1984).

Adrenalectomy was able to normalize the body temperature of ob/ob mice maintained at 28 °C (Figure 23) and was able to significantly decrease the hypothermia characteristic of ob/ob mice exposed to cold. This result is consistent with the work of Tokuyama and Himms-Hagen (1987 and 1989) but disagrees with Saito and Bray (1984) who found no improvement by ADX of the hypothermia of ob/ob mice exposed to 6 °C for 30 minutes. Dubuc and Carlisle (1988) also found no significant effect of ADX on ob/ob mice exposed to 8 °C for up to 5 hours. It is possible that ADX ob/ob mice are able to tolerate mild cold (14 °C) but not more severe cold exposure.

The effects of ADX on the hypothermia of cold exposed ob/ob mice

have been found to be diet dependent (Kim and Romsos, 1987; Warwick and Romsos, 1988). Mice fed a high fat diet (66 % fat) were able to improve cold exposed hypothermia (4 °C for 2 hours) whereas ob/ob mice fed a stock diet show no effect of adrenalectomy on cold exposed hypothermia. The Purina chow #5012 used in our experiment is considered to be a high carbohydrate diet (53.2 % CHO) thus, our results do not agree. The reason for this is not clear but may be related to the relatively low housing temperature (23-25 °C) used by Romsos's group compared our housing temperature of 28°C.

At 28 °C ADX decreased the weight of BAT, presumably by decreasing lipid and increased total BAT protein by 52 % in ob/ob mice but had no effect on lean mouse BAT. The increase in ob/ob total BAT protein was accompanied by an increase in BAT UCP (Table 11 and Figure 27) whereas there was no significant change in BAT UCP of lean mice at 28 °C. Kim and Romsos (1988) also found no effect of ADX on the amount of BAT or on BAT protein in lean mice, however, they were unable to show an increase in BAT protein of ob/ob mice.

Adrenalectomy had no effect on GDP-binding to isolated BAT mitochondria of lean or ob/ob mice at 28 °C. This is consistent with the lack of effect of ADX on lean mouse BAT UCP However, ADX was able to normalize the level of UCP in BAT of the ob/ob mouse without significantly increasing GDP-binding to isolated BAT mitochondria at 28 °C. Kim and Romsos (1988) also saw no effect of ADX on BAT GDP-binding of lean and ob/ob mice fed a high-glucose diet at 23-25 °C but they did observe an increase in BAT GDP-binding in ob/ob mice fed a high starch diet at 23-25 °C. It is possible that the discrepancies in these

results could also be due to the differences in environmental temperature.

Adrenalectomy increased both GDP-binding and the amount of BAT UCP in lean mice exposed to cold. Thus it seems that corticosterone inhibits the thermogenic and trophic response of BAT to cold in lean mice and ADX removes this effect. Corticosterone treatment is known to inhibit BAT GDP-binding in lean mice (Tokuyama and Himms-Hagen, 1987 and 1989; Galpin et al., 1983). Bouillaud et al. (1988) have recently found several areas of the 5' region of the UCP gene that were partially homologous to previously described glucocorticoid regulatory elements, thus it is possible that corticosterone could have a direct effect on the expression of UCP.

I found that the improved hypothermia of the ADX cold exposed ob/ob mouse was accompanied by a marked improvement in BAT thermogenic activity. Cold exposed ADX ob/ob mice had greater BAT protein (Table 11), levels of BAT UCP (Table 11 and Figure 27) GDP-binding (Figure 26) and BAT T5'D activity (Table 11 and Figure 28) than sham operated control ob/ob mice exposed to 14 °C for 6 hours. Thus adrenalectomy was able to improve many of the defects of the cold exposed ob/ob mouse. The increase in BAT UCP in response to cold exposure was significantly greater than the response to cold exposure of lean mice. This illustrates that the defective thermogenic response of the ob/ob mouse does not lie in BAT itself but more likely in some hormonal or sensory mechanism since the ob/ob mouse is capable of a normal thermogenic response to cold exposure when adrenalectomized.

Vander Tuig et al., (1984) have shown that ADX was able to increase

the rates of noradrenaline turnover (NATO) more than 2 fold in BAT of ob/ob mice (fed Purina rodent chow) to rates nearly equal to those observed in lean mice without affecting NATO in BAT of lean mice maintained at 23-25 °C. This result explains why ob/ob mice at 28°C were able to increase BAT UCP levels whereas lean animals were not. However, an increase in NATO in ob/ob mice at 28 °C is not consistent with the lack of effect of ADX on GDP-binding and T5'D activity in ob/ob mice at 28 °C. The reason for the dissociation of increased sympathetic activity and increased GDP-binding and BAT T5'D activity is not clear, possibly some as yet unknown factor must also be present in order for NA to have a maximal effect. Noradrenaline turnover has not been measured in ADX cold exposed mice however one may speculate that ADX would further increase NATO in both lean and ob/ob mice since GDP-binding, BAT UCP levels and BAT T5'D activity are all increased further upon cold exposure.

Adrenalectomy had no significant effect on serum thyroid hormone levels of lean or ob/ob mice at 28 °C. This is consistent with a lack of effect of ADX on BAT T5'D activity at 28 °C. Kim and Romsos (1988) however, report that ADX ob/ob mice fed a high starch diet were able to increase plasma thyroxine levels but not T_3 levels whereas there was no effect of ADX on thyroid hormones of lean mice. Lean sham mice exposed to 14 °C for 6 hours were able to significantly increase serum T_3 levels whereas ob/ob mice were not. Adrenalectomy of cold exposed ob/ob mice normalized not only the increase in BAT T5'D activity but also the increase in serum T_3 (Figure 25).

The role of glucocorticoids in extrathyroidal production of serum

T_3 is complex. During the fetal and neonatal period glucocorticoids, T_4 and insulin must all be present for fetal mouse liver cells to maximally express Type I thyroxine 5'-deiodinase activity in culture (Sato et al., 1984). In the adult rat treatment with dexamethasone (Cavalieri et al., 1984) or corticosterone (McCann et al., 1984) caused a significant decrease in Type I T5'D activity in liver and kidney. In fact the stress-induced increase in corticosterone was also able to decrease Type I T5'D activity in both liver and kidney (Bianco et al., 1988). Glucocorticoids were shown to have no significant effect on Type II T5'D activity in pituitary (McCann et al., 1984) and in brown adipose tissue (Silva and Larsen, 1986b). Adrenalectomy was also shown to have no significant effect on BAT T5'D activity in rats (Silva and Larsen, 1986b). Thus our results agree with those of Silva and Larsen (1986b). However, Silva (1988) has shown that both NA and T_3 derived from BAT T5'D activity are necessary for maximal expression of BAT UCP. In this experiment 28 °C ADX ob/ob mice were able to significantly increase BAT UCP levels without an increase in BAT T5'D activity even in the presence of an increase in sympathetic activity. However, it must be noted that an even greater increase in BAT UCP levels was found when ADX ob/ob mice were exposed to cold and was accompanied by a normalization of BAT T5'D activity.

Adrenalectomy is known to decrease serum insulin and glucose in ob/ob mice (Wittmers and Haller, 1983; Tokuyama and Himms-Hagen, 1987 and Kim and Romsos, 1988) and is able to reverse insulin resistance in muscle from ob/ob mice (Ohshima et al., 1984). Thus an improvement in insulin sensitivity combined with an increase in sympathetic activity

may more fully explain the improved thermogenic response of BAT T5'D activity to cold exposure in ob/ob mice.

In conclusion, the following table summarizes the effects of ADX on lean and ob/ob mice at 28 °C and exposed to 14 °C for 6 hours:

	LEAN		OB/OB	
	28 °C	COLD EXP	28 °C	COLD EXP.
BODY WEIGHT	-	-	↓ IMPROVE	↓ NORM
WAT WEIGHT	-	-	↓	↓
BAT WEIGHT	-	-	↓	↓
BODY TEMP	-	-	↑ NORM	↑ IMPR
CORTICOSTERONE	↓	↓	↓	↓
T_3	-	↑	-	↑ NORM
GDP-BINDING	-	↑	-	↑ NORM
UCP	-	↑	↑ NORM	↑ NORM
T5'D ACT.	-	-	-	↑ NORM

thus ADX is able to improve (IMPR) or normalize (NORM) many of the defects of the ob/ob mice discussed in the introduction and in Chapter 1, Part 1, Experiment 2. This treatment demonstrated that the ob/ob mouse is capable of a normal thermogenic response to cold exposure. The mechanisms necessary for the increase in sympathetic activity, GDP-binding, BAT T5'D activity and BAT UCP synthesis are all in working order The defect in the ob/ob mouse seems to lie in the central sensing mechanisms for environmental temperature and could be related to an excessive sensitivity to corticosterone.

CHAPTER 1:

THE EFFECT OF COLD ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 2: TREATMENTS IMPROVING DEFECTIVE BAT T5'D ACTIVITY IN OB/OB MICE.

EXPERIMENT # 2: GRADUAL ACCLIMATION TO MILD COLD.

BACKGROUND AND OBJECTIVE:

Previous work in our laboratory (Hogan and Himms-Hagen, 1980) had shown that gradual acclimation of ob/ob mice to mild cold (14 °C) induces an almost normal tissue growth, GDP-binding to isolated BAT mitochondria, mitochondrial proliferation, and mitochondrial ultrastructure. The objective of this experiment was to examine the relationship between BAT T5'D activity and the improvement in thermogenic activity of gradually cold acclimated BAT of ob/ob mice.

METHOD:

Female C57BL/6J lean (+/?) and genetically obese (ob/ob) mice arrived at 7-8 weeks of age. They were individually caged and given Purina rodent chow #5012 ad libitum and free access to tap water. The mice were housed at 27 °C with a 12:12 lighting schedule with lights on at 07:00. This experiment was performed from Dec. 1985 to Feb. 1986. A group of 8 lean and 8 obese mice were gradually acclimated to cold by moving them to a 22 °C room for two weeks then the room was cooled to 18 °C and the animals were allowed to acclimate to that temperature for 2 weeks. Finally the animals were moved to a 14 °C cold room and again

2 weeks. Finally the animals were moved to a 14 °C cold room and again acclimated for 2 weeks. Food intake was measured every week, therefore there were 2 measurements made per mouse at each temperature. Body weights were measured at the beginning of the experiment at 27 °C, two weeks later at 22 °C, 2 weeks later at 18 °C, one week later at 14 °C and finally a measurement was made when the animals were killed, approximately one week later.

A group of 8 lean and 8 obese mice that had been housed at 27 °C for 6 weeks were exposed to 14 °C for 12 hours starting at 21:00. A 12 hour food intake was also measured for all animals. Control 27 °C, cold exposed (12 hours at 14 °C) and gradually cold acclimated animals were killed at 09:00 in the appropriate room as described previously. BAT homogenates and mitochondria were prepared as described in methods section # 5 and #6. Protein was estimated as described in section #7, GDP-binding was measured as described in section #8 and BAT T5'D activity was measured as described in section #15.

RESULTS:

FOOD INTAKE:

Control obese mice consume more total metabolizable food energy than control lean mice under all conditions studied. Gradual acclimation to cold caused a significant increase in food intake of lean mice 39 %, 55 %, 69 %, as the temperature was lowered. Obese mice however, did not significantly change their food intake compared to control ob/ob animals at any temperature. At 14 °C lean mice

TABLE 13

EFFECT OF GRADUAL COLD ACCLIMATION ON 24 HOUR FOOD INTAKE OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CONTROL 27 °C n = 16	ACCLIMATED n = 16	CONTROL 27 °C n = 14	ACCLIMATED n = 14
24 HOUR INTAKE (Kcal)				
TWO WEEKS AT:				
22 °C	11.3 ± 0.4	15.7* ± 0.4	20.0§ ± 0.4	22.1§ ± 1.0
18 °C	9.7 ± 0.6	15.0* ± 0.7	17.0§ ± 0.7	18.3§ ± 1.0
14 °C	10.9 ± 0.5	18.4* ± 0.7	18.1§ ± 1.2	16.2 ± 1.4

Values are means ± SEM for the number (n) of measurements per group. The symbols are as follows, * represents a significant effect of temperature and § represents a significant effect of obesity. Statistics were measured using repeated measures ANOVA followed by Tukey's HSD post-hoc test ($p < 0.05$).

acclimated to cold consumed the same metabolizable energy as obese mice at 27 °C and at 14 °C (see Table 13). The 12 hour food intake data (Table 14) also shows that lean acclimated mice consume more metabolizable energy than lean controls whereas acclimated ob/ob mice consume the same total Kcal as control ob/ob mice. Lean mice exposed to 14 °C for 12 hours almost double their food intake where as ob/ob mice reduce their food intake to less than half that of ob/ob control animals. Thus, gradual acclimation to cold was able to normalize the hyperphagia of ob/ob mice.

BODY WEIGHT GAIN, BODY WEIGHT, WAT WET WEIGHT AND BODY TEMPERATURE:

Gradual acclimation to cold caused a significant reduction in body weight gain (Figure 29) compared to control ob/ob mice and this was reflected in a significantly lower body weight at the end of the experiment (Table 14). Lean mice gradually acclimated to cold had a tendency towards higher body weight gain (Figure 29) but a significant effect was seen only at the end of the experiment (Table 14) where only a 12 % increase in body weight was observed. The increase in body weight seen in lean acclimated mice was accompanied by a 47 % decrease in the amount of gonadal WAT (Table 14). Obese mice have significantly more gonadal WAT at all times however, acclimation to cold also caused a decrease in the amount of gonadal WAT in ob/ob mice acclimated to cold. Acute exposure to 14 °C caused a small but significant decrease in body temperature in lean mice and in ob/ob mice, a significant hypothermia was observed. Gradual acclimation to cold allows lean mice to maintain a body temperature similar to 27 °C control animals whereas ob/ob mice are able to improve but not to normalize their body

TABLE 14

EFFECT OF COLD EXPOSURE AND COLD ACCLIMATION ON LEAN AND OB/OB MICE

	LEAN			OB/OB		
	CONTROL 27 °C n = 8	EXPOSED 14 °C n = 8	ACCLIMATED n 8	CONTROL 27 °C n = 7	EXPOSED 14 °C n = 7	ACCLIMATED n - 7
12 Hour Food Intake Kcal	7.1 ± 0.4	14.1* ± 0.9	10.4* ± 0.6	12.3§ ± 0.8	4.4§* ± 2.1	11.3 1.3
Body Weight g	20.1 ± 0.3	20.1 ± 0.5	22.5* ± 0.6	50.5§ ± 1.7	50.9§ ± 1.2	38.2§* ± 2.3
WAT Wet Weight g	0.32 ± 0.03	0.27 ± 0.04	0.17* ± 0.03	4.31§ ± 0.36	4.38§ ± 0.37	3.00§* ± 0.27
Body Temperature °C	36.9 ± 0.2	34.9* ± 0.5	36.7 ± 0.2	35.0§ ± 0.2	21.9§* ± 2.2	33.0§ * ± 0.6
BAT Wet Weight g	0.18 ± 0.01	0.16 ± 0.01	0.29* ± 0.02	1.52§ ± 0.12	1.30§ ± 0.07	1.23§ ± 0.08

Values are means ± SEM for the number (n) of animals per group. The symbols are as follows: * indicates a significant effect of treatment (cold acclimation or cold exposure) and § indicates a significant effect of obesity. Statistics were measured using two-way ANOVA followed by Tukey's HSD post-hoc test.

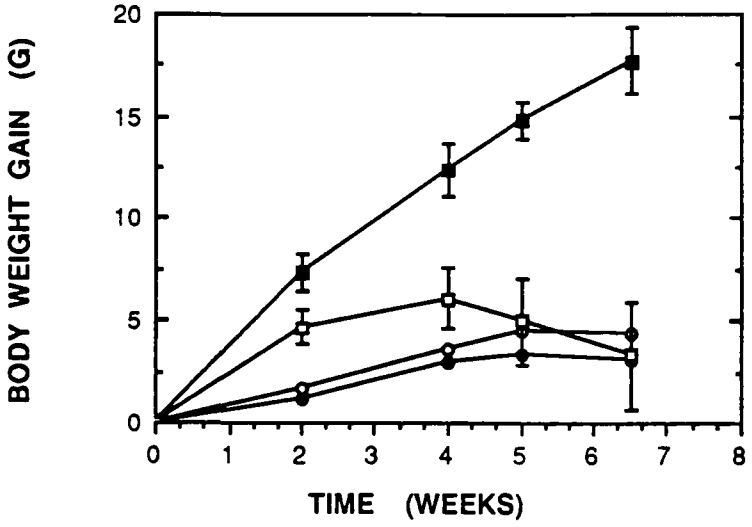


FIGURE 29: BODY WEIGHT GAIN PER WEEK OF GRADUALLY COLD ACCLIMATED LEAN AND OB/OB MICE. Body weight gain (per week) of control 27 °C ob/ob mice (n = 7. solid squares), cold acclimated ob/ob mice (n = 7, open squares), control 27 °C lean mice (n = 8, solid circles) and cold acclimated lean mice (n = 8 open circles). Cold acclimated ob/ob mice gain significantly less weight than control ob/ob mice from 2 weeks of exposure. The data represents means ± SEM for the number (n) of animals per group. The data was analyzed using 2-way ANOVA and Tukey's HSD post-hoc test ($p < 0.05$).

temperature at 14 °C if they are gradually cold acclimated.

EFFECT ON BAT:

Obese mice have more BAT than lean mice at all times studied. The loss of body weight and gonadal WAT observed in cold acclimated ob/ob mice was not accompanied by a change in the amount of BAT (Table 14).

Obese mice do not have significantly more BAT protein than lean mice at 27 °C. When lean mice are exposed to cold for 12 hours they significantly increase BAT protein 66 %, obese mice however, are unable to increase BAT protein in response to cold exposure. Cold acclimated lean mice are also able to increase BAT protein but not significantly more than cold exposure for 12 hours. In contrast, ob/ob mice are able to significantly increase BAT protein 3.8 fold to a value more than twice that of lean cold acclimated mice (Figure 30b). Thus gradual cold acclimation was able to promote growth of BAT in both lean and ob/ob mice.

GDP-BINDING:

Obese mice have significantly lower basal GDP-binding values than lean mice. When lean mice are exposed to cold for 12 hours they are able to increase GDP binding 180 % . This hign level of binding is maintained when lean animals are gradually acclimated to cold. In contrast obese mice were able to significantly double GDP-binding to isolated BAT mitochondria but the actual value achieved was only half the value of lean mice at 27 °C. In contrast when obese mice are acclimated to cold they are able to increase GDP-binding 10 fold

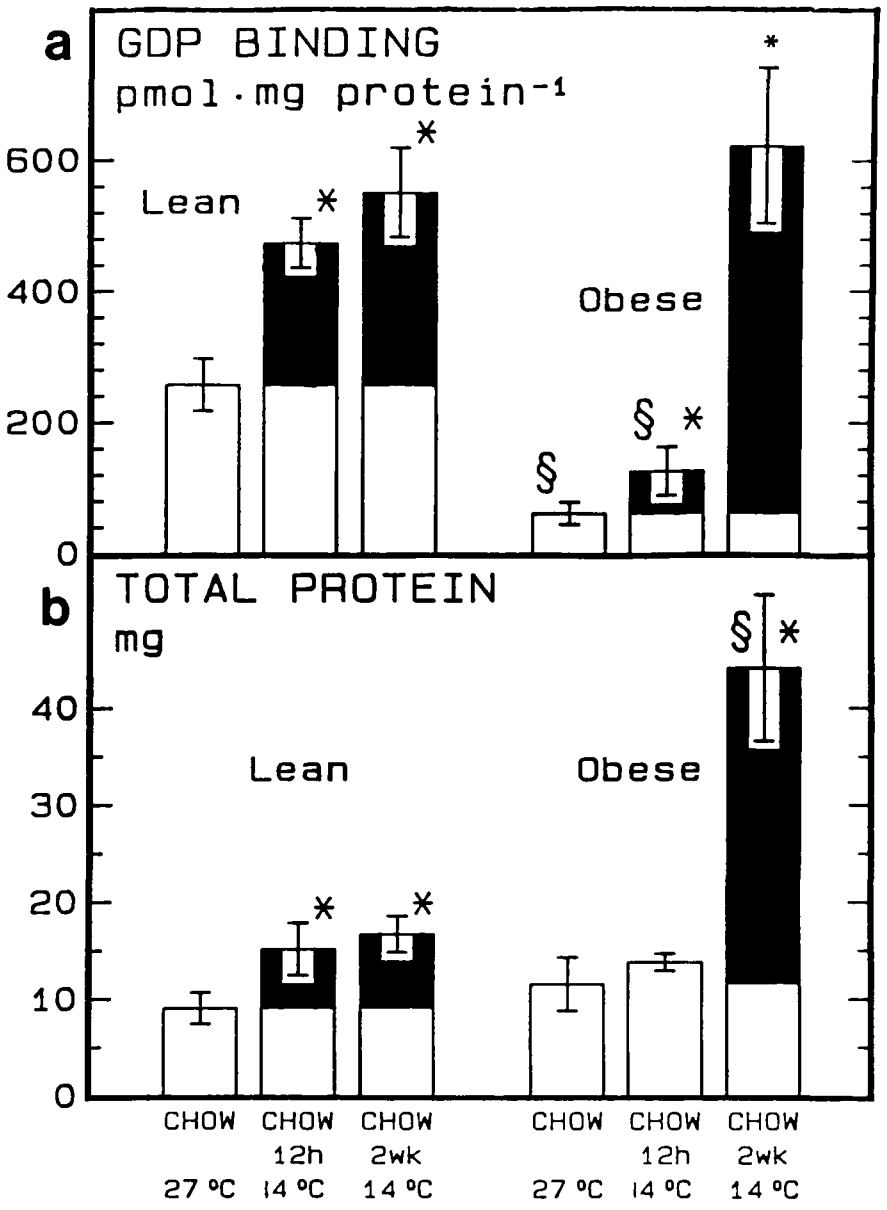


FIGURE 30: EFFECT OF COLD EXPOSURE AND COLD ACCLIMATION ON GDP-BINDING (30A) AND BAT TOTAL PROTEIN (30b) OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 15. Black portions of bars represent a significant increase due to treatment compared to same animal type control. Symbols are as follows: significant effect of treatment * and significant effect of obesity §.

compared to 27 °C controls. The value attained by the cold acclimated ob/ob mice was not significantly different from the increase in GDP-binding seen in lean cold acclimated mice. Thus gradual cold acclimation was able to normalize the increase in thermogenic state of BAT of the ob/ob mouse.

BAT T5'D ACTIVITY:

Obese mice have significantly higher basal BAT T5'D total activity (but not specific activity) than lean mice (Figure 31 a and b). When lean mice are exposed to 14 °C for 12 hours, a significant 12 fold increase was observed in specific activity. whereas a 20 fold increase was observed in total activity. This high level of BAT T5'D activity was not maintained in the lean animals that were gradually acclimated to cold. The specific activity was not significantly different from basal levels whereas the total activity was 2 fold higher that basal levels. In contrast, obese mice were unable to increase BAT T5'D specific activity in response to 12 hour cold exposure whereas they were able to significantly increase BAT T5'D total binding 2.5 fold. However. when obese mice are gradually acclimated to cold they are capable of increasing BAT T5'D specific activity 60 % and BAT T5'D total activity 6.5 fold (Figure 31 a and b).

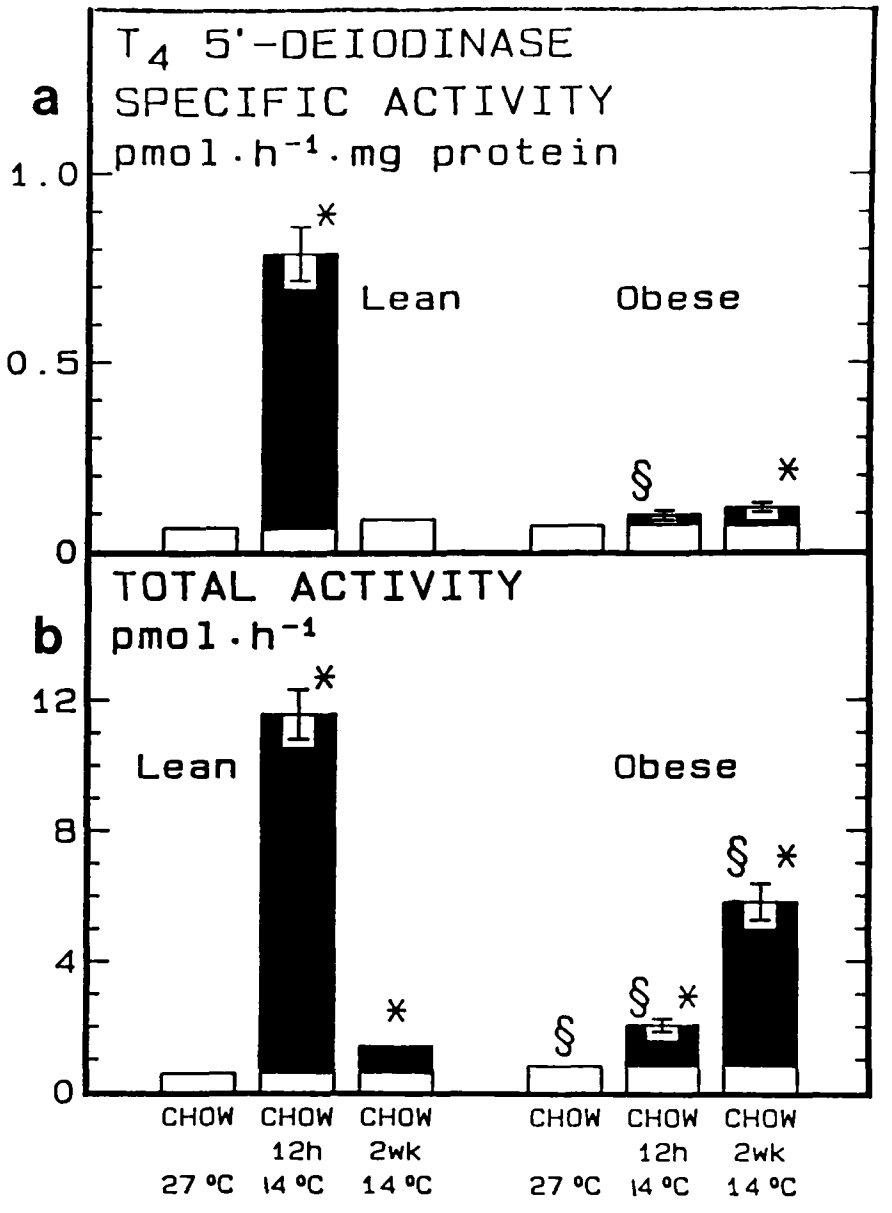


FIGURE 31: EFFECT OF COLD EXPOSURE AND COLD ACCLIMATION ON BAT T5'D SPECIFIC ACTIVITY (31a) AND TOTAL ACTIVITY (31b) OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Table 15. Black portions of bars represent a significant increase due to treatment compared to same animal type control. Symbols are as follows: significant effect of treatment * and significant effect of obesity §.

TABLE 15

EFFECT OF COLD EXPOSURE AND COLD ACCLIMATION ON LEAN AND OB/OB MICE: GRAPH DATA

	LEAN			OB/OB		
	CONTROL 27 °C n 8	EXPOSED 14 °C n = 8	ACCLIMATED n 8	CONTROL 27 °C n 7	EXPOSED 14 °C n = 7	ACCLIMATED n 7
GDP-Binding pmol/mg prot	257.1 ± 40.0	473.4* ± 37.8	551.5* ± 67.8	61.6§ ± 7.0	125.9§* ± 15.0	621.1* ± 72.5
BAT Protein mg	9.1 ± 0.6	15.1* ± 1.0	16.7* ± 0.7	11.5 ± 1.1	13.8 ± 0.4	44.1§* ± 3.1
T5'D Spec. Act. pmol/h/mg prot.	0.07 ± 0.01	0.84* ± 0.08	0.09 ± 0.03	0.06 ± 0.02	0.08§ ± 0.03	0.10* ± 0.01
T5'D Tot. Act. pmol/h	0.63 ± 0.08	11.51* ± 0.72	1.29* ± 0.09	0.92§ ± 0.05	2.28§* ± 0.11	5.98§* ± 0.75

Values are means ± SEM for the number (n) of animals per group. The symbols are as follows: * indicates a significant effect of treatment (cold acclimation or cold exposure) and § indicates a significant effect of obesity. Statistics were measured using two-way ANOVA followed by Tukey's HSD post-hoc test.

DISCUSSION:

The most important observation from this experiment was that gradual acclimation of obese mice to cold was able to improve the response of BAT T5'D activity to cold. The increase in BAT T5'D total activity (Figure 31 b) was due partly to a small but significant increase in BAT T5'D specific activity but was mostly due to the impressive increase in BAT growth in the ob/ob mouse in response to gradual cold acclimation as measured by an increase in total BAT protein (Figure 30 b).

Lean mice were able to double BAT T5'D total activity but this increase was relatively unimpressive compared to the stimulation elicited by cold exposure. In fact there was no significant difference between the T5'D specific activity of 27 °C control lean mice and cold acclimated lean mice. Thus the high levels of BAT T5'D activity induced by cold exposure (this experiment and Chapter 1, Part 1 Experiment #2) returns to near basal levels in lean mice after cold acclimation even if the animals continue to live at 14 °C. This observation is similar to that of Eley and Himms-Hagen (1989a) who acclimated lean mice to 14 °C for 2 weeks but is different from the persistent cold-induced elevation of BAT T5'D activity seen in rats and hamsters (Kopecky et al., 1986; Park and Himms-Hagen, 1988)).

The high level of BAT T5'D activity in the cold acclimated ob/ob mouse was accompanied by a normalization of GDP-binding levels (Figure 30a and Hogan and Himms-Hagen, 1980). In contrast, lean mice were able to maintain high levels of GDP-binding in the cold acclimated state without high levels of BAT T5'D activity. Eley and Himms-Hagen

(1989a) also observed persistently high levels of GDP-binding and serum T_3 accompanied with near basal levels of BAT T5'D activity in lean mice acclimated to 14 °C for 2 weeks. Thus, in the cold acclimated state, BAT T5'D activity is not the source of the elevated serum T3 found in lean mice (Eley and Himms-Hagen, 1989a).

The growth of BAT in cold acclimated lean and ob/ob mice (Figure 30b) measured by an increase in BAT protein in the cold acclimated state is known to be induced and maintained by the sympathetic nervous system (Himms-Hagen, 1986a). Sympathetic nervous system activity would be expected to remain elevated in BAT of individually caged lean and ob/ob mice living at 14 °C (Knehans and Romsos, 1982, 1983; Zaror-Behrens and Himms-Hagen, 1983 and Ashwell and Dunnett, 1985). This elevated SNS activity is reflected by high levels of GDP-binding (Figure 30a, Hogan and Himms-Hagen, 1980; Eley and Himms-Hagen, 1989a and Ashwell et al., 1983) and an increase in BAT UCP in lean mice (Ashwell et al., 1983; Desautels et al.,1986). I did not measure BAT UCP levels in cold acclimated ob/ob mice but if we assume an increase in GDP-binding can be positively related to an increase in BAT UCP (as seen in the adrenalectomy experiment) in the cold acclimated state we could predict that BAT UCP levels would have increased in the cold acclimated state.

Recent reports by Silva and co-workers have shown that the full expression of the uncoupling protein gene in response to acute stimulation requires the presence of both NA and T_3 produced from BAT T5'D activity (Silva, 1988). Noradrenaline is the primary signal which stimulates the transcription rate of the UCP gene and T_3 acts to

amplify 4-5 fold the response to NA but does not prolong the half life of the mRNA (Bianco et al., 1988). Recently, Nedergaard et al., (1989) have shown that the level of specific UCP mRNA in lean mice acclimated to cold for 3 months returned to near basal levels upon acclimation to cold. However, after about 2-3 days in the cold, there was an increase in the rate of degradation of UCP mRNA (Jacobsson et al.,1987) but there was also a significant increase in the total amount of RNA in the tissue, so that when the total amount of thermogenin mRNA was calculated a large increase was seen within the first 4 hours of cold exposure and this high level persisted throughout the time in the cold. Thus there seems to be a dissociation of BAT T5'D activity from the maintenance of elevated total UCP mRNA and UCP levels in BAT mitochondria of cold acclimated lean mice since the activity of BAT T5'D activity decreases 24 hours after cold exposure and is back to near basal levels in the cold acclimated state. Therefore it is possible that BAT T5'D activity is important in the acute activation of UCP mRNA but not in the acclimated state. However, T_3 is well known for its long term effects on increasing total RNA levels and protein synthesis in many tissues such as liver (Barsano and DeGroot, 1983). Other as yet unknown factors must alsb be important since cold acclimated rats with denervated BAT were able to increase BAT UCP levels without an increase in SNS activity or any activation of BAT T5'D activity (Park and Himms-Hagen, 1988).

However, in ob/ob mice the high levels of total BAT T5'D activity seem to be related to high levels of thermogenic activity (GDP-binding) and BAT growth (BAT protein). It is quite possible that the ob/ob

mouse has a much slower response to cold acclimation than lean mice; the response to cold acclimation was similar to the acute response of lean mice to cold, thus it is possible to speculate that had the ob/ob mice remained at 14 °C for perhaps 4 more weeks a similar response as that of lean mice might be expected. It is possible that the low thermogenic capacity and the atrophied state of BAT of ob/ob mice at 27 °C delays the response to cold exposure (see previous section) but upon gradual cold acclimation ob/ob mice are able to detect the environmental signals and increase SNS activity and BAT thermogenic function.

CONCLUSION:

Cold acclimation of ob/ob mice was able to improve cold induced hypothermia (Table 14 and Trayhurn and James, 1978), normalize food intake (Table 13) and body weight gain (Figure 29) and was able to supramaximally stimulate BAT growth (Figure 30b), GDP-binding (Figure 30a), and BAT T5'D total activity (Figure 31b). Thus the effects of cold acclimation are very similar to the effects of adrenalectomy on cold exposed ob/ob mice (see previous experiment). Cold acclimation is known to selectively increase the insulin-sensitivity of BAT in rats *in vivo* (Smith *et al.*, 1986; Vallerand *et al.*, 1989) and *in vitro* (Howland and Bond, 1987). The insulin sensitivity of BAT in ob/ob mice was also improved by cold acclimation thus resulting in a more normal response to their activated sympathetic nervous system (Seydoux *et al.*, 1982). These effects of cold acclimation and adrenalectomy demonstrate that BAT of the ob/ob mouse is capable of a normal thermogenic response under the appropriate conditions.

SUMMARY OF CHAPTER 1

Obese mice have the same or higher basal BAT T5'D activity depending on the time of year studied. This finding was also observed by Kaplan and Young (1987). The reason for the higher values found in the ob/ob mice is not clear but may be a long term adaptation to the hyperinsulinemic state of the ob/ob mouse.

Cold exposure causes a rapid increase in GDP-binding to isolated BAT mitochondria of lean mice within 1 hour reflecting unmasking of GDP-binding sites on BAT UCP since the amount of BAT UCP did not increase for at least 24 hours. The activity of BAT T5'D has a lag phase of approximately 1 hour before a dramatic increase in activity was observed peaking at 12-16 hours, decreasing at 24 hours and near basal levels in the cold acclimated state. From this data we observe that the acute unmasking of GDP-binding sites in lean BAT UCP does not require T_3 generation by BAT T5'D activity. However, growth of the tissue including an increase in BAT UCP occurs after the dramatic increase in BAT T5'D activity in lean mice thus, T_3 may be necessary for this process. When BAT has reached its maximum growth and thus level of BAT UCP, the activity of BAT T5'D in lean mice drops to near basal levels. This implies that BAT T5'D activity and thus a continuous supply of T_3 is not necessary for the maintenance of high levels of BAT UCP. This could be partly due to the long half life of the UCP in the cold acclimated state (Nedergaard *et al.*, 1989; Park and Himms-Hagen, 1988).

In contrast, the ob/ob mouse does not increase GDP-binding to isolated BAT mitochondria for at least 6 hours upon cold exposure. The small increase in GDP-binding observed in ob/ob mice most likely represents unmasking of binding sites since there was no significant increase in BAT UCP for at least 24 hours of cold exposure. This data shows that ob/ob mice are capable of unmasking binding sites on BAT UCP. The activity of BAT T5'D in ob/ob mice remains near basal levels for at least 6 hours of cold exposure. After this time a variable response in activity was noted and seemed to be related to the central sensing mechanisms controlling the ability to maintain body temperature. This data is consistent with the idea that ob/ob mice are refractory to the normal NATO observed in the cold (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983) and that both a normal response to noradrenaline and levels of BAT T5'D activity would be necessary to see an increase in BAT UCP levels.

Two treatments: adrenalectomy and gradual acclimation to cold known to be able to improve the sensitivity of ob/ob BAT to NA and insulin were able to increase BAT T5'D activity, BAT growth and levels of BAT UCP These two treatments demonstrate that ob/ob BAT is capable of a normal thermogenic and trophic response to cold. A defect in the central sensing mechanism of this environmental stimulus seems to be the most likely "primary" defect of the ob/ob mouse.

The next chapter will study another stimulus known to activate BAT thermogenesis, namely diet.

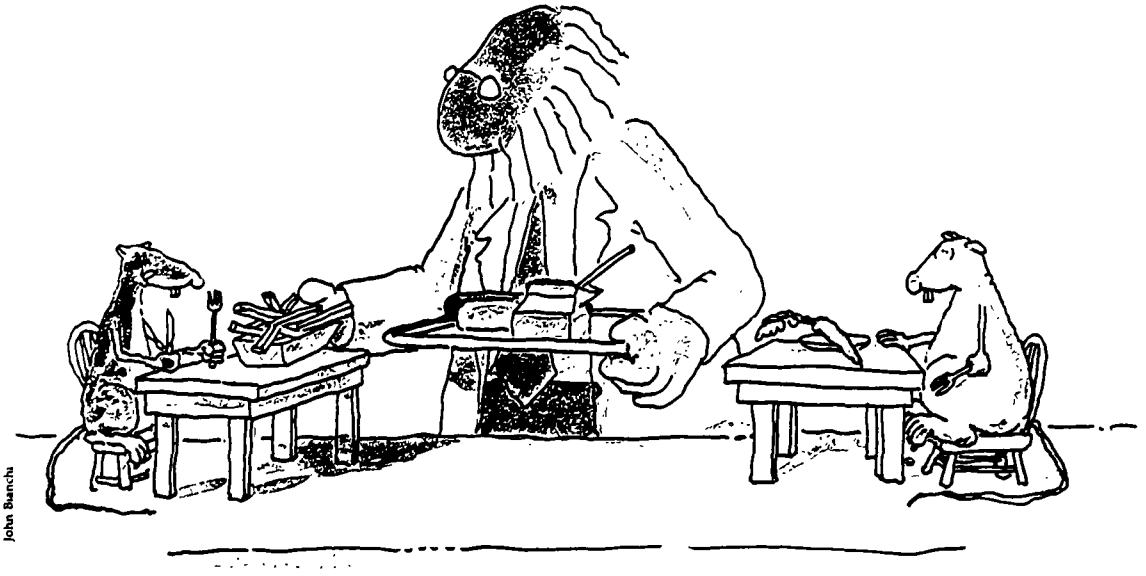
CHAPTER 2:

EFFECT OF DIET ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 1: EFFECT OF FEEDING A HIGH FAT "CAFETERIA" DIET TO LEAN AND OB/OB MICE.

PART 2: EFFECT OF FEEDING A HIGH CARBOHYDRATE DIET (SUCROSE) DIET TO LEAN AND OB/OB MICE.

PART 3: EFFECT OF FASTING AND FOOD RESTRICTION ON LEAN AND OB/OB MICE.



CHAPTER 2:

EFFECT OF DIET ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 1: EFFECT OF FEEDING A "CAFETERIA" DIET ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

BACKGROUND:

The two major stimuli of BAT thermogenesis are exposure to cold and feeding a palatable high fat known as the "cafeteria diet". In chapter 1, the stimulatory effect of cold on BAT T5'D activity was discussed. In this chapter I will explore the possibility that feeding a cafeteria diet may also have a similar stimulatory effect on BAT T5'D activity in lean and ob/ob mice. This is plausible because both cold and diet are thought to stimulate BAT through similar mechanisms, (Rothwell and Stock, 1980) namely the action of noradrenaline on β-adrenergic receptors in BAT (see introduction for more details). Previous work in this laboratory (Himms-Hagen et al., 1986) has shown that feeding a palatable cafeteria diet to ob/ob mice was able to increase BAT noradrenaline turnover, GDP-binding to isolated BAT mitochondria and increase the characteristically low body temperature of ob/ob mice to near normal levels. The involvement of BAT T5'D activity in the stimulation of BAT metabolism in response to feeding the cafeteria diet had not been previously studied.

OBJECTIVE:

The objective of this experiment was to study the effect of feeding a "cafeteria" diet on BAT T5'D activity in lean and obese (ob/ob) mice.

METHODS:

Female C57BL/6J lean (+/?) and obese (ob/ob) mice arrived at 6 weeks of age and were housed in like pairs at 27 °C with a 12 hour light/dark cycle with lights on at 6:00 am. They were fed Purina rodent chow #5012 and tap water ad libitum. At 9 weeks they were housed 4 like animals per cage. One-half of the animals were offered a palatable cafeteria diet at 2 pm every day for 3 weeks. The control animals also received chow at this time. The cafeteria diet was fed on a rotating schedule of 3 menus:

Menu I: Processed cheddar cheese, pecans (unsalted), short bread cookie, chocolate and chow.

Menu II: Processed swiss cheese, almonds (unsalted), chocolate chip cookie, chocolate and chow.

Menu III: Cheddar cheese, walnut (unsalted), oatmeal cookie, chocolate and chow.

Food intake was measured twice a week in order to study the food intake of each menu twice. Digestible energy content and nutrient composition of the various foods was obtained from food tables (Pennington and Church, 1980). Body weight was also measured twice a week and body weight gain was calculated.

The animals were killed at 12 weeks of age at 9:00 am by cervical dislocation, blood was collected, rectal temperatures were measured and gonadal fat was removed cleaned and weighed. Interscapular and subscapular BAT was removed, cleaned, and weighed. BAT homogenates were prepared first using a glass-teflon homogenizer and then with an all-glass homogenizer. Homogenates were frozen in liquid nitrogen and stored at - 70°C. The BAT T5'D assay was measured as described in methods section # 15. Serum thyroid hormones were measured using commercially available kits as described in methods section #13.

RESULTS:

Food Intake:

Ob/ob mice are hyperphagic and consume more total metabolizable energy than lean mice regardless of the type of diet (Table 16). Animals fed a cafeteria diet did not consume more total energy than animals fed the chow diet. However, both lean and ob/ob mice did change the composition of their diet. The cafeteria diet selected by both lean and ob/ob mice was low in protein and carbohydrates with a much greater portion of their diet derived from fat. The fat was mostly saturated animal fat (cheese, chocolate and cookies) with some polyunsaturates derived from nuts.

Weight Gain and Gonadal WAT Weight.

Ob/ob mice weigh more than lean mice. Feeding a cafeteria diet caused an increase in final body weight in both lean and ob/ob mice (Table 17). This greater final body weight was a result of an increased rate of body weight gain in both lean and ob/ob mice (Figure 32).

TABLE 16

EFFECT OF CAFETERIA FEEDING ON FOOD INTAKE OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CHOW n=12	CAFETERIA n=12	CHOW n=12	CAFETERIA n=12
Total Kcal	51.0 ± 3.0	55.3 ± 1.8	77.3§ ± 5.2	84.1§ ± 3.0
Protein Kcal	13.4 ± 0.8 (26.2 %)	6.5* ± 0.4 (11.7 %)	20.3§ ± 1.4 (26.2 %)	10.4§* ± 1.0 (12.4 %)
CHO Kcal	31.6 ± 1.9 (62.0 %)	15.7* ± 1.2 (28.4 %)	47.9§ ± 3.3 (62.0 %)	19.1* ± 1.8 (22.7 %)
Fat Kcal	6.0 ± 0.4 (11.8 %)	33.2* ± 1.6 (60.0 %)	9.1§ ± 0.6 (11.8 %)	54.6§* ± 3.4 (65.0 %)

Food intake was measured twice a week such that each of 3 menus were measured twice. Values are means ± SEM of 6 measurements for two cages of animals per group. * indicates a significant effect of cafeteria diet and § indicates a significant effect of obesity. The percentages in brackets are calculated using total Kcal as 100 %. The statistics were measured using a two-way ANOVA followed by Tukey's HSD post-hoc test.

TABLE 17

EFFECT OF CAFETERIA FEEDING ON BODY WEIGHT, GONADAL WAT, BODY TEMPERATURE, BAT WET WEIGHT AND PROTEIN OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CHOW n=8	CAFETERIA n=8	CHOW n=8	CAFETERIA n=8
Final Body Wt.	20.6 ± 0.5	25.2* ± 1.1	51.6§ ± 1.3	58.5§* ± 1.4
Gonadal WAT g	0.44 ± 0.05	1.10* ± 0.17	5.09§ ± 0.27	6.33§* ± 0.22
Rectal Temp °C	36.9 ± 0.3	37.5 ± 0.1	33.5§ ± 1.5	37.1* ± 0.2
BAT Wet Weight g	0.25 ± 0.01	0.23 ± 0.02	1.35§ ± 0.14	1.63§ ± 0.19
Total BAT Protein mg	9.3 ± 0.8	10.5 ± 1.0	13.9§ ± 1.7	16.4§ ± 1.9

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of cafeteria diet and § indicates a significant effect of obesity. The statistics were measured using two-way ANOVA followed by Tukey's HSD post-hoc test.

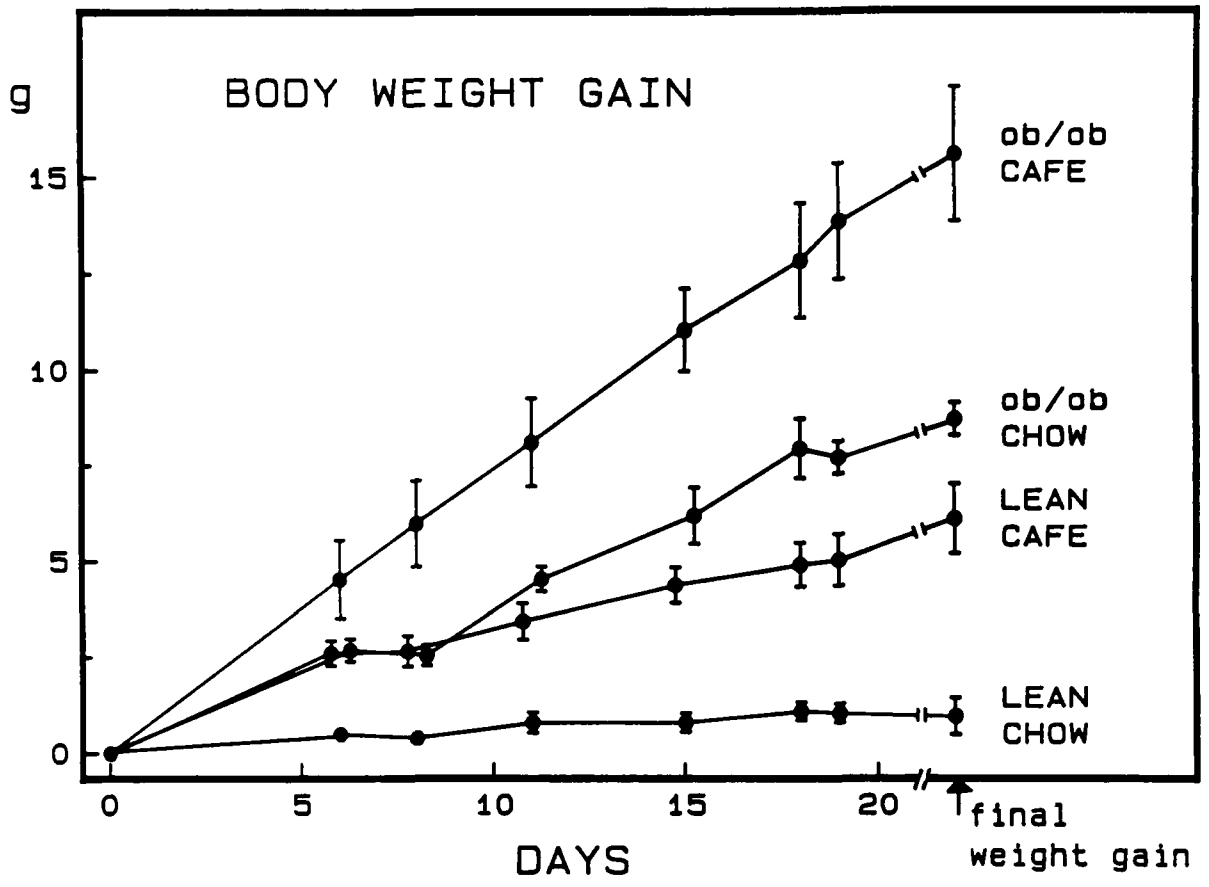


Figure 32: EFFECT OF FEEDING A CAFETERIA DIET ON BODY WEIGHT GAIN OF LEAN AND OB/OB MICE. Both lean and ob/ob mice gained significantly more body weight than their chow fed counter parts from the first measurement made at 6 days. The values are means ± SEM for n = 8. The statistics were measured by repeated measures ANOVA.

Both lean and ob/ob mice increased the amount of gonadal WAT indicating that the fat from the "cafeteria diet" was stored as adipose tissue.

Body Temperature

Obese (ob/ob) mice have characteristically lower body temperatures than lean mice when fed chow (Table 17). Feeding a cafeteria diet significantly increases the body temperature of ob/ob mice to the same level as that of lean mice. Feeding a cafeteria diet does not affect body temperatures of lean mice.

BAT Wet Weight and Total BAT Protein:

Ob/ob mice have a greater amount of BAT and total BAT protein than lean mice. Feeding a cafeteria diet did not significantly increase BAT wet weight or BAT total protein in lean or ob/ob mice (Table 17).

BAT T5'D Activity:

Ob/ob mice have higher basal total T5'D activity than lean mice. Feeding a cafeteria diet to lean and ob/ob mice for 3 weeks had no effect on BAT T5'D activity (Figure 33).

Serum Thyroid Hormones:

There was no significant difference in serum T_3, T_4 or $T3/T_4$ ratio between lean and ob/ob mice fed chow. Cafeteria feeding tended to increase serum T_3 levels but not significantly in both lean and ob/ob mice. Cafeteria feeding did not affect serum T_4 levels in lean mice but caused a significant decrease in serum T_4 in ob/ob mice which resulted in an increase in T_3/T_4 ratio (Table 18).

TABLE 18

EFFECT OF CAFETERIA FEEDING ON SERUM THYROID HORMONE LEVELS OF LEAN AND OB/OB MICE

	LEAN		OB/OB	
	CHOW n=8	CAFETERIA n=8	CHOW n=8	CAFETERIA n=8
T_3 ng/dl	67.4 ± 7.0	87.4 ± 4.3	82.5 ± 5.4	99.3 ± 10.0
T_4 µg/dl	7.2 ± 0.7	6.5 ± 0.5	7.2 ± 0.2	4.4 §* ± 0.4
T_3/T_4 %	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	2.3 §* ± 0.3

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of cafeteria diet and § indicates a significant effect of obesity. The statistics were measured by two-way ANOVA followed by Tukey's HSD post-hoc test.

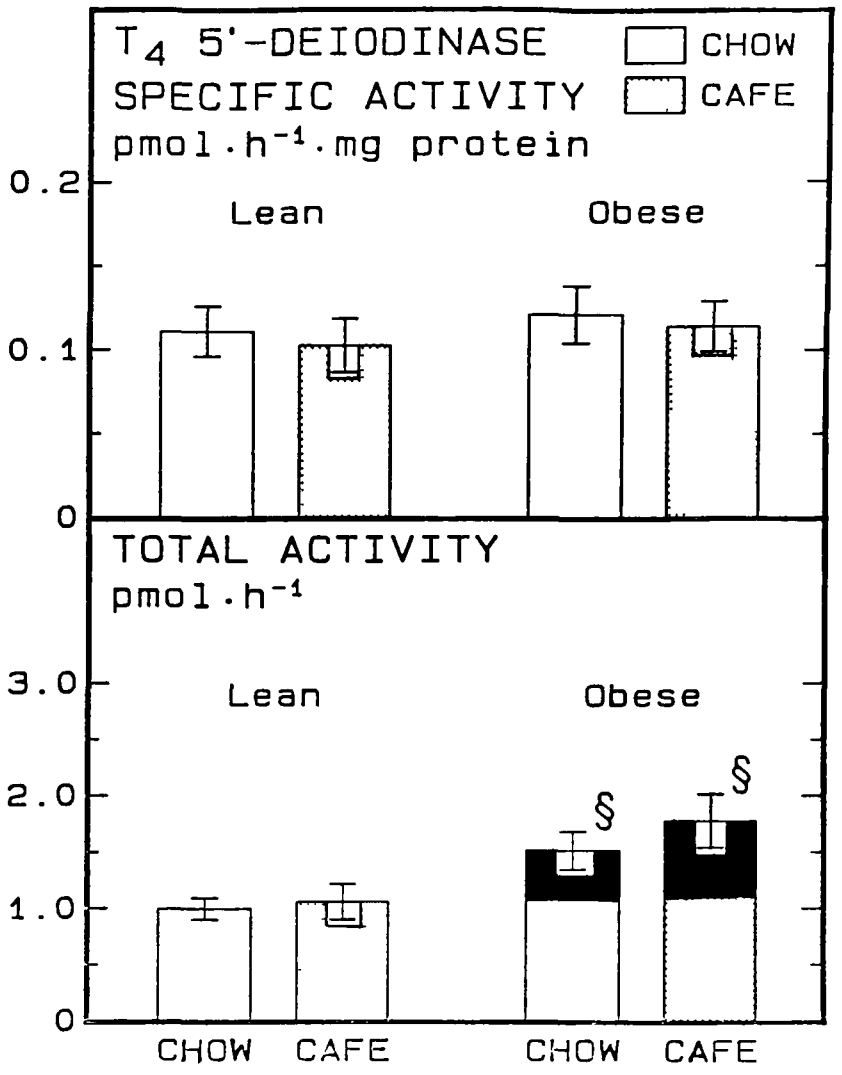


Figure 33: EFFECT OF A CAFETERIA DIET ON BAT T5'D SPECIFIC AND TOTAL ACTIVITY IN LEAN AND OB/OB MICE. There was no effect of diet or obesity on BAT T5'D specific activity (33a) and there was no significant effect of diet on BAT T5'D total activity (33b). There was a significant effect of obesity on total activity which was represented by the darkened portions of the bars and the symbol §. The statistics were measured by two-way ANOVA followed by Tukey's HSD post-hoc test.

DISCUSSION:

The most important finding from this study was that unlike the impressive stimulatory effect of cold on BAT T5'D activity seen in Chapter 1. there was no effect of feeding a palatable cafeteria diet for 3 weeks on BAT T5'D activity of lean or ob/ob mice (Figure 33). This result is in agreement with Wu et al. (1987) who studied the effect of a 3 week cafeteria diet on lean and obese Zucker rats and also found no effect of the diet on BAT T5'D activity. Previous work in our laboratory had shown an increase in noradrenaline turnover in ob/ob mice fed a cafeteria diet for 1 week and an increase in GDP-binding to isolated BAT mitochondria in both lean and ob/ob mice fed a cafeteria diet for 3 weeks, which suggests that the cafeteria diet does have a stimulatory effect on BAT of lean and ob/ob mice (Himms-Hagen et al., 1986). Subsequent work in our laboratory using the same dietary protocol but studying BAT thermogenesis at various times during the day showed no effect of the cafeteria diet on BAT T5'D activity in lean mice at any time of the day studied (Eley and Himms-Hagen, 1988b). However, cafeteria feeding increased GDP-binding to isolated BAT mitochondria at most times of the day studied in lean mice indicating an activation of BAT thermogenesis. Thus as with cold acclimation we see a dissociation between T5'D activity and GDP-binding in lean mice and unlike cold acclimation, we also see the same dissociation in ob/ob mice.

I did not find a statistically significant increase in serum T_3 in lean or ob/ob mice at 9:00 am. Eley and Himms-Hagen, (1988b), did not find a significant increase in serum T_3 in the morning, confirming our

finding, however, they did find a significant increase in serum T_3 at all other times of the day studied in lean mice. They also found no effect of cafeteria feeding on serum T_4 levels in lean mice. I find that there was a significant decrease in serum T_4 in ob/ob mice in response to a cafeteria diet. This decrease could be caused by a decrease in secretion of T_4 from the thyroid gland, an increase in clearance of T_4, or an increase in deiodination elsewnere.

Recent studies have implicated liver (Ma et al., 1987) and not BAT (Ma et al., 1988) as the major site of DIT in cafeteria-fed rats. The evidence for this finding consists of the observations that partial hepatectomy effected a much larger reduction in the resting oxygen consumption of cafeteria-fed rats than that of their chow fed counterparts, and substantially reduced the characteristic inhibitory effect of propranolol on the elevated metabolic rate of the cafeteria fed rats. It is possible that the source of the increase in serum T_3 could be deiodination in the liver. Hillgartner and Romsos (1985), have noted a significant decrease in liver T_4 5'-deiodinase in ob/ob mice compared to lean mice at 25 °C. However the effect of feeding a cafeteria diet on liver T5'D actvity in lean and ob/ob mice has not been studied.

The cafeteria diet usually describes a diet low in protein and carbohydrate and high in fat (see Table 16). Rats fed low protein diets are able to increase heat production and increase noradrenaline turnover in BAT (Kevonian et al., 1984). Hillgartner and Romsos (1987), compared kinetic parameters of iodothyronine 5'-deiodination in hepatic and renal microsomes in rats fed low protein diets. They found

that a low protein diet had little effect on either the liver or the kidney iodothyronine 5'-deiodinase in spite of an increase in serum T_3 with no change in serum T_4.

Thus, the relationships between an increase in serum T_3, an increase in noradrenaline turnover in BAT and changes in peripheral deiodination are very complex. Possibly the time I and others have chosen to study the effect of diet on T5'D activity was inappropriate. The acute effect of cold on BAT T5'D activity was most evident between 12-16 hours after cold exposure and upon cold acclimation there was no stimulatory effect evident in lean mice. Possibly by studying BAT T5'D activity 3 weeks after the feeding regime has started I have overlooked an effect that may have occurred earlier in the study. It is also possible that noradrenaline is not the only factor that is involved in the cold stimulated increase in BAT T5'D activity, possibly some other factor must be present with noradrenaline in order for complete stimulation to occur.

Feeding a cafeteria diet, however is able to improve many abnormalities of the genetically obese mouse (Himms-Hagen et al., 1986). This improvement is not related to an increase in BAT T5'D activity under the conditions of this study.

Unlike rats, lean and obese C57BL/6J mice do not increase their energy intake when offered a "cafeteria" high fat diet (Table 16; Eley and Himms-Hagen, 1988b; Himms-Hagen et al., 1986). They change the composition of their diet such that the proportion of their diet derived from protein and carbohydrate are reduced whereas fat comprises 60-65 % of the diet. Richard et al., (1988) fed albino mice a high fat

diet consisting of 2 parts peanut butter and one part vegetable fat shortening supplemented with vitamins and minerals such that the diet was 66 % fat. Mice fed this diet increased their energy intake 45 % . The authors suggest that the degree of hyperphagia is important in the development of adaptive DIT. Thus there are strain differences in the degree of hyperphagia induced by a high fat diet in mice.

There are many disadvantages in using a cafeteria diet to study diet induced thermogenesis (Moore, 1987). The animals are allowed to select their diet from a number of palatable foods that are varied to encourage the animals to overeat. A balanced diet (in our case Purina rodent chow #5012) is available but the animals are not usually interested and consume very few calories from this source. This leads to a diet that is high in fat, low in protein and carbohydrates and in some cases deficient in vitamins and minerals. Individual animals may also have specific food preferences, thus, each animal may not be consuming the same diet, causing variation in the data collected.

The next section (Part 2), will attempt to address some of these problems and improve the design of the study of the effect of diet on BAT T5'D activity in lean and ob/ob mice.

CHAPTER 2:

EFFECT OF DIET ON BAT T5'D ACTIVITY OF LEAN AND OB/OB MICE.

PART 2: EFFECT OF FEEDING A HIGH CARBOHYDRATE (SUCROSE) DIET ON BAT T_4 5'-DEIODINASE ACTIVITY IN LEAN AND OB/OB MICE.

BACKGROUND:

In rats, feeding a high carbohydrate diet is known to increase: the noradrenaline stimulated increase in oxygen consumption (Rothwell, et al., 1981), BAT noradrenaline turnover (Walgren et al., 1987; Young et al., 1982), forskolin stimulated BAT adenylate cyclase activity (Scarpace et al., 1987), and GDP-binding to isolated BAT mitochondria (Rothwell and Stock, 1984). Thus, a high carbohydrate diet has a stimulatory effect on BAT of the rat. Knehans and Romsos (1984) fed lean and ob/ob mice a high carbohydrate diet composed of free access to chow and a 30 % sucrose solution. They were able to observe changes in oxygen consumption and noradrenaline turnover indicating that a high carbohydrate diet may also have a stimulatory effect on BAT of lean and ob/ob mice. The advantages of this type of feeding regime include ease of food intake measurement and the likelihood that each animal will consume a diet of similar composition.

A high carbohydrate diet is also known to increase serum T_3 levels in mice (Shafrir et al., 1984). This increase is thought to be due to

extrathyroidal deiodination since the diet had no effect on serum T_4 levels. The exact contribution of the various tissue deiodinases to the increase in serum T_3 levels in response to a high carbohydrate diet in mice is not known.

OBJECTIVES:

The objective of this experiment is to study the effect of feeding a high carbohydrate (30 % sucrose) diet on BAT T5'D activity in lean and obese (ob/ob) mice. This objective will be accomplished in two experiments:

Experiment #1: Lean mice were fed the high carbohydrate diet for various times: 0.5, 1.5, 2.5, 3.5 days, 1 week and 2 weeks to determine the optimum time to study the effect of a high carbohydrate diet on BAT T5'D activity.

Experiment #2: Both lean and ob/ob mice were studied at the optimum times determined in the first experiment. The effect of feeding a high carbohydrate diet on food intake, body weight, body temperature, BAT wet weight, protein, GDP-binding, T5'D activity and serum thyroid hormone levels were studied.

METHODS:

Female C57Bl/6J lean (+/?) and/or obese (ob/ob) mice 8-9 weeks old were housed either 2 per cage (Experiment #1) or singly (Experiment #2)

at 28 °C with a 12 hour light/dark cycle with lights on at 7:00 am. The animals were fed either purina rat chow (#5012) and tap water ad libitum or they were offered a 30 % sucrose solution in a graduated water bottle in addition to the chow and tap water ad libitum.

Food intake of chow and 30 % sucrose was measured at 8:00 am every day by noting the volume of sucrose remaining in each graduated water bottle and calculating the amount (g) consumed. A fresh 30 % sucrose solution was provided each day. Since there was no effect of time in Experiment #1, the data were pooled and calculated as 24 hour food intake. In Experiment #2, there was a significant effect of time, thus data was reported as 12 hour and 36 hour food intake.

Animals were killed at 8:00 am by cervical dislocation and blood was collected. Rectal temperatures were measured and gonadal WAT was removed, cleaned and weighed. Interscapular and subscapular BAT was removed, cleaned, weighed and homogenized as described in methods section #5. BAT mitochondria were prepared as described in methods section #6. Protein was measured as described in section #7 GDP-binding to isolated BAT mitochondria was measured as described in methods section #8. BAT T5'D activity was measured as described in methods section #15. Serum thyroid hormones were measured by RIA using the method of Larsen (1976) as described in methods section #14.

Statistics were measured using ANOVA followed by Scheffe's post-hoc test. Food intake measurements in Experiment #1 were measured using repeated measures ANOVA.

RESULTS:

EXPERIMENT 1

Food Intake:

There was no significant effect of time on the 24 hour food intake measurements of lean mice fed chow or chow and 30 % sucrose for 2 weeks. For this reason the data were pooled and the effect of 30 % sucrose feeding was studied. Lean animals offered a 30 % sucrose solution in addition to chow consumed the same amount of metabolizable energy as did animals offered chow alone (Table 19). Lean mice offered a high carbohydrate diet ate less chow and changed the composition of their diet such that there was a 30 % increase in the contribution of carbohydrates to the total number of calories consumed.

Body Weight, WAT Wet Weight, and Body Temperature:

A significant increase in body weight was observed with time after 2 weeks of feeding both chow and the high carbohydrate diet. This indicates growth of the animals. A significant increase in body weight due to the effect of sucrose feeding was noted after 2.5 and 3.5 days but not after 1 and 2 weeks. WAT wet weight did not change significantly with time, however after 3.5 days and 1 week, there was a significant increase in WAT wet weight due to 30 % sucrose feeding compared to chow fed controls. This increase was not sustained and there was no significant difference found at 2 weeks. Body temperature was increased at 2.5 and 3.5 days after 30 % sucrose feeding. However, this increase was only transient and was not sustained for 1 or 2 weeks (Table 20).

TABLE 19

EFFECT OF 30 % SUCROSE FEEDING ON 24 HOUR FOOD INTAKE.

	CHOW n = 24	SUCROSE n = 24
Total Energy Consumed (Kcal)	22.6 ± 0.9	23.7 ± 0.6
Kcal Derived From Carbohydrates	12.0 ± 0.5	19.7* ± 0.6
% of Diet Derived From Carbohydrates	53 %	83 %

Values are means ± SEM for the number (n) of measurements made. Animals were caged in pairs and food intakes were measured at 08:00 am. There was no effect of time on food intake therefore, the data were pooled. * indicates a significant effect of 30 % sucrose feeding. The data were analyzed by ANOVA and Scheffe's Test.

TABLE 20

EFFECT OF 30 % SUCROSE FEEDING WITH TIME ON:

BODY WEIGHT, WAT WET WEIGHT AND BODY TEMPERATURE

	0.5 DAY		1.5 DAYS		2.5 DAYS		3.5 DAYS		1 WEEK		2 WEEKS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4
Body Weight g	17 0 ± 0 4	17 5 ± 0 5	18 7 ± 0 7	19 0 ± 0 3	18 0 ± 0 3	19 0* ± 0 3	17 4 ± 0 3	18 4* ± 0 4	18 6 ± 0 2	19 1 ± 0 6	18 6¶ ± 0 8	19 4¶ ± 0 3
WAT Wet Weight g	0 26 ± 0 01	0 30 ± 0 07	0 28 ± 0 02	0 33 ± 0 03	0 33 ± 0 02	0 39 ± 0 02	0 20 ± 0 04	0 35* ± 0 03	0 24 ± 0 01	0 39* ± 0 06	0 37 ± 0 01	0 42 ± 0 04
Body Temp °C	37 7 ± 0 1	37 5 ± 0 4	36 9 ± 0 2	37 0 ± 0 3	36 8 ± 0 2	37 5* ± 0 2	36 8 ± 0 4	37 5* ± 0 3	37 5 ± 0 1	37 8 ± 0 1	37 7 ± 0 1	37 9 ± 0 1

Values are means ± SEM for the number (n) of lean animals per group. * indicates a significant effect of 30 % sucrose feeding. ¶ indicates a significant effect of time compared to overnight value. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

TABLE 21

EFFECT OF 30 % SUCROSE FEEDING WITH TIME ON LEAN MICE:

EFFECT ON THYROID HORMONES.

	0 5 DAYS		1 5 DAYS		2.5 DAYS		3.5 DAYS		1 WEEK		2 WEEKS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4
T_4 µg/dl	2 ± 0 1	2 6* ± 0 1	2 2 ± 0 2	1 7 ± 0 1	2 3 ± 0 2	2 7 ± 0 2	2 4 ± 0 1	2 1 ± 0 2	2 2 ± 0 1	2 5 ± 0 1	2 1 ± 0 1	1 8 ± 0 1
T_3 ng/dl	55 ± 5 0	84 8 ± 14 2	75 9 ± 5 9	98 8* ± 9 3	87 5 ± 3 9	119 4* ± 4 4	82 6 ± 5 6	74 5 ± 3 8	71 8 ± 7 9	81 7 ± 5 9	76 3 ± 6 4	71 8 ± 9 0
T_3/T_4 Ratio %	3 08 ± 0 31	3 18 ± 0 36	3 29 ± 0 28	6 12* ± 0 84	3 84 ± 0 33	4 50 ± 0 27	3 51 ± 0 29	3 62 ± 0 31	3 34 ± 0 31	3 64 ± 0 03	3 62 ± 0 17	3 87 ± 0 62

Values are means ± SEM for the number (n) of lean animals per group. * indicates a significant effect of 30 % sucrose feeding. ¶ indicates a significant effect of time compared to overnight value. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

Serum Thyroid Hormones:

After an overnight feeding of 30 % sucrose lean mice significantly increased serum T_4 levels but did not alter serum T_3. After 1.5 days however, serum T_4 decreased and remained similar to chow fed controls while serum T_3 and the ratio of T_3/T_4 increased. This increase in serum T_3 was maintained for 24 hours after which there was no effect of sucrose feeding on serum thyroid hormone levels (Table 21).

Effect on Brown Adipose Tissue:

Feeding a high carbohydrate diet to lean mice for 14 days had no effect on BAT wet weight or on BAT protein (Table 22). There was a significant 2-3 fold increase in both total and specific BAT T5'D activity after 12 hours of feeding 30 % sucrose to lean mice (Table 22 and Figure 33). This stimulation of activity was not sustained inasmuch as, 24 hours later the stimulation was only 50 % and 24 hours after that there was no significant effect of sucrose feeding on BAT T5'D activity (Table 22 and Figure 33). There was no initial effect of sucrose feeding on GDP-binding to isolated mitochondria of lean mice. However, GDP-binding was significantly increased after 2.5 and 3.5 days of sucrose feeding but this effect was not sustained over the 2 weeks of this study (Table 22).

The results of Experiment #1 indicated that there was an acute effect of a high carbohydrate diet on BAT T5'D activity. For this reason I chose to study the effect of a high carbohydrate diet on lean and ob/ob mice after 12 hours and 36 hours of feeding.

TABLE 22

EFFECT OF 30 % SUCROSE FEEDING WITH TIME ON LEAN MICE:

EFFECT ON BROWN ADIPOSE TISSUE.

	0.5 DAY		1.5 DAYS		2.5 DAYS		3.5 DAYS		1 WEEK		2 WEEKS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4
BAT Wet Weight g	0.21 ± 0.02	0.26 ± 0.02	0.26 ± 0.02	0.33 ± 0.03	0.28 ± 0.03	0.34 ± 0.02	0.27 ± 0.03	0.33 ± 0.04	0.25 ± 0.04	0.26 ± 0.01	0.27 ± 0.02	0.25 ± 0.01
BAT Protein mg	8.5 ± 0.6	10.1 ± 0.5	8.4 ± 0.3	6.7 ± 0.4	8.0 ± 0.8	8.4 ± 0.2	9.4 ± 0.6	9.0 ± 0.7	8.7 ± 0.1	7.9 ± 0.4	8.4 ± 0.4	8.3 ± 0.6
TD SpAct pmol/h/mg	0.05 ± 0.005	0.14* ± 0.02	0.04 ± 0.004	0.07*¶ ± 0.003	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.002	0.04 ± 0.003	0.05 ± 0.003	0.06 ± 0.005	0.07 ± 0.005
TD TotAct pmol/h	0.41 ± 0.04	1.38* ± 0.21	0.32 ± 0.03	0.46*¶ ± 0.05	0.46 ± 0.08	0.61 ± 0.05	0.78 ± 0.13	0.64 ± 0.04	0.37 ± 0.03	0.43 ± 0.04	0.50 ± 0.07	0.53 ± 0.03
GDP-Binding pmol/mg P	75.3 ± 9.5	96.8 ± 12.7	65.8 ± 2.8	53.5 ± 7.5	62.0 ± 9.3	109.3* ± 6.2	74.5 ± 15.6	125.3* ± 20.9	75.5 ± 19.1	107.0 ± 19.1	63.0 ± 12.0	80.0 ± 11.0

Values are means ± SEM for the number (n) of lean animals per group. * indicates a significant effect of 30 % sucrose feeding. ¶ indicates a significant effect of time compared to overnight value. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

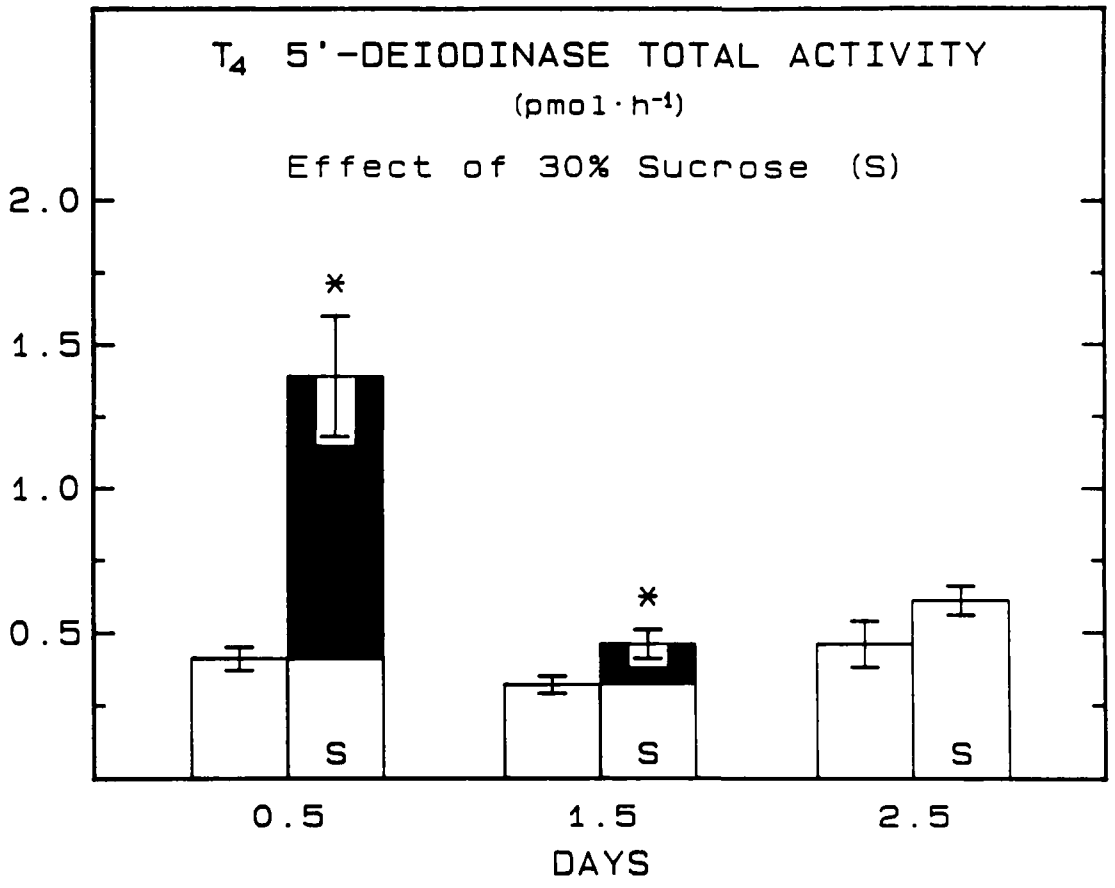


Figure 34: THE EFFECT OF FEEDING 30 % SUCROSE ON BAT T5'D ACTIVITY OF LEAN MICE. Lean mice were fed either chow (clear bars) or 30 % sucrose and chow (bars with S) for 0.5, 1.5, or 2.5 days (see Table 24 for more details). A significant effect of sucrose was symbolized by * and the dark portions of the bars. The data were analyzed by two-way ANOVA followed by Scheffe's post-hoc test.

EXPERIMENT #2

Food Intake:

Obese (ob/ob) mice consume almost twice as much metabolizable energy as lean mice when fed chow alone at both times studied (Table 23). Both lean and ob/ob mice consume more total metabolizable energy in a 36 hour time period than a 12 hour time period. When fed 30 % sucrose neither lean mice nor ob/ob mice overeat. There was no significant effect of sucrose on total metabolizable energy consumed. There was a trend toward a lower amount of metabolizable energy consumed by ob/ob mice fed sucrose. In fact ob/ob mice consumed the same total metabolizable energy as lean mice when fed sucrose. Both lean and ob/ob mice changed the composition of their diets to increase the proportion of carbohydrate, this was at the expense of chow which contained the source of protein, fat and vitamins and minerals. Lean mice increased the amount of carbohydrate 10 % between the 12 hour and 36 hour measurements whereas the ob/ob mouse consumed a maximum amount of the 30 % sucrose solution at the first measurement of food intake.

Body Weight, Gonadal WAT Wet Weight and Body Temperature:

Obese (ob/ob) mice weigh more than lean mice and have more gonadal WAT. These parameters are not changed by feeding a high carbohydrate diet for 36 hours. Obese mice have lower body temperatures than lean mice. Feeding a 30 % sucrose supplement caused a small but significant increase in body temperature in lean mice but had no effect on ob/ob mice (Table 24).

TABLE 23

EFFECT OF 30 % SUCROSE FEEDING ON FOOD INTAKE OF LEAN AND OB/OB MICE

	LEAN				OB/OB			
	12 HOURS		36 HOURS		12 HOURS		36 HOURS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=3
Total Energy Kcal	6.8 ± 0.4	10.0 ± 0.8	13.6¶ ± 1.6	20.6¶ ± 2.0	12.4§ ± 0.8	10.0 ± 1.6	25.4§¶ ± 0.3	22.5¶ ± 2.0
Kcal from Carbohydrates	3.6 ± 0.2	7.4* ± 0.6	7.2¶ ± 0.9	17.6¶* ± 1.8	6.6§ ± 0.4	9.4*§ ± 1.0	13.5§¶ ± 0.5	20.4*¶ ± 1.6
% of Diet from Carbohydrates	53 %	74 %	53 %	85 %	53 %	94 %	53 %	91 %

Values are means ± SEM for the number (n) of animals per group. The animals were caged 1 per cage. Food intakes were measured for 12 hours (8:00 pm to 8:00 am) and after 36 hours (8:00 pm to 8:00 am). * indicates a significant effect of 30 % sucrose feeding, ¶ indicates a significant effect of time and § indicates a significant effect of obesity. The data were analyzed by three-way ANOVA followed by Scheffe's post-hoc test.

TABLE 24

EFFECT OF 30 % SUCROSE FEEDING ON: BODY WEIGHT, WAT WET WEIGHT, AND BODY TEMPERATURE OF LEAN AND OB/OB MICE

	LEAN				OB/OB			
	12 HOURS		36 HOURS		12 HOURS		36 HOURS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=3
Body Weight g	17 5 ± 0 6	17 5 ± 0 6	17 4 ± 0 5	19 1 ± 0 6	36 8§ ± 1 5	34 5§ ± 2 0	39 1§ ± 1 6	36 1§ ± 1 0
WAT Wet Weight g	0 35 ± 0 01	0 24 ± 0 04	0 28 ± 0 04	0 36 ± 0 03	1 90§ ± 0 14	1 77§ ± 0 13	1 99§ ± 0 11	2 01§ ± 0 15
Body Temp °C	37 1 ± 0 6	38 2* ± 0 1	37 8 ± 0 1	38 4* ± 0 2	36 2§ ± 0 7	36 6§ ± 0 6	36 2§ ± 0 3	36 7§ ± 0 2

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of 30 % sucrose feeding and § indicates a significant effect of obesity. The data were analyzed by three-way ANOVA followed by Scheffe's post-hoc test.

TABLE 25

EFFECT OF 30 % SUCROSE FEEDING ON SERUM THYROID HORMONES OF LEAN AND OB/OB MICE

	LEAN				OB/OB			
	12 HOURS		36 HOURS		12 HOURS		36 HOURS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=3
T_4 µg/dl	2 3 ± 0 1	2 3 ± 0 1	2 3 ± 0 1	2 6 ± 0 3	2 1 ± 0 1	2 1 ± 0 1	2 1 ± 0 1	2 1 ± 0 1
T_3 ng/dl	77 9 ± 3 2	92 5* ± 6 9	75 3 ± 2 7	96 0* ± 3 7	77 2 ± 4 1	89 1* ± 5 0	72 1 ± 1 4	94 2* ± 7 2
$T_3/T4$ Ratio %	3 47 ± 0 14	4 03* ± 0 26	3 33 ± 0 26	3 82 ± 0 62	3 68 ± 0 20	4 31* ± 0 25	3 52 ± 0 10	4 48* ± 0 29

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of 30 % sucrose feeding and § indicates a significant effect of obesity. The data were analyzed by three-way ANOVA followed by Scheffe's post-hoc test.

Serum Thyroid Hormones:

Obese mice fed a chow diet are not hypothyroid, they have normal levels of thyroid hormones compared to lean mice. When both lean and ob/ob mice are fed a high carbohydrate diet serum T_3 levels are increased. This increase is reflected in an increase in the T_3/T_4 ratio since serum T_4 levels do not change (Table 25).

Effect on Brown Adipose Tissue:

Obese (ob/ob) mice have more BAT than lean mice, however, ob/ob mice do not have more BAT protein than lean mice (in this experiment). A high carbohydrate diet had no effect on BAT wet weight or BAT protein.

Obese mice had higher basal levels of both specific and total BAT T5'D activity than lean mice (Table 26 and Figure 35). There was no significant effect of feeding a 30 % sucrose diet for 12 hours on specific or total T5'D activity of lean or ob/ob mice (Table 26). However, after 36 hours, there was a significant increase in total BAT T5'D activity in both lean and ob/ob mice. The lean mice were able to increase total BAT T5'D activity by 30 % whereas, ob/ob mice increased T5'D activity by 45 % (Figure 35).

Obese mice have lower GDP-binding levels than lean mice at all times studied. There was no significant effect of a high carbohydrate diet on GDP-binding at either time studied.

TABLE 26

EFFECT OF 30 % SUCROSE FEEDING ON BROWN ADIPOSE TISSUE OF LEAN AND OB/OB MICE

	LEAN				OB/OB			
	12 HOURS		36 HOURS		12 HOURS		36 HOURS	
	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=4	CHOW n=4	SUCROSE n=3
BAT Wet Weight g	0.24 ± 0.02	0.22 ± 0.02	0.25 ± 0.04	0.28 ± 0.04	0.97§ ± 0.02	0.83§ ± 0.08	0.89§ ± 0.06	1.00§ ± 0.11
BAT Total Protein mg	9.6 ± 0.4	9.2 ± 0.6	10.2 ± 0.9	10.9 ± 0.5	11.1 ± 0.9	9.8 ± 0.4	11.1 ± 1.0	11.8 ± 1.4
TD SpAct pmol/h/mg P.	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.18§ ± 0.01	0.16§ ± 0.004	0.16§ ± 0.005	0.21§*¶ ± 0.01
TD TotAct pmol/h	1.10 ± 0.12	1.16 ± 0.06	1.05 ± 0.12	1.38* ± 0.09	1.95§ ± 0.21	1.53§ ± 0.10	1.72§ ± 0.11	2.51§*¶ ± 0.27
GDP Binding pmol/mg P.	81.3 ± 9.9	85.0 ± 4.4	77.0 ± 12.3	90.8 ± 14.6	27.8§ ± 1.4	30.5§ ± 2.0	34.8§ ± 8.0	66.3 ± 18.5

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of 30 % sucrose feeding, ¶ indicates a significant effect of time and § indicates a significant effect of obesity. The data were analyzed by three-way ANOVA followed by Scheffe's post-hoc test.

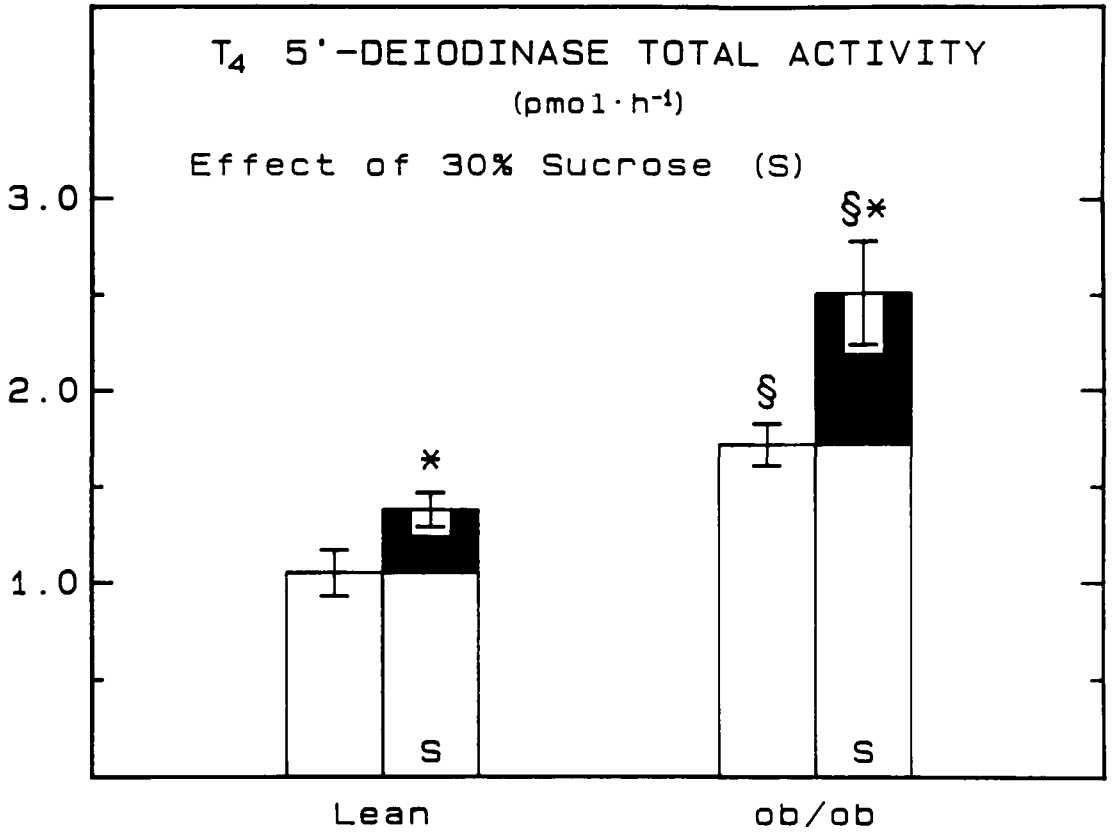


Figure 35: EFFECT OF FEEDING 30 % SUCROSE ON BAT T'5D ACTIVITY OF LEAN AND OB/OB MICE. Lean and ob/ob mice were fed chow (clear bars) or chow and 30 % sucrose (bars with S) for 36 hours (1.5 days) (see Table 26 for more details). A significant effect of sucrose was symbolized by * and the darkened portions of the bars. A significant effect of obesity was symbolized by §. The statistics were measured by three-way ANOVA followed by Scheffe's test.

DISCUSSION:

The most important observation from these two experiments is that a high carbohydrate diet (30 % sucrose) can acutely increase BAT T5'D total activity in both lean and obese (ob/ob) mice (Figure 35). In the first experiment animals were caged in pairs and in the second experiment animals were caged singly. The lean chow fed controls in Experiment #2 (Figure 35) had twice the level of BAT T5'D activity compared to the lean chow fed controls in Experiment #1 (Figure 34). It is possible that the animals that are caged singly are exposed to a relatively colder environment than animals that are caged in pairs (Jennings et al., 1986). Thus, singly caged animals would have a cold stimulated increase in BAT T5'D activity. In Experiment #1, lean mice increased BAT T5'D activity 2-3 fold in response to 12 hours of high carbohydrate feeding (Figure 34). The modest stimulation of BAT T5'D activity found in the lean animals in Experiment #2 compared to the 2-3 fold activation found in Experiment #1 could be explained by the possibility that the control animals were already cold stimulated thus, the sucose stimulated increase was relatively smaller even though the absolute values attained were similar.

Our results are similar to those of Glick et al. (1985) who meal fed rats a high carbohydrate diet and observed a 2 fold increase in BAT T5'D activity 3 hours after the start of the meal. They were also able to observe a 50 % increase in hepatic T5'D activity 3 hours and 24 hours after the test high carbohydrate meal. Silva and Larsen (1986), found a 2-3 fold stimulation in BAT T5'D activity in rats fed a

high carbohydrate diet for 48 hours or longer. However, our results differ in that the effect of sucrose feeding on BAT T5'D activity was not sustained for longer than 36 hours in Experiment #1. It is interesting to note that GDP-binding significantly increases after the increase in BAT T5'D activity indicating that the production of T_3 in BAT could be a prerequisite for an increase in GDP-binding when mice are stimulated with a high carbohydrate diet. In lean mice, the stimulatory effect of a high carbohydrate diet is minimal since the increase in GDP-binding is not maintained for 2 weeks. In rats, Rothwell and Stock (1984) found a 2-fold stimulation of BAT GDP-binding by 10 % sucrose feeding for 2 weeks. Glick et al. (1985) also showed a 50 % increase in GDP-binding 3 hours after a high carbohydrate test meal, this increase was sustained for at least 24 hours. A cafeteria diet is able to stimulate GDP-binding to isolated BAT mitochondria in lean mice for at least 3 weeks (Himms-Hagen et al., 1986; Eley and Himms-Hagen, 1988b). However, we do not know if there was an acute effect of cafeteria diet on BAT T5'D activity prior to the increase in GDP-binding in these studies.

In the second experiment (Figure 35) both lean and ob/ob mice were able to show a modest increase in BAT T5'D activity (30 % and 45 % respectively). In this experiment there was no significant effect of feeding a high carbohydrate diet on the GDP-binding of lean or ob/ob mice at the two acute time points studied in this experiment. It is possible that if the study were extended for a longer period of time an increase in GDP-binding might have been found.

It is well known that a high carbohydrate diet stimulates BAT noradrenaline turnover in rats (Walgren et al., 1987; Young et al., 1982). The increase in BAT noradrenaline turnover may be related to the increase in BAT T5'D activity (Silva and Larsen, 1986) since noradrenaline is thought to mediate the increase in BAT T5'D activity (Silva and Larsen, 1983).

In both experiments a high carbohydrate diet increased serum T_3 levels without affecting serum T_4 levels in lean and ob/ob mice. Obese mice were able to increase serum T_3 levels to the same extent as lean mice.

Knehans and Romsos (1984) fed singly caged lean and ob/ob mice housed at 23-25 °C a 30 % sucrose solution with free access to stock diet for 3 to 4 days. They found that the fractional rates of noradrenaline turnover in BAT of lean mice were unaffected by the sucrose supplement but lean mice were able to increase oxygen consumption. However, in obese mice, fractional rates of noradrenaline turnover in BAT were increased by 43 % in response to supplemental sucrose with no effect on oxygen consumption. In my study we observed an increase in both lean and ob/ob BAT T5'D activity after 36 hours of 30 % sucrose feeding, but found no effect in lean mice at 3.5 days. This result agrees with the lack of stimulation of 30 % sucrose feeding in lean mice on BAT noradrenaline turnover. Unfortunately, I did not measure BAT T5'D activity in ob/ob mice after 3-4 days of 30 % sucrose supplementation. It is possible that in lean mice the increase in serum T_3 noted in our study might be sufficient to increase oxygen consumption whereas, the ob/ob mouse maybe refractory to the increased

serum T_3 and increased noradrenaline turnover in response to sucrose supplementation and thus, did not increase oxygen consumption (Knehans and Romsos, 1984) I also found that sucrose supplementation had no effect on body temperatures of ob/ob mice whereas in lean mice a significant increase was found. Knehans and Romsos (1984) did not measure body temperatures.

When rats are offered a cafeteria diet (Rothwell et al., 1982) or a high carbohydrate diet (Rothwell et al., 1981; Ackroff and Sclafani, 1988) they become hyperphagic and increase total metabolizable energy intake. In contrast lean and obese (ob/ob) mice offered a palatable cafeteria diet do not overeat (Himms-Hagen et al., 1986; Eley and Himms-Hagen, 1988b; Chapter 2 part #1). In this study, 30 % sucrose feeding did not cause lean or ob/ob mice to overeat. In fact. 30 % sucrose supplementation had a tendency to decrease food intake compared to chow in ob/ob mice. Knehans and Romsos (1984) also noted a decrease in the total metabolizable energy consumed by ob/ob mice when offered a 30 % sucrose diet.

It is well known that feeding a high carbohydrate diet increases serum T_3 in rats (Gavin et al., 1988), pigs (Dauncey et al., 1983) and in humans (Danforth et al., 1979; Welle et al., 1984). Carbohydrate feeding in the rat increases liver T_4 5'-deiodinase activity (Gavin et al., 1981; Gavin et al , 1988) and BAT T5'D activity which was assessed by measurement of tissue T_3 and T_4 (Gavin et al., 1988). Gavin et al., (1988) conclude that the increase in serum T_3 associated with glucose feeding is due to increases in both liver and BAT T5'D activity. An increase in liver T5'D activity could explain the increase in serum T_3

seen in Experiment #2 after 12 hours of sucrose feeding when there was no effect of 30 % sucrose on BAT T5'D activity at this time point.

Feeding a high carbohydrate diet is well known not only for increasing serum T_3 levels but also for increasing the activity of many lipogenic enzymes such as: malic enzyme, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, and fatty acid synthase in rat liver (Oppenheimer and Schwartz, 1986; Oppenheimer et al., 1987; see Hoch, 1988 for review). In fact, T_3 seems to be able to multiply an intracellular signal derived from the metabolism of glucose which is able to increase mRNA for these lipogenic enzymes (Mariash and Oppenheimer, 1985). Shafrir and Trostler, (1984) found that feeding a high carbohydrate diet to lean mice significantly increased liver pyruvate kinase, NADP-malate dehydrogenase, acetyl-CoA carboxylase, and glycerol phosphate dehydrogenase whereas the increases seen in the ob/ob mouse with a high carbohydrate diet were much smaller since ob/ob mice had higher basal levels. In rats, feeding a high sucrose diet greatly increased the rate of lipogenesis seen in BAT (Thompson and Grigor, 1987). This observation is consistent with the high amounts of the regulatory enzymes of fatty acid synthesis found in BAT (McCormack and Denton, 1977). Thus, although I did not measure BAT lipogenesis, a high carbohydrate diet is likely to increase BAT lipogenesis in lean and ob/ob mice. I did not find an increase in BAT wet weight with sucrose feeding, but a change in the physical appearance of the tissue (an increase in lipid rendering homogenization of the tissue more difficult) indicated lipogenesis had indeed occurred.

In conclusion, both lean and ob/ob mice are able to respond to a high carbohydrate diet by increasing BAT T5'D activity and serum T_3 levels. Unlike the effect of cold, the ob/ob mouse does not have a defective diet-induced increase in BAT T5'D activity under the conditions studied.

CHAPTER 2:

EFFECT OF DIET ON BAT T5'D ACTIVITY IN LEAN AND OB/OB MICE.

PART 3: EFFECT OF A 48 HOUR FAST AND A 60 % RESTRICTED DIET ON BAT T5'D ACTIVITY IN LEAN AND OBESE (OB/OB) MICE.

BACKGROUND:

In previous sections, the stimulation of BAT thermogenesis by cold and diet was found to be positively related to an increase in BAT T5'D activity. BAT can also become inactive and atrophy in a number of physiologically important situations such as fasting in rats (Rothwell et al., 1984) and mice (Desautels, 1985), and food restriction in rats (Rothwell and Stock, 1982). Also lactation (Trayhurn and Richard, 1985) or the return of animals housed below thermoneutrality to a thermoneutral environment (Ashwell et al., 1983; Desautels and Himms-Hagen, 1980) can inactivate BAT thermogenic activity.

Food restricted lean and obese mice reduce their body temperature in the early morning and become torpid (Himms-Hagen, 1985). At this time their BAT is inactive as measured by low levels of GDP-binding (Himms-Hagen, 1985). However, food restricted lean and ob/ob mice were able to quickly (15-30 minutes) arouse from torpor by activating BAT thermogenesis (increased GDP-binding) and raise their body temperature usually at the time of their daily meal (Himms-Hagen, 1985). Arousal from torpor is very difficult to study because it happens very quickly. I chose to study the restricted mice during torpor when one would

expect BAT to be inactive.

Food restriction and fasting are known to decrease serum T_3 (Silva and Larsen, 1986c). Fasted rats not only have reduced serum T_3 levels but also have a significant 40 % reduction in liver T5'D activity (Gavin and Moeller, 1983) whereas the activity of the kidney T5'D was unaffected by fasting (Ferguson et al., 1985).

In this section the relationship between a decrease in BAT thermogenesis caused by a 48 hour fast or by torpor induced by feeding a 60 % restricted diet and BAT T5'D activity will be studied in lean and genetically obese (ob/ob) mice. A 60 % restricted diet was chosen since it had been previously shown to significantly reduce body weight in ob/ob mice (Himms-Hagen, 1985).

OBJECTIVE:

The objective of this study was to measure BAT T5'D activity in lean and obese mice under conditions when BAT thermogenesis is reduced, such as a 48 hour fast and feeding a 60 % restricted diet.

METHOD:

Female lean (+/?) and genetically obese (ob/ob) mice arrived in January, 1986 from Jackson Labs at 4-5 weeks of age. They were grouped into cages of 3 phenotypically similar animals and housed at 28 °C with a 12:12 lighting schedule with lights on at 7:00 am. The animals were assigned to one of three groups: control animals which were fed Purina rodent chow #5012 ad libitum; 48 hour fasted animals, which were treated as controls except they were fasted for 48 hours immediately

before their death; and a 60 % food restricted group which were fed 60 percent of the amount of chow that the control group consumed at 3 pm every day. All animals had free access to tap water. The animals followed this feeding schedule for 10 months. Body weights were measured every week for 25 weeks. During the final 2 weeks of the study food intake measurements were made and body weights were again measured.

The 48 hour fasted animals had their food removed at 8:00 am two days before their death and food intake measurements were made on the remaining control animals. All the animals in a cage were killed on the same day at 8:00 am by cervical dislocation. Blood was collected, rectal temperatures and body weight were measured. Gonadal WAT was removed, cleaned and weighed. Interscapular and subscapular BAT was removed, cleaned, weighed and homogenized as described in method section #5. BAT mitochondria was measured as described in methods section #6 and GDP-binding to isolated BAT mitochondria was measured as described in methods section #8. BAT protein was estimated as described in section #7. Serum thyroid hormones were measured using a RIA (Larsen, 1976). BAT T5'D activity was measured as described in section #15.

Body weight and food intake measurements were analyzed by repeated measures ANOVA, the rest of the data were analyzed by two-way ANOVA followed by Scheffe's post-hoc test.

RESULTS:

Food Intake:

Obese mice consume more total metabolizable energy than lean mice. Both lean and ob/ob mice consume more food energy in a 48 hour time period than in a 24 hour time period. Thus the 48 hour fasted animals had a caloric deficit (Table 27).

Body Weight:

Obese (ob/ob) mice weigh more than lean mice regardless of treatment (Figure 36). Obese mice restricted to 60 % chow consumed by control animals gained considerably less weight compared to the chow ad libitum fed controls. This effect was significant after 1 week of the restricted diet. By the end of the study restricted ob/ob mice had approximately half the body weight of the obese control animals (Figure 36 and Table 28). The effect of a restricted diet on lean animals was less dramatic. Restricted lean mice weigh significantly less than ad lib. fed controls after 6 weeks of the diet and by the end of the experiment they had lost 20 % of the weight of the ad lib. fed lean controls. The 48 hour fasted animals were fed ad libitum and thus did not have significantly different body weights before the 48 hour fast (Figure 36). However, during the 48 hour fast both lean and ob/ob mice lost weight (Table 28). Although ob/ob mice lost more weight than lean mice during the 48 hour fast, when expressed as a percentage, lean mice lost proportionately more weight than ob/ob mice (20 % vs 13 %). This loss of body weight during the 48 hour fast was reflected in a 36 % decrease in the amount of gonadal white adipose tissue in lean mice. Obese mice, however, did not lose gonadal white adipose tissue

TABLE 27

24 HOUR AND 48 HOUR FOOD INTAKE OF LEAN AND OB/OB MICE

	24 HOUR FOOD INTAKE		48 HOUR FOOD INTAKE	
	LEAN CHOW n=4	OB/OB CHOW n=4	LEAN CHOW n=2	OB/OB CHOW n=2
CONTROL				
Total Kcal Intake	21.4 ± 2.7	36.5§ ± 3.3	43.5¶ ± 6.0	77.9§¶ ± 3.5
60 % RESTRICTED				
Kcal	12.8 ± 0.5	21.9 ± 0.5	26.1 ± 0.9	46.7 ± 1.0

Values are means ± SEM for the number (n) of food intake measurements made during a 24 hour period one and two weeks prior to the 48 hour fast and during the 48 hour fast. The animals were housed 3 phenotypically similar animals per cage. ¶ indicates a significant effect of time (48 hours vs 24 hours) and § indicates a significant effect of obesity. Data were analyzed by ANOVA followed by Scheffe's post-hoc test.

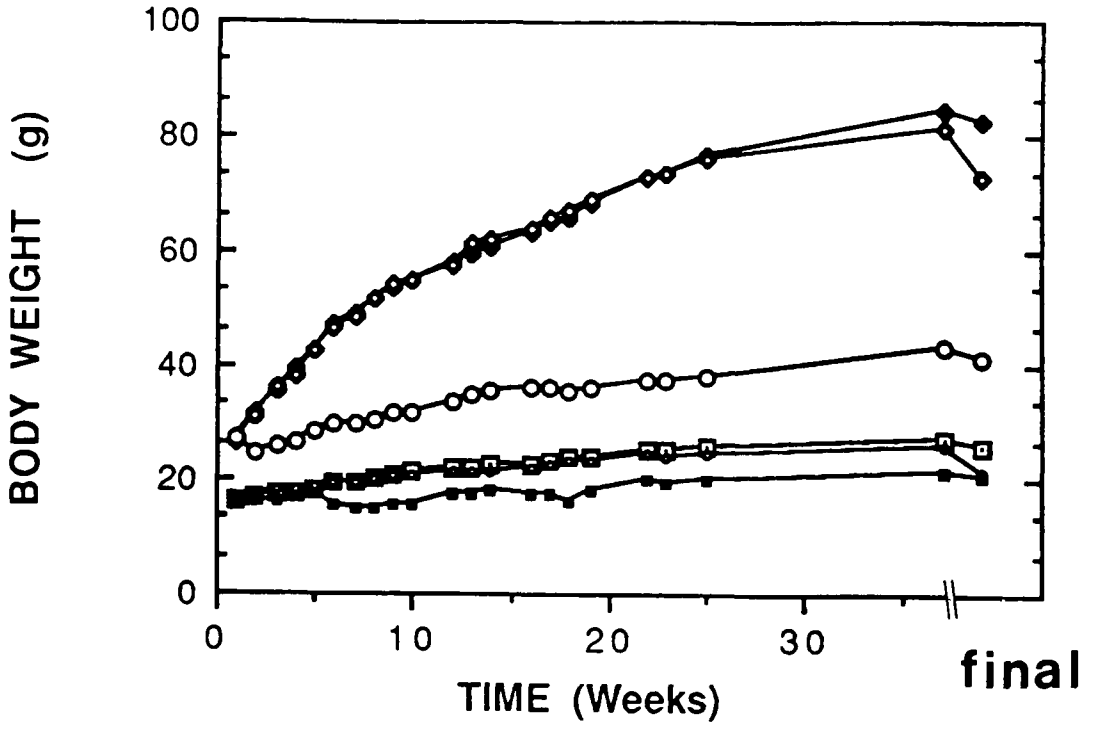


Figure 36: EFFECT OF 60 % FOOD RESTRICTION AND A 48 HOUR FAST ON BODY WEIGHT OF LEAN AND OB/OB MICE. Lean (solid squares) and ob/ob mice (open circles) were fed 60 % of their like controls for 39 weeks. A group of lean (small open diamonds) and ob/ob (large open diamonds) were fed as controls except they were fasted for 48 hours before they were killed. The values are means of 5-6 mice. Food restriction caused a significant decrease in both lean and ob/ob mice (see text for more detail). The statistics were measured by repeated measures ANOVA.

during the fast compared to ad libitum control ob/ob mice. The animals that were restricted to 60 % of the control diet had less gonadal white adipose tissue. Lean animals reduced the amount of white adipose tissue to 40 % of controls, while ob/ob mice were able to reduce the amount of white adipose tissue to 70 % of controls.

Body Temperature:

Obese mice have lower body temperatures than lean mice under all conditions studied. A 48 hour fast significantly reduced the body temperature of both lean and obese mice. Lean mice fed a 60 % restricted diet reduced their body temperature but not significantly. Obese mice on the other hand reduced their already low body temperature by 2 degrees when fed a 60 % restricted diet and were thus considered to be torpid at the time of day studied (08:00) (Table 28).

Serum Thyroid Hormones:

Obese mice are euthyroid compared to lean control animals. When lean and ob/ob mice are fasted for 48 hours they significantly decrease serum T_4 and T_3 to the same extent without significantly changing the T_3/T_4 ratio. When lean and ob/ob mice are on a restricted diet they decrease serum T_3 with no significant change in serum T_4 or T_3/T_4 ratio. The decrease in serum T_3 in ob/ob mice caused by the restricted diet was slightly greater (48 %) than that of lean mice (32 percent) (Table 29, Figure 37).

Effect on Brown Adipose Tissue:

Obese (ob/ob) mice have a greater wet weight of BAT with a greater protein content than lean mice regardless of treatment (Table 30). Fasting does not affect BAT wet weight or total protein content in

TABLE 28

EFFECT OF 60 % FOOD RESTRICTION AND 48 HOUR FAST ON BODY WEIGHT, WAT WEIGHT AND BODY TEMPERATURE ON LEAN AND OB/OB MICE

	LEAN			OB/OB		
	CONTROL n = 6	48 HOUR FAST n = 6	60 % RESTRICTED n = 6	CONTROL n 5	48 HOUR FAST n = 6	60 % RESTRICTED n = 6
Body Weight g	25 8 ± 1 4	21 6 ± 0 8	20 5* ± 1 1	82 9§ ± 4 4	73 0§ ± 2 3	41 5§* ± 1 6
48 Hour Weight Loss g	1 0 ± 0 3	5 1* ± 0 6	0 7 ± 0 1	1 0 ± 0 9	10 5§* ± 2 5	1 8§ ± 0 2
WAT Wet Weight g	0 96 ± 0 19	0 61* ± 0 06	0 36* ± 0 08	3 98§ ± 0 26	3 60§ ± 0 47	2 69§* ± 0 16
Body Temp °C	36 7 ± 0 2	34 5* ± 0 3	35 1 ± 0 8	33 5§ ± 0 9	32 0§* ± 0 5	31 3§* ± 0 3

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of diet compared to control and § indicates a significant effect of obesity. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

TABLE 29

EFFECT OF 60 % FOOD RESTRICTION AND 48 HOUR FAST ON SERUM THYROID HORMONES OF LEAN AND OB/OB MICE.

	LEAN			OB/OB		
	CONTROL n 6	48 HOUR FAST n = 6	60 % RESTRICTED n = 6	CONTROL n = 5	48 HOUR FAST n = 6	60 % RESTRICTED n = 6
T4 µg/dl	2.8 ± 0.3	0.9* ± 0.1	2.1 ± 0.3	2.0 ± 0.1	1.2* ± 0.1	1.9 ± 0.1
T_3 ng/dl	95.3 ± 4.6	53.1* ± 4.1	65.1* ± 9.2	83.0 ± 5.2	49.4* ± 4.0	42.9§* ± 6.3
T_3/T_4 Ratio %	3.52 ± 0.40	6.12 ± 0.75	3.57 ± 0.65	4.38 ± 0.51	4.23 ± 0.36	2.08 ± 0.30

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of diet compared to control and § indicates a significant effect of obesity. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

TABLE 30

EFFECT OF 60 % FOOD RESTRICTION AND A 48 HOUR FAST ON BROWN ADIPOSE TISSUE OF LEAN AND OB/OB MICE.

	LEAN			OB/OB		
	CONTROL n = 6	48 HOUR FAST n = 6	60 % RESTRICTED n = 6	CONTROL n = 5	48 HOUR FAST n = 6	60 % RESTRICTED n = 6
BAT Wet Weight g	0 26 ± 0 02	0 26 ± 0 02	0 31 ± 0 03	3 63§ ± 0 23	3 53§ ± 0 36	1 50§* ± 0 11
BAT Total protein mg	10 7 ± 0 3	7 7* ± 0 8	9 6 ± 0 3	23 8§ ± 0 9	27 1§ ± 1 4	18 6§* ± 1 2
TD SpAct pmol/h/mg Prot	0 038 ± 0 005	0 042 ± 0 004	0 028 ± 0 001	0 047 ± 0 009	0 031 ± 0 003	0 040 ± 0 005
TD TotAct pmol/h	0 41 ± 0 05	0 32 ± 0 02	0 27 ± 0 01	1 14§ ± 0 24	0 84§ ± 0 10	0 76§ ± 0 12
GDP Binding pmol/mg Prot	77 8 ± 4 0	40 7* ± 2 1	43 6* ± 9 0	32 0§ ± 1 2	27 8§ ± 3 4	28 0§ ± 2 2

Values are means ± SEM for the number (n) of animals per group. * indicates a significant effect of diet and § indicates a significant effect of obesity. The data were analyzed by ANOVA followed by Scheffe's post-hoc test.

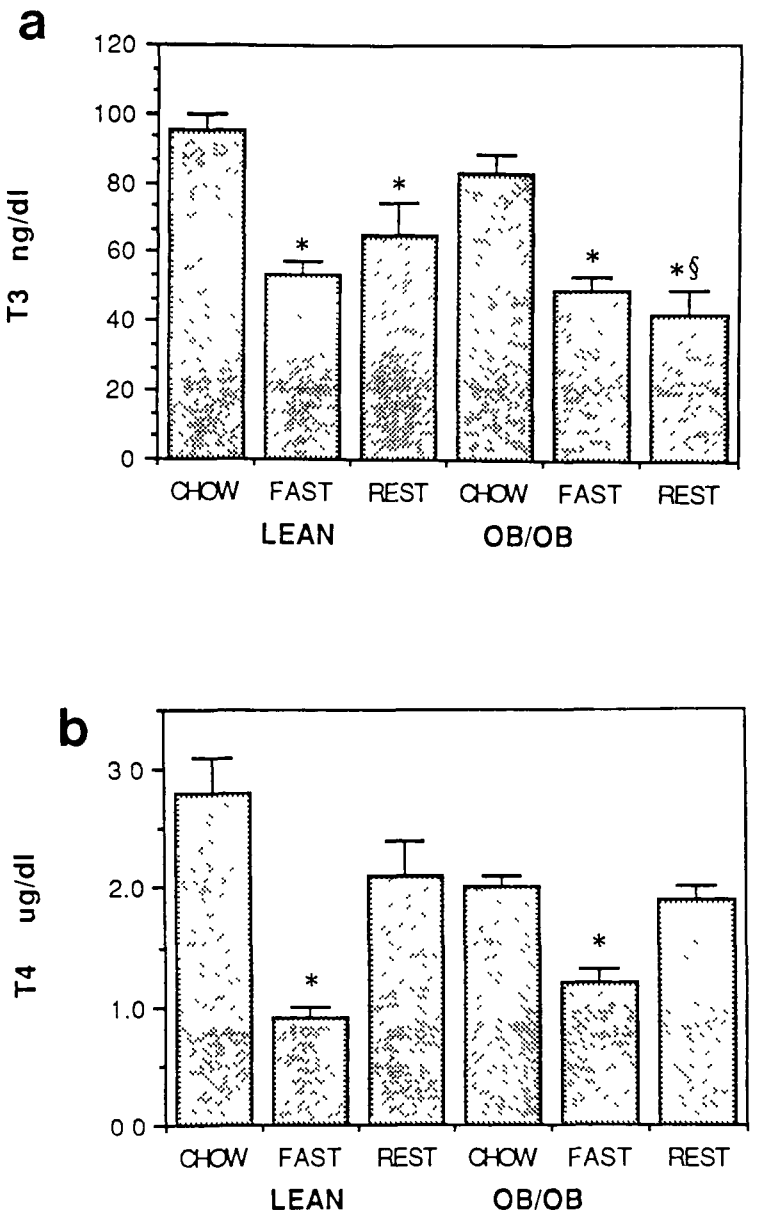


Figure 37: EFFECT OF 48 HOUR FAST AND 60 % FOOD RESTRICTION ON SERUM THYROID HORMONE LEVELS. The values are means ± SEM for the number of animals in Table 29. A 48 hour fast caused a significant reduction in serum T_3 (37a) in both lean and ob/ob mice. A 60 % restricted diet also caused a significant reduction but ob/ob mice had significantly lower levels compared to lean restricted animals (§). A significant effect of treatment is symbolized by *. In contrast, only a 48 hour fast was able to significantly reduce serum T_4 levels in lean and ob/ob mice (37b).

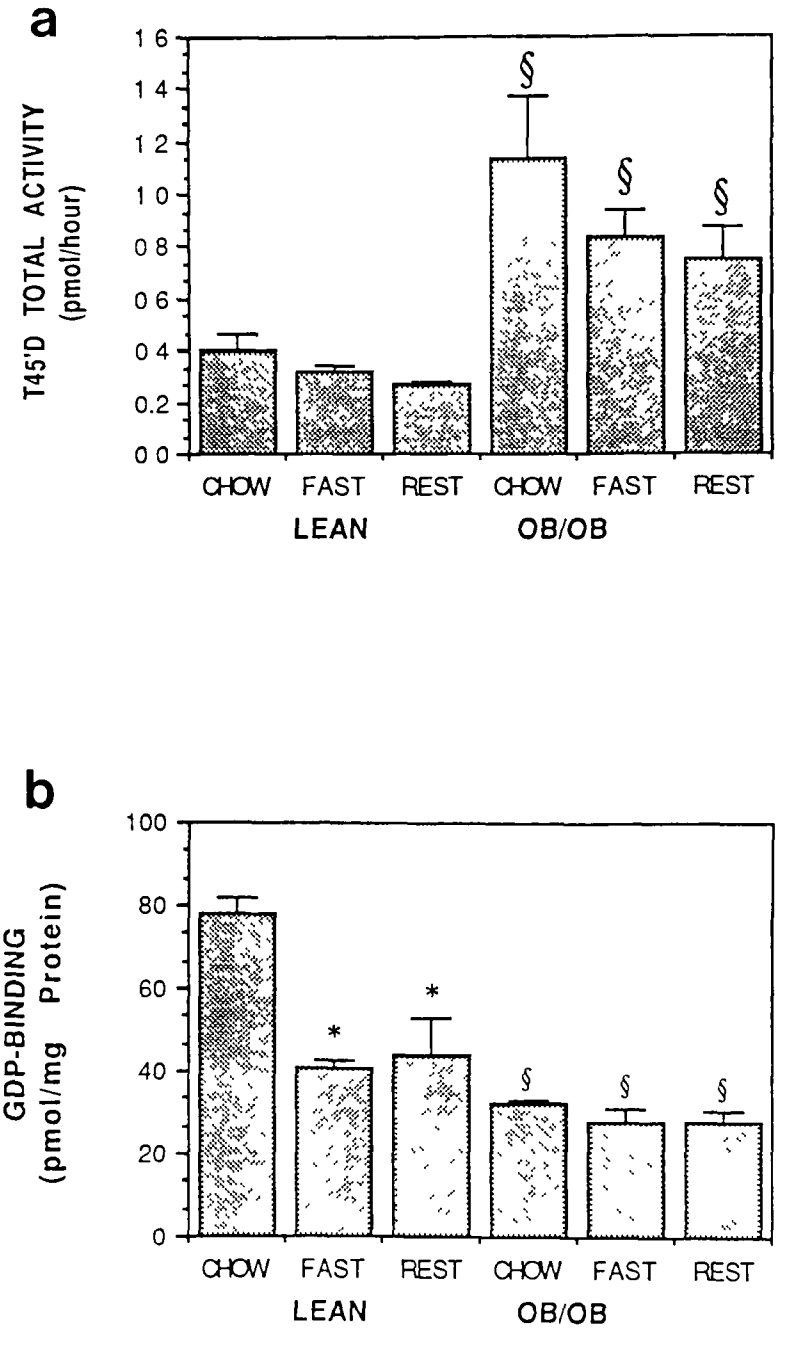


Figure 38: EFFECT OF 48 HOUR FAST AND 60 % FOOD RESTRICTION ON TOTAL T5'D ACTIVITY AND GDP-BINDING IN LEAN AND OB/OB MICE. The values are means ± SEM for the number of animals in Table 30. A significant effect of treatment was symbolized by § and a significant effect of treatment was denoted by *. See text for more detail.

ob/ob mice. However, lean mice do not decrease the weight of BAT but significantly reduced the amount of BAT protein by 25 % .

When following a restricted diet, obese mice significantly decrease both the weight of BAT and total BAT protein where as there was no significant effect of the restricted diet on these parameters in lean mice.

There was no effect of obesity or diet on the specific activity of BAT T5'D activity (Table 30). Obese mice had higher levels of BAT T5'D total activity (Table 30, Figure 38a) due to the significantly higher level of BAT total protein. There was no effect of a 48 hour fast or a 60 % restricted diet on BAT T5'D total activity.

Obese (ob/ob) mice have significantly lower basal levels of GDP-binding to isolated BAT mitochondria than lean mice (Table 30. Figure 38b). Lean mice significantly decrease GDP-binding in response to a 48 hour fast (48 %) and a 60 % restricted diet (44 %). There was no significant effect of diet on GDP-binding in obese ob/ob mice.

DISCUSSION:

The most important observation from this study is that when BAT is thermogenically inactive, as measured by a decrease or low levels of GDP-binding, as it is during a 48 hour fast or during torpor induced by a 60 % restricted diet, BAT T5'D activity is not significantly different from basal levels in lean or ob/ob mice. Thus, I find a dissociation of BAT T5'D activity and BAT thermogenesis. My results are in agreement with Silva and Larsen, (1986a) who found no effect of an overnight fast on rat BAT T5'D activity. However, Murakami et al.

(1988) found a significant decrease in BAT T5'D activity in rats fasted for 3 days. Wu and Wright (1987) fasted albino mice for 3 days and found a 5 fold increase in BAT T5'D specific activity (37 ± 8 vs 195 ± 19 fmol/h/mg protein). I studied the effect of a 48 hour fast. It is possible that had I studied the animals after a 3 day fast I might have found similar results. The authors suggest this finding is an example of dissociation of BAT T5'D activity and BAT thermogenesis. They also speculate that the T_3 produced in BAT during starvation may be to help offset the reduction in hepatic T5'D (Gavin and Moeller, 1983) and thus an adaptive mechanism to maintain serum T_3. Unfortunately, the authors did not report serum thyroid hormone levels, in any case, I found that a fasting-induced 40-44 % reduction in serum T_3 was not sufficient to stimulate BAT T5'D activity in lean or ob/ob mice.

Fasting is known to decrease sympathetic nervous system activity to various organs including BAT in rats (Young and Landsberg, 1981; Young et al., 1982). In mice (Knehans and Romsos, 1983) a decrease in NA content but not NATO was found in response to 24 hour fast. Seydoux et al., 1982, found a decrease in sensitivity to NA in 2 day fasted lean mice. Noradrenaline is thought to stimulate BAT T5'D activity (Silva and Larsen, 1983) through mechanisms involving mRNA synthesis and protein synthesis (Silva and Larsen, 1986a). Thus if sympathetic activity is reduced in BAT one would not expect an increase in BAT T5'D activity in response to fasting. However, hypothyroid rats have high levels of BAT T5'D activity only after 9 days after thyroidectomy (Silva and Larsen, 1986b). It is possible that as serum thyroid hormones reach a certain critical low level, BAT T5'D activity may be

stimulated in order to maintain serum T_3 levels and prevent death.

GDP-binding to isolated BAT mitochondria decreased by approximately 50 % in lean mice after a 48 hour fast and a 60 % restricted diet. The already minimal basal levels of GDP-binding found in obese mice were not decreased further by either treatment (Table 30). These results are consistent with the work of Himms-Hagen, (1985), Trayhurn and Jennings (1986; 1988), and Desautels (1985). It is known that both adrenergic stimulation and T_3 concurrently are necessary for the full expression of the UCP gene (Silva, 1988). Trayhurn and Jennings (1986) found that the 50 % decrease in GDP-binding to isolated BAT mitochondria in fasted lean mice was accompanied by a 50 % decrease in UCP as measured by RIA. As mentioned previously, lactation is associated with reduced BAT thermogenesis (Trayhurn and Richard, 1985; Villarroya et al., 1986) and with reduced iodothyronine 5'-deiodinase activity in brown adipose tissue (Giralt et al., 1986); these results are consistent with a direct relationship between BAT thermogenesis and BAT T5'D activity.

CHAPTER 2: SUMMARY AND CONCLUSION

In this chapter I examined the effect of diet on BAT T5'D activity in lean and obese mice. I found that although a cafeteria diet is known to stimulate BAT thermogenesis in lean and obese (ob/ob) mice, after 3 weeks of feeding the diet, there was no effect on BAT T5'D activity of either lean or obese mice. This indicates that high BAT T5'D activity is not necessary for long term adaptation of lean and ob/ob mice to the cafeteria diet. It is possible that BAT T5'D

activity could be more important during the acute stage of a response to a dietary stimulus. Thus, I studied the effect of a 30 % sucrose diet on BAT T5'D activity of lean and obese mice after 12 and 36 hours. A high carbohydrate diet was able to modestly and transiently stimulate both lean and ob/ob mouse BAT T5'D activity confirming the hypothesis that BAT T5'D activity was more important in the acute stages of a dietary stimulus. These experiments also demonstrated that diet is a much weaker stimulus for BAT T5'D activity than exposure to cold. Unlike the acute response to cold, the acute response of BAT T5'D activity to diet was not defective in the ob/ob mouse. The last part of this chapter demonstrated that when BAT is thermogenically inactive during a 48 hour fast or during torpor induced by a 60 % restricted diet, BAT T5'D activity remains at basal levels in lean and obese (ob/ob) mice.

Sympathetic activity is increased in BAT in response to cold, cafeteria diet, and a high carbohydrate diet and sympathetic activity is not increased in BAT during starvation (Knehans and Romsos, 1983). It seems that there is a close relationship between sympathetic activity and BAT T5'D activity. In the next chapter I will examine this relationship more directly.

HS

THYROID HORMONE METABOLISM IN BROWN ADIPOSE TISSUE OF LEAN AND GENETICALLY OBESE (OB/OB) MICE

by

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

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CHAPTER 3:

EFFECT OF ADRENERGIC AGENTS ON BAT THYROXINE 5'-DEIODINASE ACTIVITY OF LEAN AND OBESE (OB/OB) MICE.

BACKGROUND:

The experiments described in this chapter were performed from April to August 1987. At that time it was well known that subcutaneous injections of noradrenaline (NA) in rats, stimulated basal BAT T5'D at least 10 fold (range 6 - 30) in rats (Silva and Larsen, 1983). The effect of noradrenaline was thought to be mediated by α_1-adrenergic receptors since prazosin (an α_1- antagonist) was able to block the noradrenaline stimulated increase in BAT T5'D (Silva and Larsen, 1983). Prazosin was also able to block the increase in BAT T5'D seen with cold in rats (Silva and Larsen, 1985). The mechanism of noradrenaline stimulated increase in BAT T5'D activity involves synthesis of mRNA (Silva and Larsen, 1986a). Kaplan and Young, (1987), reported an increase in BAT T5'D in response to NA in lean and ob/ob mice. The adrenergic mechanism of this response in mice had not been previously studied.

OBJECTIVE:

The objective of these experiments was to further characterize the nature of the adrenergic activation of BAT T5'D in lean and ob/ob mice. This was accomplished by a series of three experiments. First,

determining the time course of activation of BAT T5'D after acute injection of noradrenaline in lean mice. Then the optimum dose of isoproterenol and phenylephrine was determined in lean and obese mice in the second experiment. Finally, in the third experiment, a number of adrenergic agonists and antagonists were injected into lean and ob/ob mice in order to characterize the adrenergic activation of BAT T5'D.

MATERIALS AND METHODS:

Female C57BL/6J lean (+/?) and obese (ob/ob) mice arrived at 4-5 weeks of age and were separated into individual cages. They were housed at 28 °C with a 12 hour light/dark cycle starting at 9:00 am. They were allowed free access to water and purina rodent chow #5012. The animals were studied at 8 weeks of age.

Experiment 1

The time course experiment began by injecting saline or 40 µg/100g bw (body weight) noradrenaline (arterenol, (-) norepinephrine bitartrate salt, Sigma Chemical Co.) subcutaneously into mice starting at 9:00 am. The NA was first dissolved in 0.2 % ascorbic acid and then diluted in sterile saline to 0.1 ml/10 g bw. The animals were always weighed the night before the experiment to determine the volume of injection.

The animals injected with saline were killed immediately after the injection and were called time = 0. The noradrenaline injected animals were killed after 3,6, and 12 hours. Blood was collected, rectal temperatures were measured and body weights were measured again.

Gonadal WAT was removed and weighed, and interscapular and subscapular BAT depots were removed and placed in ice cold isolation medium. BAT was cleaned of WAT and adhering muscle and homogenized in 3.5 ml isolation medium using a Polytron (Brinkmann Instruments) at setting 4 for 15 seconds. Homogenates were frozen immediately in liquid nitrogen and stored at -70 °C. BAT homogenate proteins were measured as described in Methods Section #7. T5'D was measured as described in Methods Section #15. Serum thyroid hormones were measured by RIA using the method of Larsen, (1976) as described in Methods Section #14. BAT UCP was measured as described in Methods Section #12.

Experiment 2

The second experiment was designed to determine the optimum dose of phenylephrine and isoproterenol to be injected. Previous work in our laboratory had shown that ob/ob mice were ten times more sensitive to isoproterenol than lean mice since maximum oxygen consumption measurements were obtained with much smaller doses. In fact ob/ob mice did not survive at the doses of isoproterenol which stimulated maximum responses in lean mice (Gloria Zaror-Behrens, personal communication).

At 9:00 am lean and ob/ob mice were injected s.c. with 40 µg/100 g bw NA or phenylephrine (PHE) (hydrochloride, Sigma Chemical Co.) at three concentrations: high (Hi), 40 µg/100 g bw; medium (Med), 26 µg/100 g bw; low (Lo), 13 µg/100 g bw. The phenylephrine was first dissolved in 0.2 % ascorbic acid and diluted in sterile saline. Lean mice were injected with 3 doses of isoproterenol (bitartrate, Sigma Chemical Co.): high, 40 µg/100 g bw; medium 20 µg/100 g bw; and low 6.5 µg/100 g bw. Obese (ob/ob) mice were injected with one tenth the

dose: high, 4 µg/100 g bw; medium, 2 µg/100 g bw and low 0.65 µg/100 g bw. The isoproterenol was also made up first in 0.2 % ascorbic acid and was then diluted with sterile saline.

The animals were killed 3 hours later at 12:00 noon, blood was not collected from these animals. Rectal temperatures and body weights were measured. Gonadal WAT was removed, cleaned, and weighed. Interscapular and subscapular BAT were removed, cleaned and processed as described above in the time course experiment.

Experiment 3

In order to test the effect of various adrenergic agents on lean and ob/ob BAT T5'D animals were injected s.c. with saline (VEH), 40 µg/100 g bw, noradrenaline (NA), 26 µg/100 g bw, phenylephrine (PHE), 300 µg/100 g bw, yohimbine (YOH), and 20 µg/100 g bw (lean) and 2 µg/100 g bw (ob/ob) isoproterenol (ISO) at 9:00 am. A group of animals received two injections, one ISO and one PHE but the total volume injected s.c. was similar to that of a single injection. Prazosin (PRAZ) (HCl, a gift from Pfizer Canada Inc.) was dissolved in 50 % ethanol and diluted with sterile saline. A dose of 40 µg/100 g bw was injected i.p. five minutes before injection of 40 µg/100 g bw of NA s.c.. Propranolol (PROP) (HCl, Sigma Chemical Co.) was prepared as described for prazosin. A dose of 2.3 mg/100 g bw was injected i.p. 5 minutes before injection of NA. Yohimbine was prepared first in 50 % ethanol then diluted with sterile saline and was injected i.p. 300 µg/100 g bw 30 minutes before the injection of NA. Dr. Gloria Zaror-Behrens performed the yohimbine experiment.

Three hours after injection, (at 12:00 noon), the animals were

killed and blood collected. Rectal temperatures and body weights were measured. Gonadal WAT was removed, cleaned and weighed. Interscapular and subscapular BAT was cleaned, weighed and homogenized as described in Methods Section #5. Mitochondria were isolated (Section #6) and GDP-Binding measured (Section #8). Protein was measured by the modified Lowry method (Section #7), serum T_3 and T_4 were assayed by RIA (Larsen, 1976), (Section #14) and BAT T5'D was measured as described in Section #15.

EXPERIMENT 1

RESULTS: TIME COURSE OF RESPONSE TO ACUTE NA INJECTION

Acute injection of noradrenaline into lean mice had no effect on body weight or WAT wet weight over the 12 hours studied (Table 31). Body temperature was highest 6 hours after injection and declined significantly 6 hours later. Serum T_4 also followed this pattern; the highest level was found at 6 hours post injection followed by a decline to pre-injection levels 6 hours later. Serum T_3 and T_3/T_4 ratio increased but not significantly in response to injection of NA. BAT wet weight and BAT UCP measured by RIA were not affected by an acute injection of NA. There was a small (20 %) but significant increase in BAT protein 3 hours after NA injection (Table 32). At the same time point there was a significant 7-fold increase in BAT T5'D specific activity and a 9-fold increase in total activity. This stimulated level was not maintained for longer than 3 hours (Figure 39).

TABLE 31

TIME COURSE AFTER ACUTE INJECTION OF NORADRENALINE: EFFECT ON BODY WEIGHT, BODY TEMPERATURE, WAT WET WEIGHT AND THYROID HORMONES

	SALINE TIME = 0 n = 3	NORADRENALINE TIME = 3 HOURS n = 3	NORADRENALINE TIME = 6 HOURS n = 3	NORADRENALINE TIME = 12 HOURS n = 3
Body Weight g	18.0 ± 0.6	18.7 ± 0.2	17.3 ± 1.1	17.2 ± 0.6
Body Temp. °C	37.1 ± 0.2	37.0 ± 0.1	37.8* ± 0.1	36.5 ± 0.3
WAT Wet Weight g	0.36 ± 0.05	0.43 ± 0.11	0.35 ± 0.09	0.46 ± 0.01
T_4 µg/dl	2.3 ± 0.1	2.5 ± 0.2	2.9* ± 0.1	2.0 ± 0.2
T_3 ng/dl	62.5 ± 1.7	83.0 ± 8.7	92.7 ± 19.9	88.0 ± 3.6
T_3/T_4 Ratio %	2.69 ± 0.17	3.35 ± 0.46	3.69 ± 0.83	4.37 ± 0.26

Values are means ± SEM for the number (n) of lean animals per group at various times after a single injection of noradrenaline (40 µg/100g bw s.c.). The symbol, * indicates a significant effect of time after injection of noradrenaline. Data was analyzed using two-way ANOVA followed by Scheffe's post-hoc test.

TABLE 32

TIME COURSE AFTER ACUTE INJECTION OF NORADRENALINE: EFFECT ON BROWN ADIPOSE TISSUE

	SALINE TIME = 0 n - 3	NORADRENALINE TIME = 3 HOURS n = 3	NORADRENALINE TIME = 6 HOURS n = 3	NORADRENALINE TIME = 12 HOURS n = 3
BAT Wet Weight g	0.18 ± 0.03	0.16 ± 0.01	0.17 ± 0.03	0.18 ± 0.02
BAT Total Protein mg	12.2 ± 0.3	14.6* ± 0.5	12.9 ± 0.6	13.0 ± 0.1
T5'D SpAct. pmol/h/mg prot.	0.10 ± 0.01	0.79* ± 0.05	0.19 ± 0.02	0.19 ± 0.04
T5'D TotAct. pmol/h	1.24 ± 0.05	11.52* ± 1.10	2.39 ± 0.20	2.46 ± 0.47
UCP RIA µg/mg prot	5.81 ± 1.12	5.72 ± 2.80	3.55 ± 0.69	3.66 ± 1.13

Values are means ± SEM for the number (n) of lean animals per group at various times after a single injection of noradrenaline (40 µg/100g bw s.c.). The symbol, * indicates a significant effect of time after noradrenaline injection. Data was analyzed using two-way ANOVA followed by Scheffe's post-hoc test.

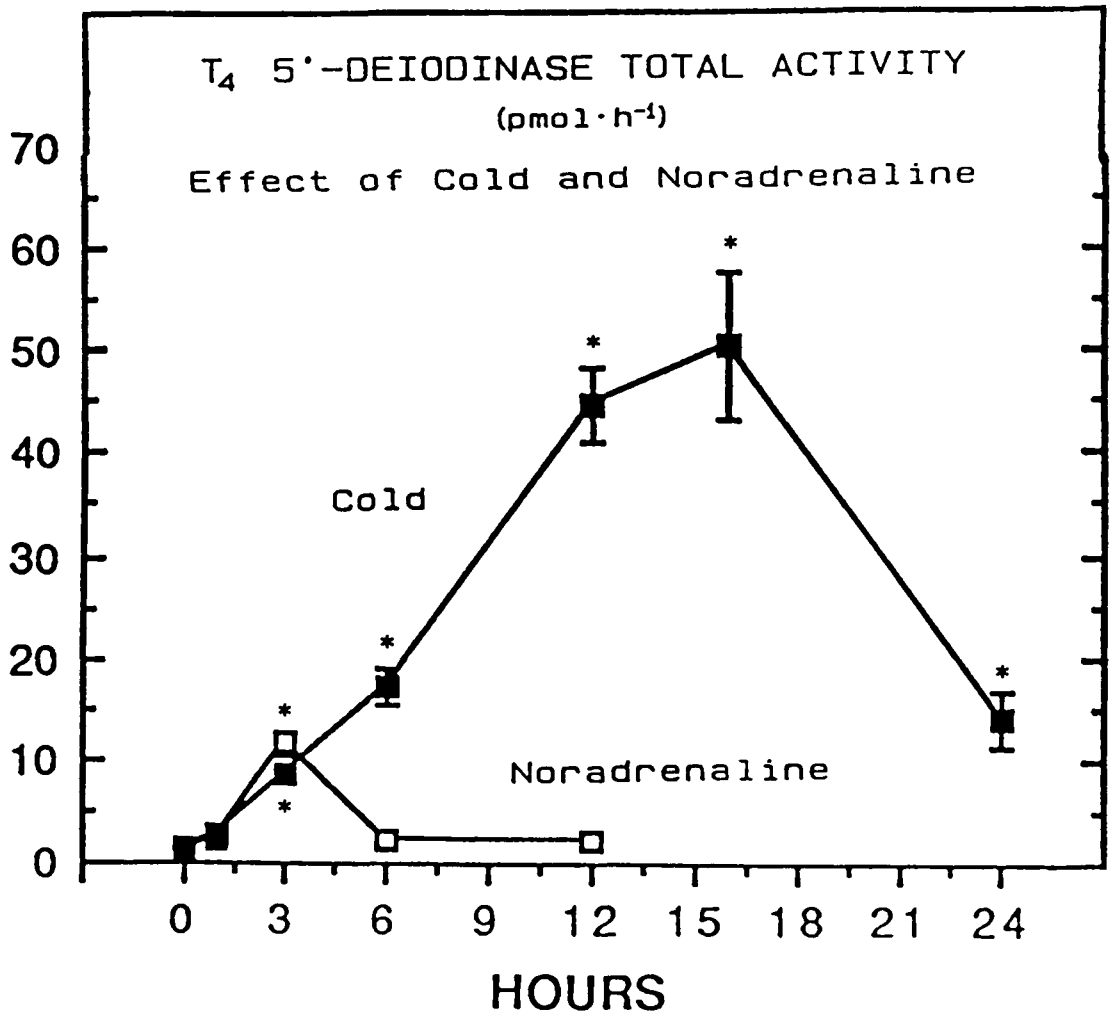


Figure 39: EFFECT OF COLD AND NORADRENALINE ON BAT T5'D TOTAL ACTIVITY IN LEAN MICE. Lean mice were either exposed to 14 °C or injected with noradrenaline (40 µg/100 g bw) at 0 hours and killed at various times there after. The values are means ± SEM. A significant effect of treatment is symbolized by *. Both cold and NA injection were able to stimulate BAT T5'D activity equally at 3 hours. However, after this time point cold was able to cause a much greater stimulation whereas NA was not.

DISCUSSION: TIME COURSE OF ACUTE NORADRENALINE INJECTION

The most important finding of this experiment was that 3 hours after an acute injection of NA, we observe an activation of both specific and total BAT T5'D activity in lean mice. This level of activation was similar to that observed by acute exposure to cold in Chapter 1, Part 1, Experiment #2. The specific activity was virtually identical (NA, 0.79 ± 0.05 vs cold, 0.77 ± 0.08) while total activity was similar (NA, 11.52 ±1.0 vs cold, 8.44 ± 0.63) (See Figure 39). Silva and Larsen, (1983,1986) also observe similar stimulation of BAT T5'D by NA and cold up to 4 hours after which cold is a much greater stimulator of BAT T5'D. However, the time course of activation of BAT T5'D by NA reported by Silva and Larsen (1983, 1986) using the same dose of NA in rats was different from our observations. Silva and Larsen observed that after acute NA injection in rats, a plateau of BAT T5'D activity was reached at 4-8 hours and by 16 hours the activity had returned to basal levels. Our results show a peak in T5'D activity 3 hours after acute injection which is not sustained and returns to basal levels by 6 hours post-injection. One explanation for such a different time course could be that lean mice have a higher metabolic rate than rats (Himms-Hagen, 1986) and therefore the half-life of NA in mice could be considerably shorter than in rats. I agree with Silva and Larsen's (1983) report that noradrenaline did not consistently alter serum thyroid hormone levels. Young et al. (1984) injected noradrenaline (100 µg/100 g bw) and various other longer acting thermogenic compounds such as fenoterol and ephedrine chronically into mice. They concluded that the inability of noradrenaline to completely

mimic the effects of cold was simply due to the rapid metabolism of noradrenaline and its short duration of action *in vivo*. Young *et al*. (1984) were also unable to see any effect of NA on metabolic rate 6 hours after an acute injection of NA.

The results of this experiment led us to choose 3 hours after injection to study the effect of various adrenergic drugs on BAT T5'D activity of lean and ob/ob mice.

EXPERIMENT 2

RESULTS: ISOPROTERENOL, PHENYLEPHRINE AND NORADRENALINE INJECTIONS IN LEAN AND OB/OB MICE.

Obese mice survived the injection of all three doses of both drugs. Obese (ob/ob) mice had higher body weights, WAT wet weights and lower body temperatures than lean mice (Tables 33 and 34). None of the drugs injected had any significant effect on these parameters. Obese mice also had greater BAT wet weights and total BAT protein values than lean mice. Obese mice also had greater basal BAT T5'D activity than lean mice. However when stimulated with noradrenaline both lean and obese mice increase BAT T5'D to the same value (Table 35 and 36). There was no significant effect of the other drugs injected on BAT T5'D.

TABLE 33

OPTIMUM DOSE OF ISOPROTERENOL AND PHENYLEPHRINE:

EFFECT ON BODY WEIGHT, BODY TEMPERATURE AND WAT WET WEIGHT

	LEAN MICE							
	Veh n = 3	Iso Hi n = 3	Iso Med n = 3	Iso Lo n = 3	NA n = 3	Phe Hi n = 3	Phe Med n = 3	Phe Lo n = 3
Body Weight g	18.2 ± 0.9	17.8 ± 0.1	18.1 ± 0.4	18.0 ± 0.4	17.7 ± 0.8	18.1 ± 0.2	17.1 ± 0.6	16.4 ± 0.6
Body Temp. °C	37.4 ± 0.2	37.0 ± 0.4	37.2 ± 0.3	36.7 ± 0.3	36.8 ± 0.1	36.9 ± 0.2	36.4 ± 0.2	36.4 ± 0.1
WAT Wet Weight g	0.46 ± 0.05	0.38 ± 0.12	0.40 ± 0.04	0.48 ± 0.08	0.58 ± 0.14	0.44 ± 0.11	0.39 ± 0.09	0.41 ± 0.10

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: Vehicle, Isoproterenol (40 µg/100g bw), Isoproterenol (20 µg/100g bw), Isoproterenol (6.5 µg/100g bw), Noradrenaline (40 µg/100g bw), Phenylephrine (40 µg/100g bw), Phenylephrine (26 µg/100g bw), Phenylephrine (13 µg/100g bw). All drugs were injected s.c. and animals were killed 3 hours later. ANOVA showed no main effects of drug, however there was a main effect of obesity for all variables. (See Table 34).

TABLE 34

OPTIMUM DOSE OF ISOPROTERENOL AND PHENYLEPHRINE: EFFECT ON BODY WEIGHT, BODY TEMPERATURE AND WAT WET WEIGHT

	ob/ob MICE							
	Veh n = 3	Iso Hi n = 3	Iso Med n = 3	Iso Lo n = 3	NA n = 3	Phe Hi n = 3	Phe Med n = 3	Phe Lo n = 3
Body Weight g §	35.5 ± 4.9	39.2 ± 0.2	37.2 ± 1.6	39.4 ± 1.4	38.4 ± 1.3	33.5 ± 3.4	33.6 ± 1.2	35.9 ± 1.9
Body Temp. °C §	35.1 ± 0.7	35.5 ± 0.1	35.7 ± 0.4	35.1 ± 0.4	35.2 ± 0.2	35.7 ± 0.5	35.3 ± 0.1	36.8 ± 0.3
WAT Wet Weight g §	2.60 ± 0.55	2.94 ± 0.25	2.61 ± 0.45	3.04 ± 0.20	2.66 ± 0.33	2.10 ± 0.34	2.46 ± 0.20	2.48 ± 0.13

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: Vehicle, Isoproterenol (4.0 µg/100g bw), Isoproterenol (2.0 µg/100g bw), Isoproterenol (0.65 µg/100g bw), Noradrenaline (40 µg/100g bw), Phenylephrine (40 µg/100g bw), Phenylephrine (26 µg/100g bw), Phenylephrine (13 µg/100g bw). All drugs were injected s.c. and animals were killed 3 hours later. ANOVA showed no main effects of drug, however, §, indicates a significant effect of obesity for all variables. (See Table 33).

TABLE 35

OPTIMUM DOSE OF ISOPROTERENOL AND PHENYLEPHRINE: EFFECT ON BROWN ADIPOSE TISSUE IN LEAN MICE

	LEAN MICE							
	Veh n = 3	Iso Hi n = 3	Iso Med n = 3	Iso Lo n = 3	NA n = 3	Phe Hi n = 3	Phe Med n = 3	Phe Lo n = 3
BAT Wet Weight g	0.14 ± 0.01	0.18 ± 0.03	0.16 ± 0.01	0.15 ± 0.02	0.18 ± 0.03	0.15 ± 0.03	0.16 ± 0.02	0.16 ± 0.01
BAT Total Protein mg	9.6 ± 0.9	10.8 ± 1.5	11.0 ± 0.6	10.7 ± 0.6	10.1 ± 0.7	8.5 ± 0.6	9.5 ± 1.1	9.7 ± 0.4
TD SpAct pmol/h/mg prot	0.08 ±0.01	0.20 ± 0.04	0.21 ± 0.06	0.16 ± 0.02	0.46* ± 0.08	0.09 ± 0.01	0.10 ± 0.01	0.08 0.01
TD TotAct pmol/h	0.76 ± 0.07	2.11 ± 0.19	2.35 ± 0.66	1.68 ± 0.14	4.49* ± 0.54	0.81 ± 0.11	0.96 ± 0.14	0.81 ± 0.14

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: Vehicle, Isoproterenol (40 µg/100g bw), Isoproterenol (20 µg/100g bw), Isoproterenol (6.5 µg/100g bw), Noradrenaline (40 µg/100g bw), Phenylephrine (40 µg/100g bw), Phenylephrine (26 µg/100g bw), Phenylephrine (13 µg/100g bw). All drugs were injected s.c. and animals were killed 3 hours later. ANOVA showed a main effect of obesity for BAT Wet Weight, BAT Total Protein and TD TotAct (See Table 36). ANOVA showed a main effect of Drug for TD SpAct and TD TotAct. * Indicates a significant effect of drug compared to vehicle from Scheffe's post-hoc test.

TABLE 36

OPTIMUM DOSE OF ISOPROTERENOL AND PHENYLEPHRINE: EFFECT ON BROWN ADIPOSE TISSUE IN OB/OB MICE

	ob/ob MICE							
	Veh n = 3	Iso Hi n = 3	Iso Med n = 3	Iso Lo n = 3	NA n = 3	Phe Hi n = 3	Phe Med n = 3	Phe Lo n = 3
BAT Wet Weight g §	1.06 ± 0.12	1.13 ± 0.09	1.07 ± 0.09	1.10 ± 0.09	1.08 ± 0.04	0.78 ± 0.06	0.92 ± 0.07	0.16 ± 0.20
BAT Total Protein mg §	17.0 ± 1.4	17.6 ± 0.6	17.3 ± 1.7	18.0 ± 1.2	17.1 ± 1.0	14.5 ± 0.1	14.7 ± 0.5	13.4 ± 1.7
TD SpAct pmol/h/mg prot	0.13 ±0.01	0.20 0.01	0.21 ± 0.02	0.17 ± 0.01	0.35* ± 0.07	0.18 ± 0.03	0.18 ± 0.01	0.16 ± 0.01
TD TotAct pmol/h	2.23§ ± 0.26	3.53 ± 0.16	3.65 ± 0.16	3.00§ ± 0.39	5.91* ± 0.98	2.63§ ± 0.41	2.58§ ± 0.12	2.16§ ± 0.41

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: Vehicle, Isoproterenol (4.0 µg/100g bw), Isoproterenol (2.0 µg/100g bw), Isoproterenol (0.65 µg/100g bw), Noradrenaline (40 µg/100g bw), Phenylephrine (40 µg/100g bw), Phenylephrine (26 µg/100g bw), Phenylephrine (13 µg/100g bw). All drugs were injected s.c. and animals were killed 3 hours later. * indicates a significant effect of drug compared to vehicle. § indicates a significant effect of obesity. The data were analyzed by two-way ANOVA followed by Scheffe's post-hoc test.

DISCUSSION: ISOPROTERENOL, PHENYLEPHRINE AND NORADRENALINE INJECTIONS IN LEAN AND OB/OB MICE.

An important observation from this experiment was that ob/ob mouse BAT T5'D can be stimulated by noradrenaline to similar values compared to lean mouse BAT T5'D. However, the increase measured in lean animals represented a 6 fold stimulation while that of the ob/ob mouse represented only a 3 fold stimulation.

One objective of this experiment was to determine the optimum dose of isoproterenol and phenylephrine to be injected into lean and ob/ob mice. Previous studies in our laboratory had shown ob/ob mice to be sensitive to isoproterenol. Obese mice were unharmed by all three doses of isoproterenol. However, there was no statistically significant effect of any dose of isoproterenol on BAT of lean or ob/ob mice. There was a trend towards a better response to the medium dose in BAT total T5'D activity, thus 20 µg/100 g bw was chosen for lean mice and 2.0 µg/100 g bw was chosen for ob/ob mice. Similarly, there was also no significant effect of phenylephrine on BAT, but there was a trend to higher levels of BAT total T5'D activity with the medium dose. Thus, 26 µg/100 g bw was chosen for both lean and ob/ob mice. The higher basal BAT T5'D total activity of ob/ob mice was observed again in this experiment.

EXPERIMENT 3

RESULTS: EFFECT OF ADRENERGIC AGENTS ON BAT T5'D ACTIVITY OF LEAN AND OB/OB MICE

Obese (ob/ob) mice had greater body weights and gonadal WAT wet weights but lower rectal temperatures than lean mice (Table 37 and 38). There was no effect of obesity on serum T_3/T_4 ratio. The adrenergic drugs injected had no significant effect on these parameters. Obese mice had greater BAT wet weights, and total BAT protein but had lower GDP-binding than lean mice (Table 39 and 40). There was no significant effect of drug on these parameters.

Thyroid hormones:

There was no significant effect of drug on serum T_3, however, ob/ob mice did have significantly lower serum T_3 levels in response to yohimbine, isoproterenol, and noradrenaline (Table 41 and 42, Figure 42). Similarly there was no effect of drug on serum T_4 but ob/ob mice had significantly lower serum T_4 levels in response to yohimbine and noradrenaline and yohimbine (Table 41 and 42, Figure 41).

BAT T5'D Activity:

There was no significant effect of obesity on BAT T5'D specific or total activity. However there was a significant effect of drug on BAT T5'D specific and total activity (Table 43). In lean mice yohimbine alone had no effect, isoproterenol showed a small but insignificant increase, phenylephrine had no effect, but the combination of

isoproterenol and phenylephrine showed a significant increase compared to vehicle. The noradrenaline stimulated increase in lean BAT T5'D specific activity was not significantly different from that of ISO + PHE. Propranolol did not significantly block the noradrenaline stimulated increase in BAT T5'D specific activity; however, there was also no significant difference between vehicle and noradrenaline + propranolol. Prazosin, on the other hand, was able to significantly block the noradrenaline stimulated increase in BAT T5'D specific activity. Yohimbine had no significant effect on the noradrenaline stimulated increase in BAT T5'D specific activity in lean mice (Table 41, Figure 42, Table 43).

There was no significant effect of obesity on total or specific BAT T5'D activity. However, there was a significant interaction between obesity and the injected drugs in the ANOVA. Therefore, the ob/ob mice had no significant effect of any drug on BAT T5'D specific activity (Table 42, Figure 42, Table 43).

The pattern for BAT T5'D total activity of lean mice was very similar to that of the specific activity. The only difference was a significant inhibition of the noradrenaline induced increase in BAT T5'D total activity by the β agonist propranolol (Table 41, Figure 43, Table 43).

In ob/ob mice BAT T5'D total activity, a similar pattern was seen; yohimbine had no effect, isoproterenol gave a small but insignificant increase, phenylephrine had no effect, however the combination of phenylephrine and isoproterenol showed a significant increase compared to vehicle. Noradrenaline showed a similar increase to the increase in

TABLE 37

ACUTE INJECTION OF ADRENERGIC AGENTS: EFFECT ON BODY WEIGHT, BODY TEMPERATURE, WAT WET WEIGHT AND T_3/T_4 RATIO OF LEAN MICE

	LEAN MICE								
	Veh n = 17	Yoh n = 3	Iso n = 6	Phe n = 6	Iso + Phe n = 10	NA n = 18	NA + Prop n = 5	NA + Praz n 9	NA + Yoh n 3
Body Weight g	17.8 ± 0.4	17.9 ± 0.6	17.3 ± 0.4	18.0 ± 0.6	17.5 ± 0.3	18.1 ± 0.4	17.8 ± 0.5	17.7 ± 0.5	18.4 ± 1.1
Body Temp. °C	36.8 ± 0.2	37.6 ± 0.2	36.9 ± 0.2	36.7 ± 0.2	36.6 ± 0.2	36.9 ± 0.1	36.6 ± 0.2	36.7 ± 0.2	37.5 ± 0.2
WAT Wet Weight g	0.36 ± 0.04	NM	0.36 ± 0.04	0.39 ± 0.05	0.23 ± 0.03	0.42 ± 0.04	0.25 ± 0.06	0.35 ± 0.04	NM
T_3/T_4 Ratio %	3.09 ± 0.20	3.12 ± 0.05	4.83 ± 0.26	3.08 ± 0.15	3.98 ± 0.34	3.62 ± 0.29	4.13 ± 0.53	4.06 ± 0.32	3.03 ± 0.16

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses) . NM indicates that the variable was not measured. ANOVA showed no main effects of drug, however there was a main effect of obesity for all variables except ratio. (See Table 38)

TABLE 38

ACUTE INJECTION OF ADRENERGIC AGENTS:

EFFECT ON BODY WEIGHT, BODY TEMPERATURE, WAT WET WEIGHT AND T_3/T_4 RATIO

	OBESE MICE								
	Veh n = 14	Yoh n = 3	Iso n 6	Phe n 6	Iso + Phe n - 10	NA n = 15	NA + Prop n : 6	NA + Praz n = 10	NA + Yoh n 3
Body § Weight g	38.1 ± 1.2	40.8 ± 1.5	38.1 ± 1.0	36.7 ± 1.5	40.1 ± 1.5	38.6 ± 0.8	36.1 ± 1.8	40.8 ± 1.5	39.8 ± 0.7
Body § Temp. °C	33.8 ± 0.6	35.5 ± 0.3	35.0 ± 0.4	35.1 ± 0.1	35.3 ± 0.2	34.3 ± 0.2	34.3 ± 0.2	34.7 ± 0.1	35.2 ± 0.3
WAT Wet § Weight g	2.32 ± 0.16	NM	2.40 ± 0.25	2.47 ± 0.10	2.48 ± 0.18	2.37 ± 0.13	2.08 ± 0.14	2.46 ± 0.14	NM
T_3/T_4 Ratio %	3.73 ± 0.45	4.51 ± 1.00	3.22 ± 0.65	3.21 ± 0.34	3.28 ± 0.17	3.44 ± 0.30	2.87 ± 0.27	3.27 ± 0.13	4.70 ± 1.23

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses). NM indicates that the variable was not measured. ANOVA showed no main effects of drug, however, § indicates a significant effect of obesity for all variables except ratio. (See Table 37). Statistics were measured by 2-way-ANOVA followed by Tukey's HSD post-hoc test.

TABLE 39

ACUTE INJECTION OF ADRENERGIC AGENTS:

EFFECT ON BROWN ADIPOSE TISSUE

	LEAN MICE								
	Veh n 17	Yoh n 3	Iso n = 6	Phe n 6	Iso + Phe n = 10	NA n - 18	NA + Prop n = 5	NA + Praz n 9	NA + Yoh n 3
BAT Wet Weight g	0.17 ± 0.01	0.21 ± 0.02	0.15 ± 0.01	0.19 ± 0.02	0.13 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.21 ± 0.03
BAT Total Protein mg	10.5 ± 0.5	11.1 ± 1.5	11.1 ± 0.6	10.4 ± 0.7	11.0 ± 0.6	11.3 ± 0.5	9.7 ± 0.5	10.1 ± 0.6	12.5 ± 0.8
GDP-Binding pmol/mg prot.	162 ± 18	130 ± 23	125 ± 36	117 ± 26	153 ± 11	184 ± 20	182 ± 33	162 ± 22	179 ± 39

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses). ANOVA showed no main effects of drug, however there was a main effect of obesity for all variables (See Table 40).

TABLE 40

ACUTE INJECTION OF ADRENERGIC AGENTS:

EFFECT ON BROWN ADIPOSE TISSUE

	OBESE MICE								
	Veh n 14	Yoh n 3	Iso n = 6	Phe n = 6	Iso + Phe n 10	NA n = 15	NA + Prop n = 6	NA + Praz n 10	NA + Yoh n = 3
BAT Wet § Weight g	0.93 ± 0.04	1.41 ± 0.39	1.09 ± 0.06	1.05 ± 0.08	1.08 ± 0.04	1.04 ± 0.05	1.06 ± 0.08	1.05 ± 0.06	0.92 ± 0.16
BAT Total § Protein mg	13.8 ± 0.8	16.0 ± 2.7	16.1 ± 0.9	14.7 ± 0.5	14.7 ± 0.9	15.7 ± 0.5	16.7 ± 0.8	15.3 ± 0.8	12.9 ± 0.9
GDP-Binding § pmol/mg prot.	56.6 ± 6.2	31.0 ± 2.0	42.3 ± 4.6	46.7 ± 5.3	56.6 ± 4.8	52.3 ± 5.3	59.6 ± 5.0	53.1 ± 2.0	36.0 ± 4.0

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses). ANOVA showed no main effects of drug, however, § indicates a significant effect of obesity. The statistics were measured by ANOVA followed by Tukey's HSD post-hoc test.

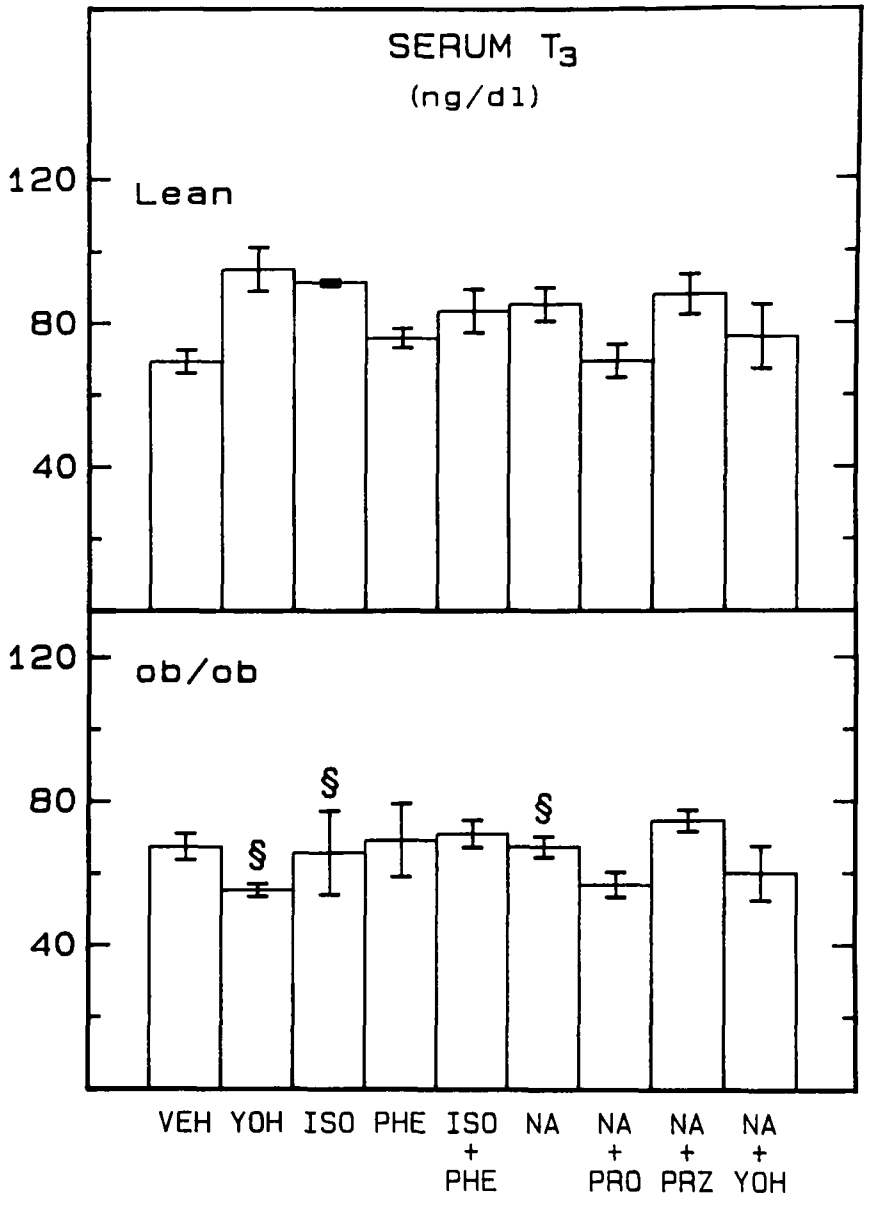


Figure 40: EFFECT OF VARIOUS ADRENERGIC DRUGS ON SERUM T_3 LEVELS OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Tables 41 and 42. A significant effect of obesity was symbolized by §. There was no significant effect of drug. Statistics were measured by ANOVA followed by Tukey's post-hoc test.

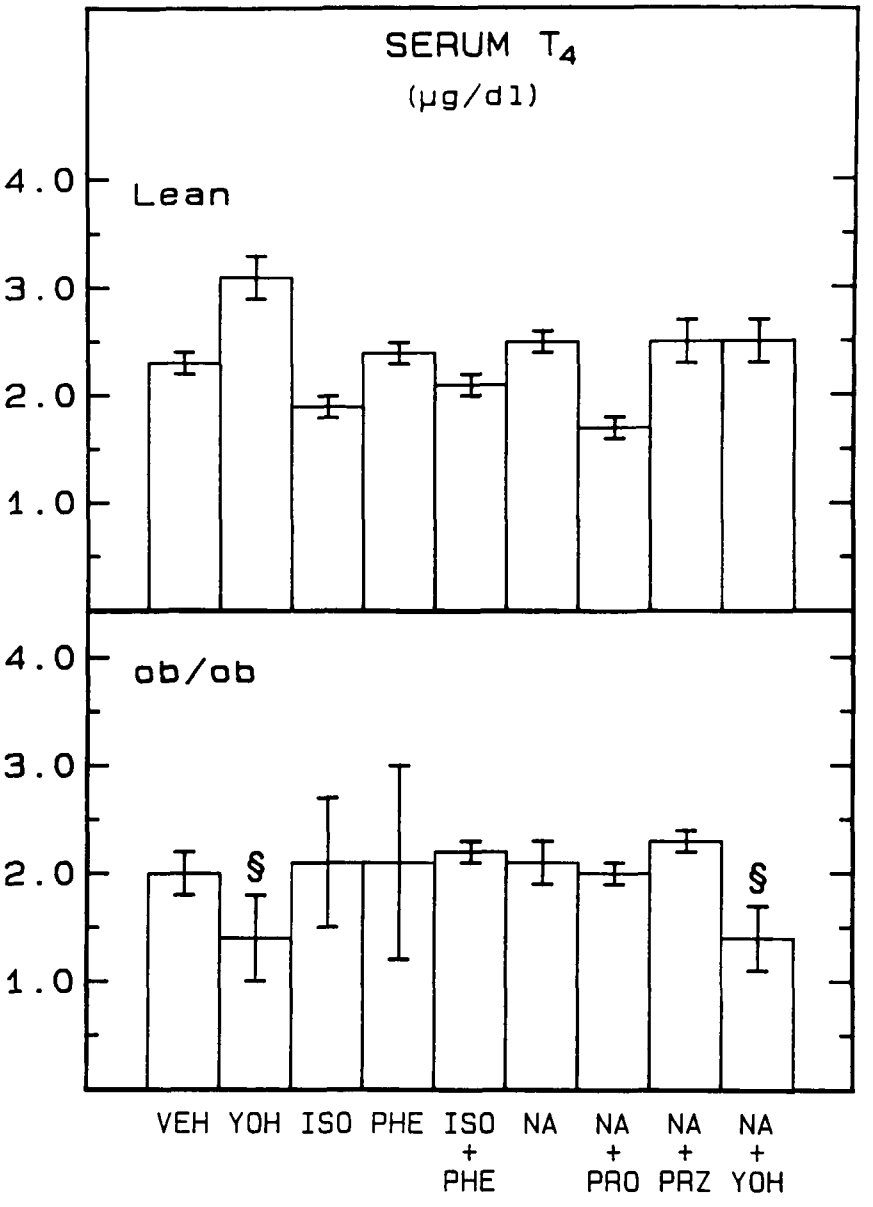


Figure 41: EFFECT OF VARIOUS ADRENERGIC DRUGS ON SERUM T_4 LEVELS OF LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Tables 41 and 42 (see Tables 41 and 42 for abbreviations). A significant effect of obesity was symbolized by §. There was no significant effect of drug. Statistics were measured by ANOVA followed by Tukey's post-hoc test.

TABLE 41

ACUTE INJECTION OF ADRENERGIC AGENTS:

EFFECT ON THYROID HORMONES AND BAT T5'D ACTIVITY

	LEAN MICE								
	Veh n 17	Yoh n = 3	Iso n = 6	Phe n 6	Iso + Phe n = 10	NA n = 18	NA + Prop n = 5	NA + Praz n = 9	NA + Yoh n = 3
Serum T_3 ng/dl	69.2 ± 3.2	95.0 ± 6.1	91.2 ± 0.9	75.6 ± 2.7	83.2 ± 6.1	85.1 ± 4.7	69.3 ± 4.6	87.8 ± 5.6	76.0 ± 9.0
Serum T_4 µg/dl	2.3 ± 0.1	3.1 ± 0.2	1.9 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	1.7 ± 0.1	2.5 ± 0.2	2.5 ± 0.2
$T_4$5'D SpAct. pmol/mg prot./h	0.08 ± 0.01	0.08 ±0.01	0.21 ± 0.03	0.10 ± 0.02	0.30* ± 0.05	0.39* ± 0.06	0.29 ± 0.09	0.19¶ ± 0.03	0.46* ± 0.06
$T_4$5'D TotAct. pmol/h	0.90 ± 0.08	0.90 ± 0.17	2.26 ± 0.32	1.08 ± 0.27	3.20* ± 0.49	4.65* ± 0.87	2.62¶ ± 0.77	1.95¶ ± 0.37	5.65* ± 0.59

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses). Statistics were measured by 2-way ANOVA followed by Tukey's HSD post-hoc test. § indicates a significant effect of obesity, * indicates a significant effect of drug compared to **Veh**, and ¶ indicates a significant effect of a blocking agent compared to **NA**. (See Table 13 for more detail).

TABLE 42

ACUTE INJECTION OF ADRENERGIC AGENTS:

EFFECT ON THYROID HORMONES AND BAT T5'D ACTIVITY

	OBESE MICE								
	Veh n = 14	Yoh n = 3	Iso n = 6	Phe n 6	Iso + Phe n = 10	NA n = 15	NA + Prop n = 6	NA + Praz n = 10	NA + Yoh n = 3
Serum T_3 ng/dl	67.3 ± 3.7	55.3§ ± 1.8	65.6§ ± 11.7	69.1 ± 10.2	70.9 ± 3.8	67.3§ ± 2.9	56.8 ± 3.5	74.6 ± 3.0	60.0 ± 7.6
Serum T_4 µg/dl	2.0 ± 0.2	1.4§ ± 0.4	2.1 ± 0.6	2.1 ± 0.9	2.2 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.3 ± 0.1	1.4§ ± 0.3
$T_4$5'D SpAct. pmol/mg prot./h	0.19 ± 0.06	0.14 ±0.04	0.17 ± 0.02	0.16 ± 0.01	0.22 ± 0.03	0.27 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.29 ± 0.02
$T_4$5'D TotAct. pmol/h	1.81 ± 0.14	2.03 ± 0.15	2.82 ± 0.38	2.30 ± 0.16	3.22* ± 0.53	4.15* ± 0.34	2.60¶ ± 0.16	2.31¶ ± 0.14	3.65* ± 0.08

Values are means ± SEM for the number (n) of animals per group. The abbreviations are as follows: vehicle, yohimbine, isoproterenol, phenylephrine, isoproterenol + phenylephrine, noradrenaline, noradrenaline + propranolol, noradrenaline + prazosin, noradrenaline + yohimbine (see method for doses). Statistics were measured by 2-way ANOVA followed by Tukey's HSD post-hoc test. § indicates a significant effect of obesity, * indicates a significant effect of drug compared to **Veh**, and ¶ indicates a significant effect of a blocking agent compared to **NA**. (See Table 13 for more detail).

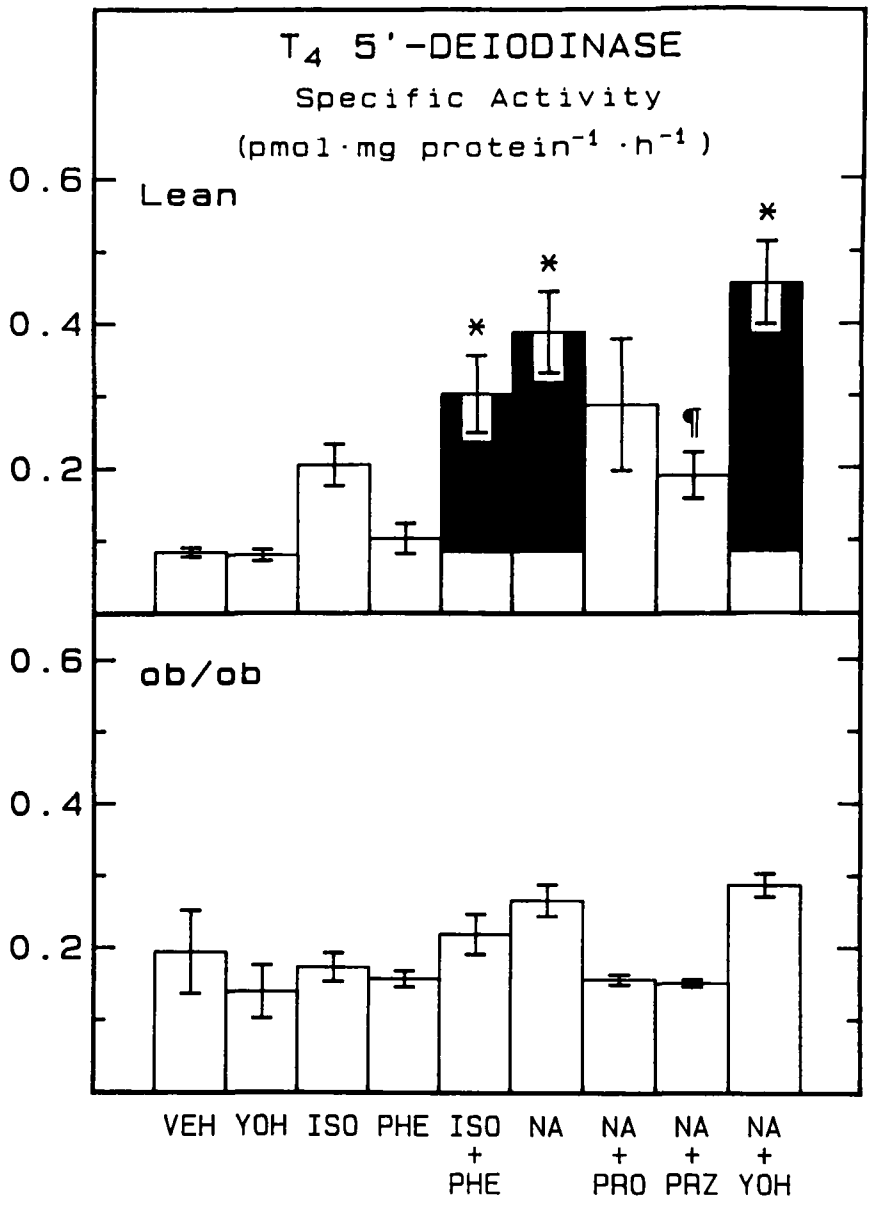


Figure 42: EFFECT OF VARIOUS ADRENERGIC DRUGS ON BAT T5'D SPECIFIC ACTIVITY IN LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Tables 41 and 42 (see Tables 41 and 42 for abbreviations). A significant effect of drug treatment compared to vehicle was symbolized by * and the darkened portions of the bars, ¶ symbolized a significant effect of a blocking agent compared to noradrenaline **NA**, other significant comparisons are listed in Table 43. There was no significant effect of drug treatment on BAT T5'D specific activity of ob/ob mice. Statistics were measured using two-way ANOVA followed by Tukey's post-hoc test.

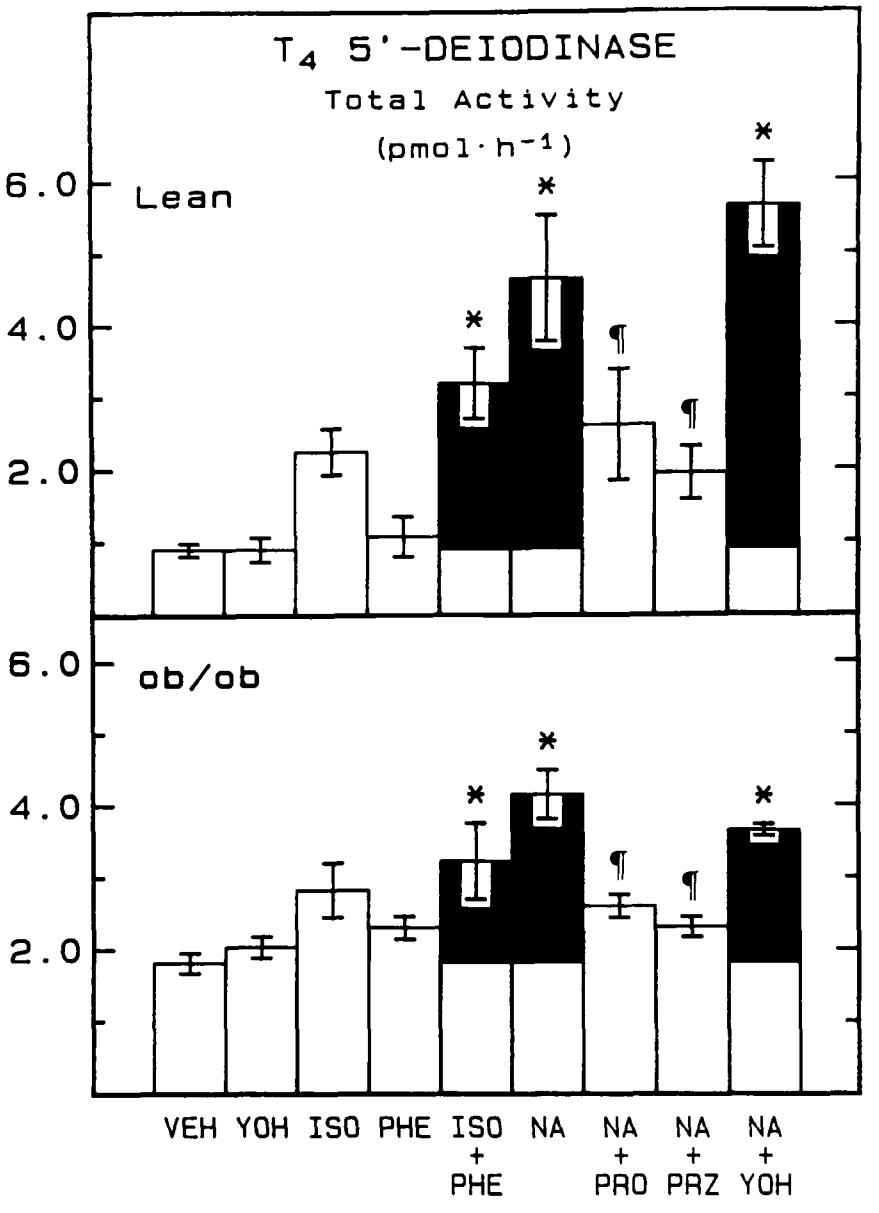


Figure 43: EFFECT OF VARIOUS ADRENERGIC DRUGS ON BAT T5'D TOTAL ACTIVITY IN LEAN AND OB/OB MICE. Values are means ± SEM for the number (n) of animals in Tables 41 and 42 (see Tables 41 and 42 for abbreviations). A significant effect of drug treatment compared to vehicle was symbolized by * and the darkened portions of the bars, ¶ symbolized a significant effect of a blocking agent compared to noradrenaline **NA**, other significant comparisons are listed in Table 43. Statistics were measured using two-way ANOVA followed by Tukey's post-hoc test.

TABLE 43

SUMMARY OF STATISTICS: SIGNIFICANT INTERACTIONS BETWEEN INJECTED DRUGS IN LEAN AND OB/OB MICE

THYROXINE 5'-DEIODINASE SPECIFIC ACTIVITY (pmol/h/mg Protein)

* Saline (**Veh**)	vs	Noradrenaline
		Phenylephrine + Isoproterenol
		Noradrenaline + Yohimbine
¶ Noradrenaline	vs	Saline
		Phenylephrine
		Isoproterenol
		Yohimbine
		Noradrenaline + Prazosin
Noradrenaline + Yohimbine	vs	Saline
		Phenylephrine
		Yohimbine
		Noradrenaline + Prazosin

THYROXINE 5'-DEIODINASE TOTAL ACTIVITY (pmol/h)

* Saline (**Veh**)	vs	Noradrenaline
		Phenylephrine + Isoproterenol
		Noradrenaline + Yohimbine
¶ Noradrenaline	vs	Saline
		Phenylephrine
		Isoproterenol
		Yohimbine
		Noradrenaline + Prazosin
		Noradrenaline + Propranolol
Noradrenaline + Yohimbine	vs	Saline
		Phenylephrine
		Yohimbine
		Noradrenaline + Prazosin

The statistics used were 2-way ANOVA followed by Tukey's HSD post-hoc test. The comparisons described above are significant at $p < 0.05$.

activity seen with ISO + PHE in ob/ob BAT T5'D total activity. Both propranolol and prazosin were able to inhibit the noradrenaline stimulated increase in BAT T5'D total activity. Yohimbine was unable to affect the noradrenaline stimulated increase in ob/ob BAT T5'D total activity (Table 42, 43 and Figure 43).

DISCUSSION: EFFECT OF ADRENERGIC AGONISTS ON BAT T5'D ACTIVITY OF LEAN AND OB/OB MICE

The most important observation from this experiment was that unlike the effect of cold, acute stimulation by noradrenaline was able to increase BAT T5'D total activity in both lean and obese (ob/ob) mice. The other principal conclusion is that increased activity of T5'D requires stimulation of both β- and α_1-adrenergic receptors in both lean and ob/ob mice. Obese mice have higher basal BAT T5'D activity, therefore, the percent increase in activity caused by noradrenaline was smaller in obese mice than in lean mice (2 fold vs 5 fold). It is also interesting to note that the results of the time course experiment (Expt. 1), which was performed in April, 1987 show a greater stimulation by acute noradrenaline injection than in this experiment, which was performed in August, 1987. Kaplan and Young, (1987), also observed a seasonal variation with their data.

The mechanism of the noradrenaline stimulated increase in BAT thermogenesis involves the interaction of noradrenaline with α_1- and β-adrenergic receptors (as discussed in the introduction). The finding by Silva and Larsen (1983) that BAT T5'D activity was controlled by noradrenaline through α_1-adrenergic receptors and mRNA synthesis was

required for this effect (Silva and Larsen, 1986a; Jones et al., 1986) ascribed this physiological function to the α_1-pathway in BAT (Nedergaard et al., 1986). In vivo there is a potentiating effect of α_1-adrenergic stimulation on thermogenesis resulting from β-receptor pathways (Foster, 1985; Ma and Foster, 1984). However, there is as yet no agreement about the exact nature of the β-adrenergic receptor. A number of workers suggest it is a β_1-adrenergic receptor (Bukowieki et al., 1978, 1980; Harris et al., 1986; Levin and Sullivan, 1986) or a mixture of β_1- and β_2-adrenergic receptors (Stribling, 1983; Rothwell et al., 1985; Scarpace et al., 1987). Some researchers find that both β_1 and β_2 agonists stimulate BAT thermogenesis equally well and interpret their findings as a third type β_{BAT}-adrenergic receptor, which is different from either β_1 or β_2 (Arch et al., 1984a,b, 1987, 1989). The finding that the β-adrenergic receptor in BAT is unique has led to the development of a number of compounds which ultimately would stimulate BAT specifically with very little stimulation in other tissues. The ultimate drug has not as yet been developed. Some compounds that are known to stimulate BAT thermogenesis are BRL 26830A (Arch et al., 1984 a,b, 1987, 1989), Ro 16-8714 (Meier et al., 1984) and Ly 79739 (Yen et al., 1984, 1988).

In my experiments, the adrenergic activation of BAT T5'D in lean and ob/ob mice was studied with the use of various adrenergic agonists and antagonists. In both lean and ob/ob mice I found a slight stimulation of activity by isoproterenol (mixed β-agonist) that was not statistically significant. Both lean and ob/ob mice have β-adrenergic receptors in BAT plasma membrane (Assimacopoulos-Jeannet et al., 1982).

The receptor number was increased 1.5 fold in purified BAT plasma membrane compared to lean controls. The authors suggest that this increased receptor number could be the consequence of an in vivo decrease in the noradrenaline concentration at the β-receptor Therefore, both lean and ob/ob mice should be able to respond to isoproterenol. Silva and Larsen, (1986a) observed that isoproterenol was able to stimulate rat BAT T5'D activity 3 fold and this modest but significant stimulation was blocked by propranolol. Isolated rat brown adipocytes do contain functional β-adrenergic receptors (Senault et al., 1984). However, Obregon et al. (1987) were unable to show a stimulation of T5'D in isolated rat brown adipocytes by isoproterenol. More recent work by Mills et al. (1989) using improved techniques was able to demonstrate a 3 fold stimulation of euthyroid rat brown adipocytes by 10 μM isoproterenol.

I did not find a stimulatory effect of phenylephrine (an agent with a higher ratio of α_1 to β-agonist activity than noradrenaline, Mohell et al., 1987) on BAT T5'D of lean or ob/ob mice. This result is not due to a lack of α_1-adrenergic receptors since both lean and ob/ob mice have typical α_1-adrenergic receptors (Zaror-Behrens and Himms-Hagen, 1986). GTG-obese mice have normal α_1-adrenergic receptors (Zaror-Behrens and Himms-Hagen, 1986) and they do not have a defective response of BAT T5'D to cold (Eley and Himms-Hagen, 1988) thus, the defective response to acute cold stimulation seen in ob/ob mice is not due to a defect in α_1-adrenergic receptors. The dose I used (26 μg/100 g bw) was similar to a dose of 50 μg/100 g bw used to study α- and β-adrenergic control of UCP mRNA in mice (Jacobsson et al., 1986). Silva

and Larsen have not published any data involving direct injection (an in vivo effect) of phenylephrine. However, Obregon, et al., 1987, show that in isolated rat brown adipocytes, phenylephrine (10 μM) is able to stimulate T5'D to the same level as norepinephrine (1 μM). However, Mills et al., (1989) were able to show and even greater stimulation with 5 μM PHE than 1 μM NA using their new system. Possibly if I had used a 10 fold higher concentration of phenylephrine I would have seen an effect. However, when both isoproterenol and phenylephrine were injected into lean and ob/ob mice at same time, an increase in BAT T5'D total activity was found. This increase was not significantly different from the noradrenaline stimulated increase in activity. In fact, the effect seemed to be additive (Table 41 and 42). A similar effect was found in hypothyroid brown adipocytes but not euthyroid brown adipocytes (Mills et al., 1989).

When propranolol (2.3 mg/100 g bw), a β-agonist, was injected together with noradrenaline, a significant decrease in BAT T5'D compared to noradrenaline alone was noted in lean and ob/ob mice. In contrast, Silva and Larsen, (1983), found no effect of 0.25 mg/100 g bw propranolol on the noradrenaline stimulated increase in BAT T5'D activity in rats. They also found no effect of 0.5 mg/100 g bw propranolol on the cold induced increase of T5'D, (Silva and Larsen, 1986). It is possible that either a higher dose of propranolol is needed to see an inhibition or that rats are different from mice in their response to propranolol. However, hypothyroid rat brown adipocytes are able to decrease their response to NA in the presence of propranolol whereas, euthyroid brown adipocytes increase their NA

stimulated activity further. The conclusion from my data is that there is an inhibitable β-adrenergic component to the noradrenaline induced stimulation of BAT T5'D activity in lean and ob/ob mice.

A significant inhibition of the noradrenaline induced increase in BAT T5'D activity was observed with prazosin (an α_1-antagonist, see Cavero and Roach, 1980 for a review), in both lean and ob/ob mice. This result agrees with the work of Silva and Larsen. (1983), who saw a 60 % inhibition with 0.2 mg/100 g bw. I also saw a 60 % inhibition but with a 5 fold lower dose, (40 µg/100 g bw). Silva and Larsen (1983, 1985) found that the cold induced increase in BAT T5'D activity could be completely blocked with prazosin but not with propranolol (0.5 mg/100 g bw), (Silva and Larsen, 1986). Thus, an α_1-component to the noradrenaline stimulated increase in BAT T5'D activity is also observed.

Both lean and ob/ob mice have typical α_2-adrenergic receptors in BAT (Zaror-Behrens and Himms-Hagen, 1989). Yohimbine, an α_2-antagonist had no effect on BAT T5'D activity in lean or ob/ob mice. When injected with noradrenaline, there was no inhibition or stimulation of the noradrenaline stimulated increase in enzyme activity. This indicates that there was no α_2 component to the noradrenaline induced increase in BAT T5'D activity. This result also agrees with the work of Silva and Larsen (1983), who also noted no effect of yohimbine on the cold stimulated increase in BAT T5'D activity.

The mechanism for the stimulation BAT T5'D by the synergistic interaction of α_1- and β- agonist is unknown. The activation of BAT T5'D by noradrenaline requires mRNA synthesis (Silva and Larsen, 1986a;

Jones et al., 1986). However, the gene for the type II BAT T5'D has not been cloned. For this reason there have not been any studies of the effects of various adrenergic drugs on BAT T5'D mRNA as has been done with BAT UCP mRNA (Jacobsson et al.,1987). Recently Barge et al. (1988) have shown that the translocation of protein kinase C from cytosol to particulate fraction is not sufficient to increase BAT T5'D activity but can modulate the α_1-adrenergic agonist-mediated response in these cells. Further research is necessary in order to determine the "second messengers" involved in the regulation of the gene for T5'D by NA acting on both β- and α_1-adrenergic receptors.

GDP-Binding:

There was no significant effect of an acute injection of adrenergic agents on BAT mitochondria GDP-binding of lean or ob/ob mice (Table 39 and 40). Peachey et al. (1988) injected 50 µg/100 g bw noradrenaline into lean and obese Zucker rats. They found an increase in GDP-binding with no change in the concentration of BAT UCP 30 minutes after the injection in both genotypes. It is possible that there could be an immediate unmasking effect of noradrenaline on isolated BAT mitochondria of lean and ob/ob mice, but at the time I studied these animals (3 hours after injection) no effect was noted. The time course of cold stimulation of BAT T5'D indicated that there was a lag-phase of approximately 1 hour. Gene transcription and protein synthesis are necessary for the cold induced increase to occur (Jones et al., 1986). Likewise, gene transcription and protein synthesis are also necessary for the noradrenaline stimulated increase in BAT T5'D (Silva and

Larsen, 1986a). Thus, in order to study T5'D activity, I was unable to optimize the conditions to study GDP-binding. Jacobsson et al.,(1986), found that in mice, the increase in vivo of UCP mRNA requires stimulation of both α- and β- adrenergic receptors and they also found a synergistic effect of isoproterenol and phenylephrine. The increase in UCP mRNA stimulated by acute 4 hour noradrenaline injection was much smaller than the increase stimulated by 4 hour cold exposure (Jacobsson et al.. 1987). In Experiment #1 noradrenaline did not affect the amount of BAT UCP, even 12 hours after the injection. Thus one would not expect to find an effect of acute noradrenaline injection on GDP-binding 3 hours after the injection. However, long term treatments of C57BL/6J lean and ob/ob mice with the β-adrenergic agonist cimaterol were able to stimulate GDP-binding to BAT mitochondria equally well in lean and ob/ob mice (Walker and Romsos, 1988). Lean and ob/ob mice of the Aston strain were both able to increase GDP-binding to the same extent in response to noradrenaline (0.64 mg/Kg, s.c. and killed 30 minutes later) and to BRL 26830A (8.4 mg/Kg, orally and killed 60 minutes later) (Wilson et al., 1984). Thus, ob/ob mice do have the capacity to respond to noradrenaline secreted in response to cold by an increase in GDP-binding.

Thyroid hormones:

Acute injection of yohimbine, isoproterenol and noradrenaline caused a decrease in serum T_3 and yohimbine and the combination of noradrenaline and yohimbine caused a decrease in serum T_4 in ob/ob mice but not in lean mice. Noradrenaline exerts a dual action on the

secretion of thyroid hormones from mouse thyroid glands. Acute injection of noradrenaline causes a small stimulation of the release of thyroxine from the thyroid gland. However, noradrenaline injected with TSH (thyroid stimulating hormone) inhibits the TSH stimulated release of T_4 (Ahrén et al., 1986). This action of noradrenaline to inhibit the TSH-induced thyroid hormone secretion is mediated mainly through α_1-adrenergic receptors with α_2-adrenergic receptors playing a minor role (Muraki et al., 1982). However, there was no effect of any adrenergic agent on serum thyroid hormone levels in lean mice. It is possible that ob/ob mice may be more sensitive to the adrenergic drugs injected thus, yohimbine may have an inhibitory effect on the secretion of T_4 and T_3 from ob/ob mouse thyroid glands. It is also possible that yohimbine, isoproterenol and noradrenaline could have caused an increase in clearance of serum T_3 and T_4 in the ob/ob mouse.

SUMMARY AND CONCLUSION

In conclusion both lean and obese (ob/ob) mice respond to an acute injection of noradrenaline by increasing BAT T5'D activity. This increase is mediated by both α- and β- adrenergic receptors. The response of BAT T5'D activity in obese (ob/ob) mice to acute injection of noradrenaline is normal. Thus ob/ob mice do have the capacity to respond to the cold stimulated increase in noradrenaline turnover (Zaror-Behrens and Himms-Hagen, 1983) but I cannot rule out the possibility that another component of the cold response other than noradrenaline is responsible for the defective response of ob/ob BAT T5'D to acute cold stimulation.

CHAPTER 4:

PRESENCE OF THYROXINE 5'-DEIODINASE ACTIVITY IN BROWN ADIPOSE TISSUE OF THE CYNOMOLGUS MONKEY

BACKGROUND:

During the spring of 1986, I had the opportunity to obtain BAT from the primate Macaca fascicularis or the cynomolgus monkey. A long-term feeding experiment in the laboratory of Dr. J. Beare-Rogers had come to an end and various researchers in the area were invited to use any tissue that was not required for the primary study. I was able to obtain BAT from the control animals.

Histological studies had shown BAT to be present in many primate species such as: silvery marmoset, pigtail macaque, baboon, moor, guenon, patus and colobus monkey, lemur, slow loris, and bush baby (Rowlatt et al., 1971). Biochemical studies had shown BAT to be active in rhesus monkeys (Swick et al., 1986) and the common marmoset, (Rothwell and Stock, 1985). However, T5'D had not been measured in BAT of any monkey.

Itoh and Hiroshige (1967) reported that BAT was not present in Macaca cynomolgus. Chaffee et al., (1969) speculate that this could be due to the tropical environment of this species. The technique used to determine that BAT was not present involved only histology. When BAT is inactive, the adipocytes fill with lipid and become unilocular, and resemble WAT. We now have rabbit antibodies prepared against hamster UCP (see Method Section # 11) that can be used to demonstrate the

presence of UCP using an immunoblot (Method Section #10) and to quantitate the amount of UCP using a solid phase RIA (Section # 12).

OBJECTIVES:

The objectives of this study were two fold:

1) To demonstrate the presence of BAT in the primate Macaca fascicularis or cynomolgus monkey and
2) To demonstrate T5'D activity in BAT of the cynomolgus monkey.

METHODS:

Five female (mean age 7.4 years) and five male (mean age 6.0 years) cynomolgus monkeys (Macaca fascicularis) were used in this study. The maximum life span of these monkeys is 8 - 12 years, thus they were considered adult monkeys. The average weight of the males was 4.7 ± 0.6 Kg and females was 3.4 ± 0.5 Kg. The animals were housed in individual cages at 24 °C, 70 % humidity and a 12 : 12, light/dark schedule with lights on at 7:00 am.

The animals were fed a diet consisting of 20 % protein (casein), 50 % carbohydrates (corn starch, dextran, sucrose), 30 % fat (lard and corn oil), 10 % fiber, vitamins and minerals. These ingredients were mixed with agar and made into 50 g dough-balls. The animals received 200 g/day of the dough balls, 500 ml/day of apple juice, a fresh apple or carrot every second day and free access to tap water. This diet met all the nutrient requirements for monkeys of this age.

The animals were killed by injection of Rogarsetic (ketamine-HCl,

Bristol Labs.) followed by exsanguination and perfusion. Dr. R. Mueller dissected tissue from the axillary, subscapular, cervical, interscapular and perirenal fat pads. He also removed samples of subcutaneous WAT. The tissue samples were placed in ice-cold buffered medium (0.25 M sucrose, 1.0 mM HEPES, 0.2 mM EDTA, potassium salt, at pH 7.2) and small slices of each tissue sample were placed in 10 % formalin for the histological studies performed by Dr. Mueller.

The tissue samples were cleaned and weighed and were homogenized in 4 times weight/volume of ice-cold buffered medium using a polytron at setting 4 for 15 seconds. The homogenates were frozen in liquid nitrogen and stored at - 70 °C.

The histology was performed by Dr. R. Mueller using the Novelli stain (Novelli, 1959). Briefly, tissue was fixed in 10 % formalin, embedded in paraffin, and sectioned. Paraffin was then removed and the sections were placed in a 60 °C solution of 1 N HCl, for 3 minutes, rinsed in water then placed in 1 % fuchsin acid for 30 seconds, rinsed in water and placed in 1 % light green aqueous solution for 1 to 3 minutes and finally rinsed in water. The tissue was dehydrated in 95 % and absolute alcohol and mounted in balsam. Using this stain, mitochondria were stained purple-red, the peripheral wall, chromatin and collagen stained green, muscle tissue stained purple and erythrocytes stained a brilliant red.

BAT T5'D activity was measured using the method described in Methods Section #15 using 60 µg of homogenate protein to start the reaction. Homogenate proteins were measured as described in Methods Section #7.

Homogenate proteins were separated using SDS-PAGE and transferred to nitrocellulose as described in Methods Section #10. Antiserum prepared using hamster UCP (Method Sections # 9, 10, 11) and ^{125}I protein A were used to detect the presence of UCP in monkey homogenates. A solid phase RIA was used to quantitate monkey BAT UCP (Methods Section #12). The monkey homogenates were diluted to 0.5 mg/ml and extracted with 0.5 % triton X-100 as described in Section #12. Mouse UCP was purified (Section #10) and used as the standard in the solid phase RIA. Antisera prepared in Section #11 was also used in the solid phase RIA for UCP.

RESULTS:

The tissue samples were distinctly yellow in colour. Subcutaneous WAT was a very brilliant yellow whereas the "BAT" samples did show lobular areas that were shades darker. The tissue looked very different from rodent BAT and upon visual inspection one would not assume the samples were BAT.

Histology:

Plates 2 and 3 show typical multilocular brown adipocytes. The Novelli stain shows many mitochondria (stained purple-red). The difference between WAT and BAT is illustrated quite clearly in Plate 2. White adipocytes are unilocular and do not possess many mitochondria.

Immunoblots:

In order to confirm the histological identification of BAT, I separated monkey homogenate proteins by SDS-PAGE, transferred the proteins to nitrocellulose and performed an immunoblot with rabbit

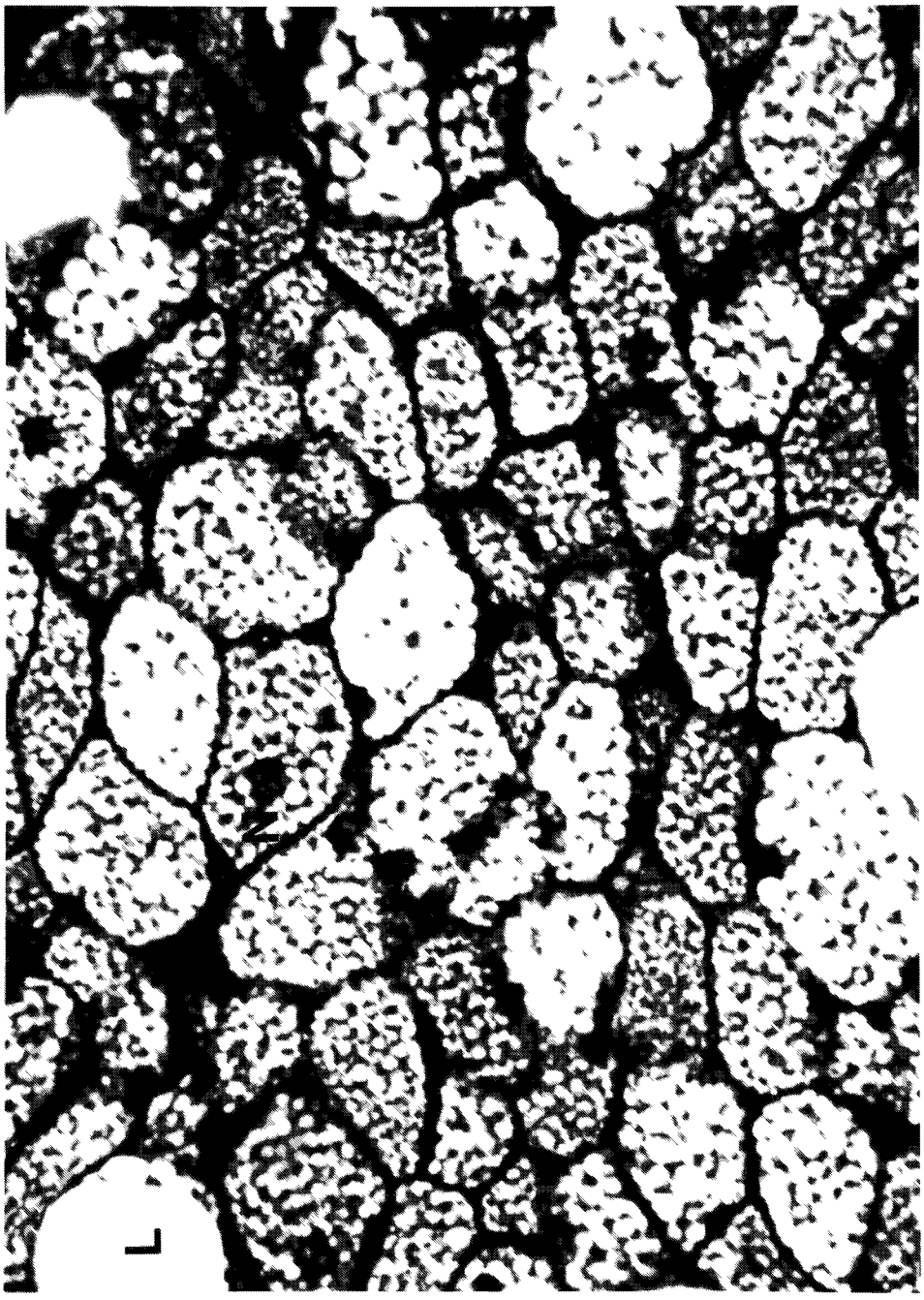
Plate 2: **NOVELLI STAIN OF WHITE ADIPOSE TISSUE AND BROWN ADIPOSE TISSUE OF THE MONKEY *MACACA FASCICULARIS*.**

WAT (W)is characterized by large unilocular lipid droplets and few mitochondria (see far left of slide). Connective tissue (C) is stained green. Typical multilocular brown adipocytes are seen on the left hand side of the slide. Brown adipocytes have numerous mitochondria which are stained purple-red by the Novelli stain. This slide is a section of interscapular BAT but other depots showed similar results. The magnification of this slide was 250 X.


O
W

Plate 3: **NOVELLI STAIN OF AXILLARY BAT OF THE MONKEY *MACACA FASCICULARIS*.**

This slide shows typical multilocular brown adipocytes enlarged to 1000 X with mitochondria stained purple-red and nuclei (N) stained blue. Large lipid droplets (L) are also seen. Other BAT depots showed similar results.


L

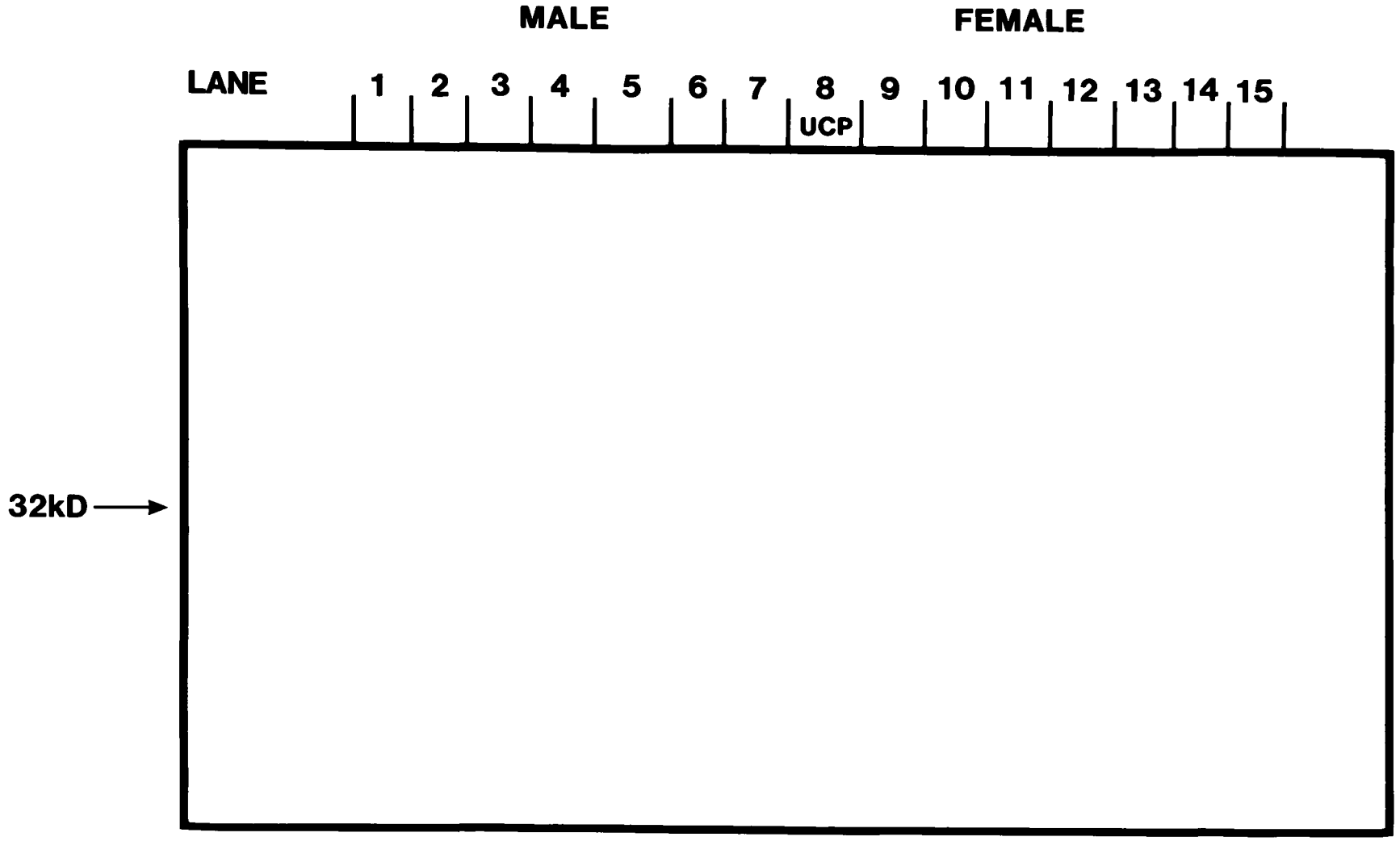
antibodies against hamster UCP. The autoradiogram shown in Plate 4 demonstrates that WAT from males and females (lane 7 and 14) showed no cross-reactivity with the antiserum. Purified rat UCP was run in lane 8 and cross reacted with the antiserum at a molecular weight of 32,000 Daltons. Monkey samples showed cross-reactivity with the antiserum. In some depots monkey BAT UCP was slightly larger than rat UCP (female interscapular, subscapular and axillary, lanes 9, 10, 11) and in some depots monkey UCP was slightly smaller than rat UCP. Some depots show more than one band, which might indicate breakdown of UCP or actually two cross-reacting proteins.

In order to compare cross-reactivities of monkey BAT UCP to that of rat, mouse and hamster BAT UCP, I separated samples from the purification procedure for mouse and hamster UCP. These samples were compared to monkey axillary, subscapular and interscapular BAT. The purification of mouse UCP is followed in lanes 2-4 of the stained SDS-gel (Plate 5). Purified rat (lane 5) and mouse (lane 4) UCP have the same electrophoretic mobility. The purification of hamster UCP was followed in lanes 6-8. Hamster UCP has an electrophoretic mobility that is slightly slower than that of rats and mice. The monkey samples (lanes 11, 12, 13) show very similar protein bands to that of hamster and mouse.

The autoradiogram of the immunoblot of the electrotransferred proteins in Plate 5 is shown in Plate 6. Unfortunately lanes 1 and 2 were not completely transferred. Mouse and rat UCP seem to have the same molecular weight. Hamster UCP has a slightly slower electrophoretic mobility, indicating a slightly greater molecular

Plate 4: **AUTORADIOGRAM OF AN IMMUNOBLOT OF MALE AND FEMALE MONKEY TISSUE.**

Monkey BAT and WAT homogenate samples were separated by SDS PAGE as follows: lane 1 (molecular weight markers), lane 2 (male interscapular BAT), lane 3 (male subscapular BAT), lane 4 (male axillary BAT), lane 5 (blank), lane 6 (male cervical BAT), lane 7 (male WAT), lane 8 (RAT UCP Standard, 6 µg), lane 9 (female interscapular BAT), lane 10 (female subscapular BAT), lane 11 (female axillary BAT), lane 12 (female perirenal BAT), lane 13 (female cervical BAT), lane 14 (female WAT), lane 15 (M.W. markers). All monkey homogenates were 50 µg. All monkey BAT samples showed immunoreactivity with the hamster UCP antiserum. The WAT samples in lanes 7 and 14 did not show immunoreactivity to hamster UCP antiserum.



MONKEY BAT (2-6, 9-13) AND WAT (7, 14)

Plate 5: **SDS PAGE OF MOUSE, RAT, HAMSTER AND MONKEY BAT AND MONKEY WAT HOMOGENATES.**

Samples from various stages of the purification of mouse and hamster BAT UCP were separated on SDS PAGE and stained with Coomasie Blue as follows: lane 1 (molecular weight markers, 12.0 µg), lane 2 (mouse BAT lubrol extract, 15.0 µg), lane 3 (mouse BAT triton X-100 extract, 20.0 µg), lane 4 (hydroxylapatite column fractions #8-23, 5 µg), lane 5 (rat UCP standard, 5 µg), lane 6 (hamster UCP standard, 5 µg), lane 7 (hamster triton X-100 extract, 20 µg), lane 8 (hamster lubrol extract 15 µg), lane 11 (male monkey axillary BAT, 15 µg), lane 12 (male monkey subscapular BAT, 15 µg), lane 13 (female monkey interscapular BAT, 15 µg), lane 14 (monkey WAT, 75 µg), lane 15 (molecular weight markers, 12 µg). See materials and methods for more details.

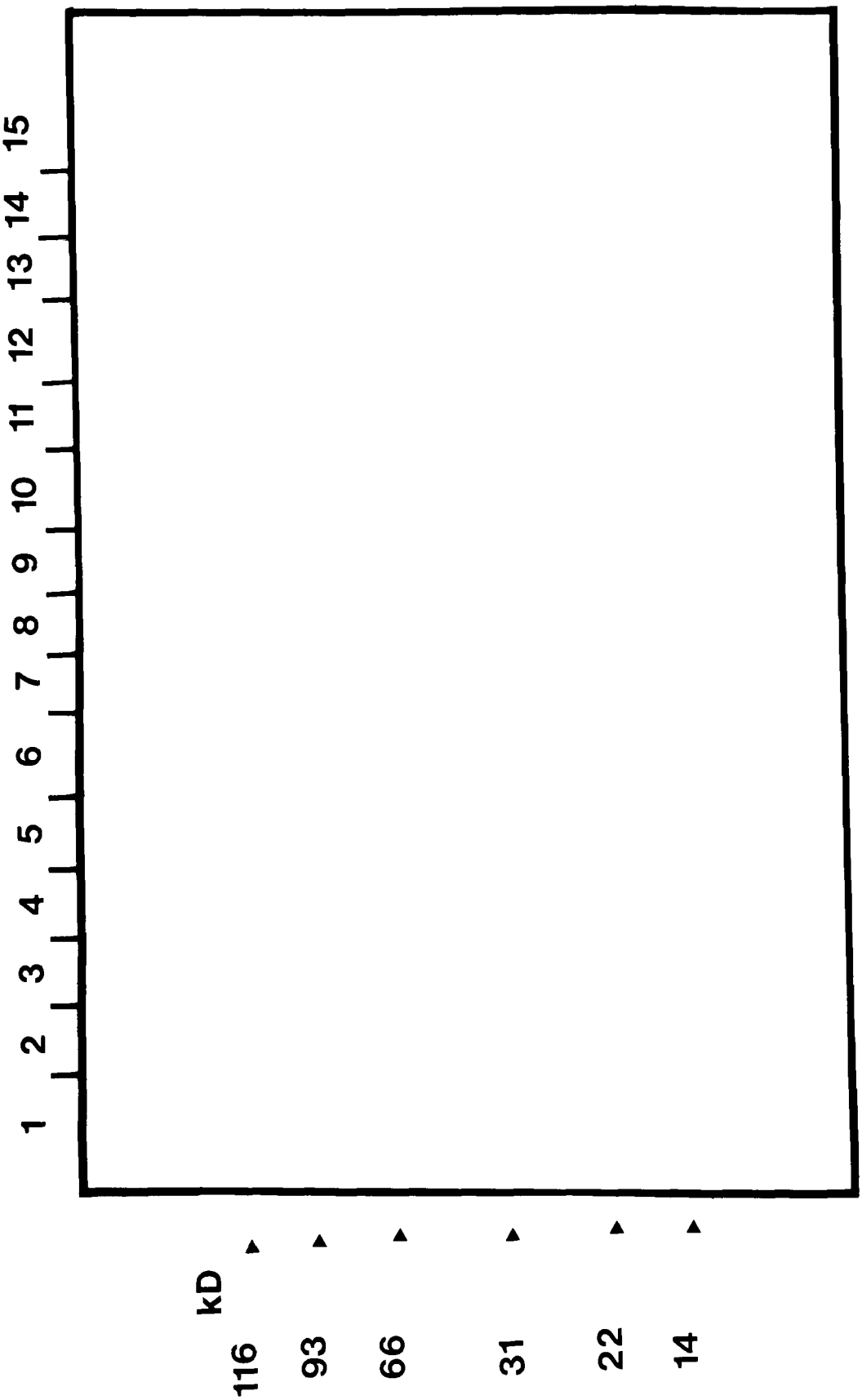

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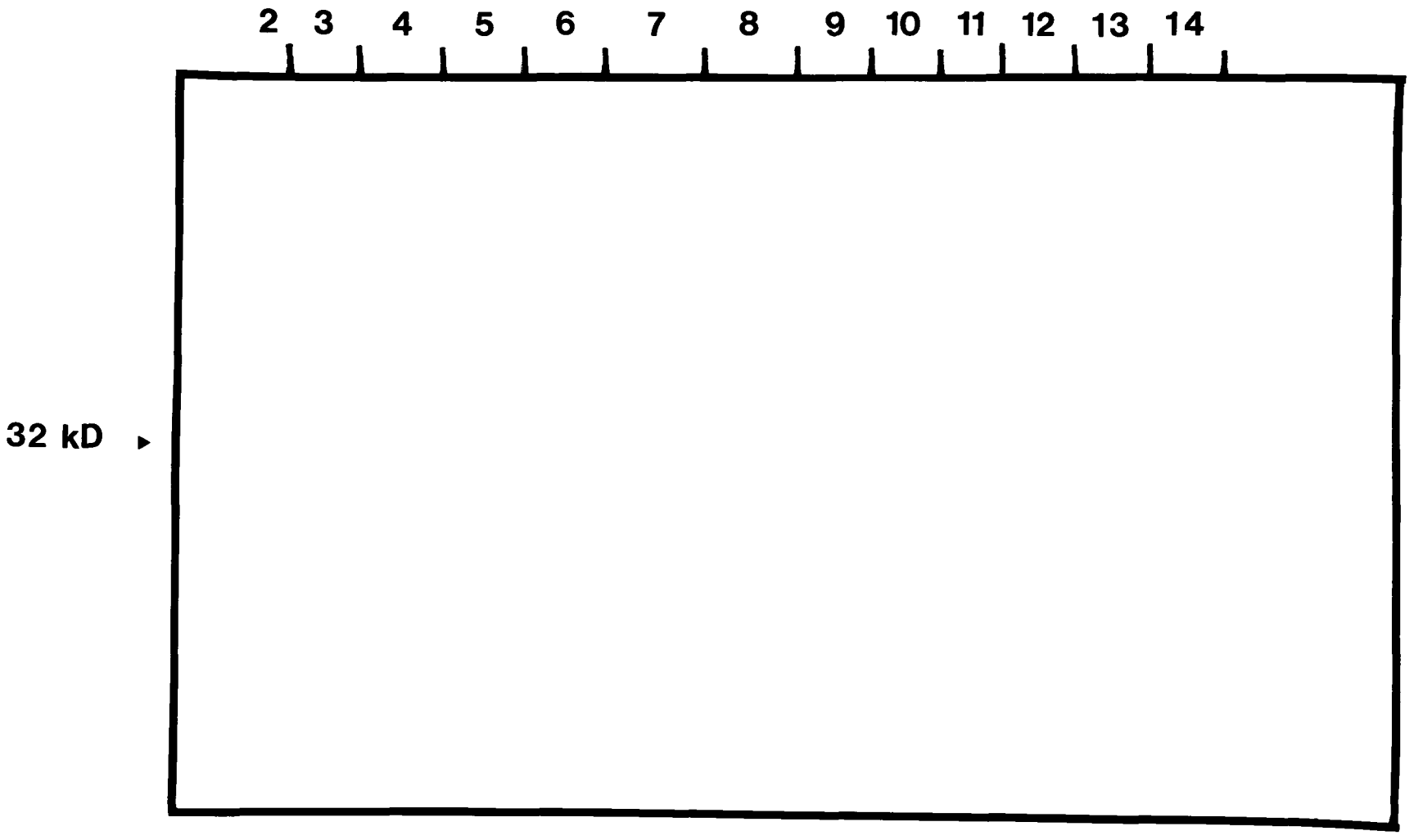
Plate 6: **AUTORADIOGRAM OF AN IMMUNOBLOT OF MOUSE, RAT, HAMSTER AND MONKEY BAT AND MONKEY WAT.**

The gel shown in Plate 5 was immunoblotted (as described in the methods section) and an autoradiogram was made by exposing for 19 hours and developing for 1 minute. Mouse and rat UCP (lanes 2,3,4 and) show similar molecular weights, whereas hamster (lanes 6, 7 and 8) have a slightly higher molecular weight. Monkey BAT (lanes 11, 12, and 13) show cross reactivity with hamster UCP antiserum, whereas monkey WAT (lane 14) did not.


2 3 4 5 6 7 8 9 10 11 12 13 14
32 kD

weight than that of rat and mouse UCP. Monkey WAT did not cross-react with the antisera. The monkey BAT samples did show cross-reactivity with the antibody at a slightly slower electrophoretic mobility, therefore, a higher molecular weight than rat mouse or hamster UCP.

T_4 5'-Deiodinase Activity:

The tissue wet weight varied considerably from perirenal to subscapular depots (Table 44). It was difficult to obtain perirenal tissue because the kidney was used by other researchers and often I was unable to obtain a sample. Protein content of the homogenates also varied for the same reason. Since the amount of tissue dissected from the animal was variable and therefore protein content was variable, the best indication of T5'D activity was the specific activity and not the total activity.

BAT T5'D activity was found in all depots studied. WAT showed only background levels of activity demonstrating that PTU-insensitive activity is not found in monkey WAT. Axillary, subscapular and interscapular BAT had high levels of T5'D with cervical and perirenal tending to have somewhat lower levels. The level of activity of T5'D in interscapular BAT of monkey is much higher than that of mouse, hamster or rat under similar temperature and housing conditions (see Figure 44).

Solid-Phase RIA:

BAT of the cynomolgus monkey contains UCP, whereas WAT only showed background levels. There was considerable variation in this assay, therefore, not all depots were significantly different from zero.

TABLE 44

BROWN ADIPOSE TISSUE OF THE MONKEY *MACACA FASCICULARIS*

	AXILLARY n=10	SUBSCAPULAR n=9	INTERSCAPULAR n=7	CERVICAL n=10	PERIRENAL n=6	WHITE ADIPOSE TISSUE n=10
Tissue Wet Weight g	0.89 ± 0.14	1.48 ± 0.22	1.36 ± 0.37	0.40 ± 0.06	0.39 ± 0.07	1.18 ± 0.15
Protein mg	37.0 ± 7.7	46.8 ± 8.3	29.9 ± 5.9	14.0 ± 3.4	9.3 ± 2.3	11.7 ± 1.5
T_4 5'-D Spec. pmol/h/mg Prot.	1.00* ± 0.14	0.95* ± 0.15	0.82* ± 0.24	0.65* ± 0.11	0.45 ± 0.14	0.01 ± 0.00
T_4 5'-D Total pmol/h	34.1 ± 6.7	52.1* ± 16.6	29.4 ± 13.5	9.9 ± 3.0	5.5 ± 2.5	0.09 ± 0.05
Uncoupling Protein UCP µg/mg Protein	4.3* ± 0.9	5.1* ± 1.0	3.0 ± 1.0	2.5 ± 0.3	4.3* ± 1.7	0.3 ± 0.1

Values are means ± SEM for the number (n) of animals per group. * indicates a value significantly different from zero ($p < 0.05$). The statistics were measured using ANOVA followed by Tukey's HSD post-hoc test.

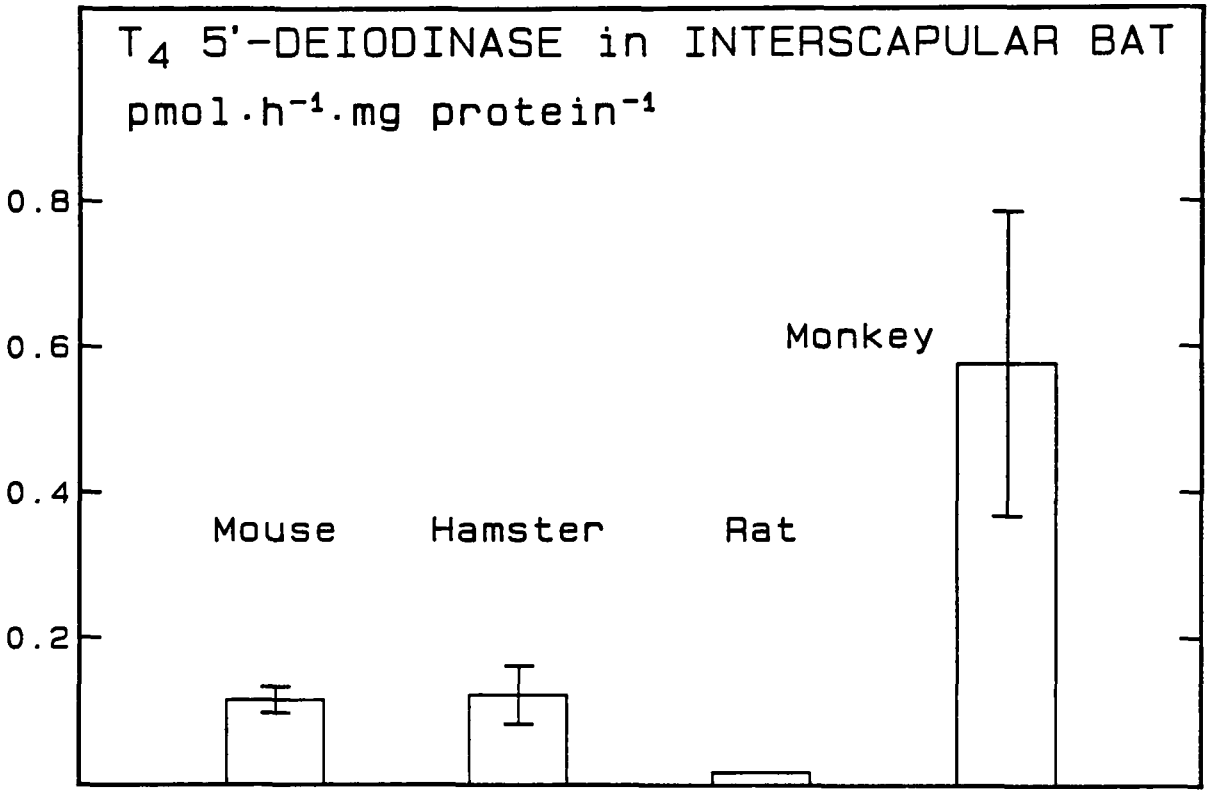


Figure 44: THYROXINE 5'-DEIODINASE ACTIVITY IN INTERSCAPULAR BAT OF THE MOUSE, HAMSTER, RAT AND MONKEY. Values are means ± SEM for the number (n) of animals as follows: lean mice (n = 4), hamster (n = 14), rat (n=4) and monkey (n = 7). The animals were housed under similar conditions; mice (28 °C), hamsters (24 °C), rats (28 °C) and monkey (24 °C). Therefore, monkey interscapular BAT had higher T5'D specific activity than any other animal studied. The data for rats and hamsters was from Kopecky et al. (1986).

DISCUSSION:

The main finding from this work is that the cynomolgus monkey does indeed have BAT in all depots studied. Histologically the tissue showed typical multilocular brown adipocytes and the border area between WAT and BAT was also very similar to that seen in the young rat (Cannon and Nedergaard, 1985; Smith and Horwitz, 1969). Itoh and Hiroshige (1967) studied the cynomolgus monkey while it was living in a tropical environment. This study was done at 24 °C. Possibly our study was able to histologically demonstrate BAT because the monkeys were in a relatively cold environment, thus activating BAT thermogenesis.

The definitive identification of BAT is to demonstrate the presence of UCP. This was done first using immunoblots (Plates 4 and 6) and then using a solid phase RIA (Table 1). My results of the immunoblots agree with that of Houstek *et al.*, (1987), who also used rabbit antibodies against hamster UCP in that we both find cross reactivity with rat and hamster UCP but the mobility of the rat antigen is slightly higher (indicating a lower molecular weight) than that of the hamster antigen. In contrast, Henningfield and Swick, (1987) used rabbit antibodies against rat UCP and found that purified UCP from hamster showed an electrophoretic mobility similar to that of rat UCP. However, when the authors electrophoresed mitochondria isolated from hamster BAT they were able to see the slower electrophoretic mobility we observed. I also observed that rat and mouse UCP was of the same molecular mass. The complete sequence of UCP has been established for both rat (Bouillaud *et al.*, 1986, 1988a; Ridley *et al.*, 1986) and

hamster (Aquila et al., 1985). The homology between the two proteins is 91.5 % (Aquila et al., 1985) and the molecular weights are 33,084 Daltons for rat UCP and 33,185 for hamster UCP which is in agreement with the results of the immunoblot in Plate 6. I observed cross reactivity of rabbit antibodies to hamster UCP with monkey BAT. In some cases the electrophoretic mobility was slower than that of rat UCP and in others the mobility was faster. In some cases I observed two bands. Henningfield and Swick, (1987), found that BAT mitochondria from the rhesus monkey (Macaca mulatta) produced single bands of slightly faster electrophoretic mobility than that of rat BAT mitochondria. Afong et al., (1985) found a similar relationship between rat BAT UCP and human infant BAT UCP.

I did observe cross-reactivity of the rabbit antibody to hamster BAT UCP to both mouse BAT UCP and monkey BAT UCP. Thus, I was able to use a mouse BAT UCP standard curve to quantitate the amount of UCP in monkey BAT homogenates using a solid phase RIA. My results (Table 44) show that WAT had only background levels of UCP where as BAT depots showed levels of BAT UCP similar to that of lean mice (Chapter 3, Table 32 and Chapter 1, Part 1, Experiment #2, Table 6). These results agree with Henningfield and Swick (1987) who measured UCP levels in BAT mitochondria of adult female monkeys (Macaca mulatta) and found values that were slightly lower than warm acclimated Charles River mice.

Another important observation from this work is that monkey BAT does possess the enzyme T_4 5'-deiodinase (Table 44). The level of specific activity of T5'D is much higher than that observed in interscapular BAT of rats, mice and hamsters under similar

environmental conditions (Figure 44). It is interesting to note that in spite of the 10 fold greater T5'D activity in monkey BAT compared to mouse BAT, both mouse and monkey had approximately the same amount of UCP. Unfortunately I was unable to obtain blood samples from these monkeys, thus, I am unable to comment about the relationship between BAT T5'D and serum T_3 levels in monkeys. However, I can speculate that since T5'D is present in BAT of the cynomolgus monkey it is possible that the deiodination of T_4 by this tissue could be important not only for optimum BAT metabolism but also for overall thyroid hormone metabolism. BAT is present in humans, this has been demonstrated using immunoblots and the solid phase RIA (Afong et al., 1985; Lean and James, 1983; Cunningham et al., 1985; Lean et al., 1987). We do not know if human BAT has an active T5'D similar to that of rats, mice, hamsters and now monkey, but it seems likely. If this is the case, then BAT in humans could serve an important role in thyroid hormone metabolism. Further research in this area is necessary before we can confirm this speculation.

GENERAL DISCUSSION AND CONCLUSION

The experiments described in this thesis have provided information about the normal regulation of BAT T5'D activity in mice at 28 °C and its relationship to BAT thermogenesis in a variety of circumstances. Also the defective regulation of BAT T5'D activity in the ob/ob mouse and its relationship to defective control of BAT thermogenesis in this genetic model of obesity was studied.

The failure of ob/ob mice to increase their BAT T5'D activity when acutely exposed to cold is related to the failure to activate BAT mitochondrial thermogenesis (see Chapter 1, Part 1) and an attenuated increase in the level of mRNA for uncoupling protein (Reichling et al., 1988). Serum T_3 likewise does not increase in the acutely cold-exposed ob/ob mouse. It is known that the sympathetic activity is increased in BAT of the acutely cold-exposed ob/ob mouse (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen, 1983). The optimum expression of the BAT UCP gene requires both noradrenaline and the endogenous production of T_3 (Silva, 1988; Bianco and Silva, 1987a,b). Noradrenaline is the major stimulus and T_3 acts to amplify the NA signal (Silva, 1988; Bianco et al., 1988). It is thus possible to conclude that the defective thermogenesis and trophic response of BAT of the ob/ob mouse to stimulation by cold is associated with, and may well be secondary to, a failure to increase endogenous T_3 production in BAT via an increased synthesis of T5'D.

Two procedures that were known to improve the defective thermogenic functioning of BAT in ob/ob mice were found to enhance the cold-induced

increase in T5'D activity.

The gradual acclimation of ob/ob mice to mild cold allows growth of BAT and an increase in BAT T5'D activity (Chapter 1, Part 2). Gradual acclimation to cold is known to stimulate sympathetic activity in BAT (Knehans and Romsos, 1983; Zaror-Behrens and Himms-Hagen,1983). Thus, ob/ob mice are eventually able to respond to the stimulation of cold by increasing BAT thermogenesis. Cold acclimation of lean mice also causes growth of BAT, however, BAT T5'D activity returns to basal levels. This indicates that the activity of BAT T5'D is important in the acute phase of cold exposure and there is a disassociation between BAT T5'D activity and BAT thermogenesis and growth in cold acclimated lean mice. Obese mice have a much slower response to cold and BAT T5'D activity is positively related to BAT growth and thermogenesis.

Adrenalectomy of the ob/ob mouse allows a normal cold-induced increase in BAT T5'D, improves the cold-induced activation of BAT thermogenesis and permits a supranormal cold-induced increase in BAT UCP level (Chapter 1, Part 2).

These two experiments in which the effect of gradual cold acclimation or of adrenalectomy was studied indicate that the ob/ob mouse is capable of a normal BAT response to cold-stimulation.

In contrast to the defective response of BAT of the ob/ob mouse to cold, the response to diet and to administered noradrenaline is normal.

Feeding a cafeteria diet for 3 weeks had no effect on BAT T5'D activity of lean or ob/ob mice (Chapter 2, Part 1). The acute effect of feeding 30 % sucrose to lean and ob/ob mice was a transient increase in BAT T5'D activity in both lean and ob/ob mice (Chapter 2, Part 2).

This indicates that ob/ob mice respond normally to a cafeteria diet (high fat) and a high carbohydrate diet.

Fasting had no affect on BAT T5'D activity of either lean or ob/ob mice but was able to suppress thermogenesis in lean mice but did not further suppress the already low thermogenic activity of ob/ob BAT. Thus, there was a disassociation of BAT T5'D activity and BAT thermogenesis in lean mice but not in ob/ob mice.

The response of ob/ob mouse BAT T5'D activity to acute injections of noradrenaline (NA) and isoproterenol and phenylephrine is normal (Chapter 3). The response of BAT T5'D to noradrenaline is mediated by both α- and β-adrenergic receptors in lean and ob/ob mice.

Since the response of BAT T5'D activity of the ob/ob mouse to injected NA is normal, it seems likely that the lack of response to endogenous NA during cold-exposure is due to the presence in the ob/ob mouse, but not the lean mouse, of some excessive inhibitory influence during acute exposure to cold. A likely candidate for this inhibitory influence is corticosterone, which is already high in comparison with the lean mouse and increases markedly in the cold-exposed ob/ob mouse (Chapter 1, Part 1. Experiment #2). Moreover, adrenalectomy markedly enhances the cold-induced increase in T5'D activity in BAT of the ob/ob mouse, and also enhances their thermogenic activation (increase in GDP-binding) as well as markedly enhancing the cold-induced increase in UCP content of BAT (Chapter 1, Part 2, Experiment #1). In other experiments from this laboratory an enhanced sensitivity and responsiveness of thermogenesis in BAT of the ob/ob mouse to corticosterone has been demonstrated (Tokuyama and Himms-Hagen, 1986,

1989). Thus, it may be suggested that the noradrenaline-induced synthesis of T5'D in the ob/ob mouse is excessively suppressed by corticosterone during acute exposure to cold. Whether this effect is exerted centrally, or peripherally or both is not clear.

The ob/ob mouse is hyperphagic but senses that it is starving and drops its body temperature in order to conserve energy during the early morning (Himms-Hagen, 1985a,b). During torpor, the body temperature is lowered and regulated at a level just above ambient temperature (Hudson, 1978; Hudson and Scott, 1979; Webb et al., 1982). The mice are mobile when they are in torpor down to about 26 °C and can recover from body temperatures as low as 16 °C (Hudson, 1978; Hudson and Scott, 1979). Most strains of mice enter torpor daily when their food supply is restricted (Hudson, 1978; Hudson and Scott, 1979; Webb et al., 1982). Arousal from torpor is extremely rapid (Hudson, 1978; Hudson and Scott, 1979) and is accompanied by an increase in BAT thermogenesis (Himms-Hagen, 1985a,b). The control of torpor in the ob/ob mouse is abnormal in that several bouts of shallow torpor occur per day even in the fed animal (Webb et al., 1982). When ob/ob mice are acutely exposed to cold they seemingly fail to thermoregulate and stimulate BAT thermogenesis but they actually deliberately enter into torpor at this inappropriate time (Himms-Hagen, 1985a,b). The control of torpor is not clear but since a restricted diet causes torpor even in lean mice (Webb et al., 1982; Himms-Hagen, 1985) the hypothalamic diet-sensing mechanism and the neural circuits involved in induction and maintenance of torpor are probably related. Thus, the ob/ob mouse may sense itself to be fasting when in fact it is hyperphagic, therefore the

hypothalamic inhibition of torpor does not occur.

The ob/ob mouse is known to have structural (Bereiter and Jeanrenaud. 1979, 1980) and chemical (Oltmans. 1983) abnormalities in its hypothalamus, possibly associated with impaired neonatal development due to refractoriness to thyroid hormone (Himms-Hagen. 1983, 1985b; Van der Kroon and Speijers. 1979). These abnormalities in the hypothalamus of the ob/ob mouse may contribute to their defective control of torpor and increased sensitivity to corticosterone.

Future directions that research on T5'D activity in BAT may take might include an investigation of the potential function of this enzyme in man. The present study which demonstrates for the first time a high level of T5'D in BAT of a primate species represents a first step in that direction.

The presence of BAT in adult man is now well-established (Lean et al., 1987; Lean and James, 1983) but the possible presence of T5'D activity in human BAT has not been studied. Indirect evidence suggests the presence in humans of a peripheral T_4 To T_3 conversion mechanism which has characteristics similar to those of BAT T5'D. Nimalasuriya et al. (1986) found a diurnal rhythm in serum T_3 which was not TSH dependent. They speculate that the diurnal rhythm could be influenced by some as yet unidentified dietary signal, which alters the efficiency of the peripheral tissue T_4 to T_3 conversion. They extended this study by using PTU, given orally to inhibit the Type I T5'D under a variety of physiological conditions (Nimalasuriya et al., 1985). Their conclusions indicate that the Type II T5'D (PTU insensitive) appears to account for the majority of circulating T_3 in low and normal T_4 states.

It is quite possible that this Type II T5'D activity could originate in BAT, and thus, BAT could be an important site of peripheral thyroid hormone metabolism. Peripheral thyroid hormone metabolism is critical for the maintenance of serum T_3 levels in a state of T_3 deficiency in man (Lum et al., 1984). Further research is needed to determine the role of BAT in thyroid hormone metabolism in humans.

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SCHOLARSHIPS

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WORK EXPERIENCE

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JUNE - SEPT. 1986	**GUEST RESEARCHER** in the laboratory of Dr. Barbara Cannon and Dr. Jan Nedergaard at the Wenner-Gren Institute at the University of Stockholm, Sweden. The subject of my research was to learn the technique of isolation of brown adipocytes and to apply the method for the measurement of the enzyme thyroxine 5'-deiodinase to this system.
SEPT - APRIL 1984 - 1986	**LABORATORY DEMONSTRATOR** for second and third year undergraduate students in biochemistry at the University of Ottawa. The courses were entitled: BCH 2936 "Introduction to Biochemistry" and BCH 3946 "Metabolism Laboratory".
SUMMER 1982 AND 1981	**SUMMER STUDENT** Nitrogen Fixation Dr. S.M. Lesley (Supervisor) Agriculture Canada
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PUBLICATIONS

PUBLISHED PAPERS

MICHAEL K. McINTOSH, CAROLYN D. BERDANIER, AND ANNA-LISA KATES (1989). Studies on 5'-deiodinase activity in rats differing in hepatic lipogenic activity. FASEB Journal in Press, April issue.

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ABSTRACTS

ANNA-LISA KATES AND JEAN HIMMS-HAGEN. The noradrenaline (NA) stimulated increase in brown adipose tissue (BAT) thyroxine 5'-deiodinase (T5'D) activity is mediated by both α and β-adrenergic mechanisms in lean and genetically obese (ob/ob) mice. UCLA Symposia on Molecular and Cellular Biology. Obesity: Towards a Molecular Approach. Keystone Colorado. April 17 - 23, 1989.

ANNA-LISA KATES AND JEAN HIMMS-HAGEN. Adrenalectomy (ADX) improves defective cold-induced stimulation of thyroxine 5'-deiodinase (TD) in brown adipose tissue (BAT) of the genetically obese (ob/ob) mouse. Eighteenth Steenbock Symposium: Hormones, Thermogenesis and Obesity. University of Wisconsin, Madison Wisconsin. June 12 - 16, 1988.

ANNA-LISA KATES, IAN R. PARK, RUDOLF MUELLER AND JEAN HIMMS-HAGEN. Presence of thyroxine 5'-deiodinase (TD) in brown adipose tissue (BAT) of the primate macaca fasicularis. FASEB, Washington D.C. March 29 - April 2, 1987.

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ANNA-LISA KATES, GLORIA ZAROR-BEHRENS AND JEAN HIMMS-HAGEN. Short photoperiod: effect on brown adipose tissue of lean and obese mice (mus musculus). International Symposium on "Living in the Cold", Lake Tahoe, California. October 6-11, 1985.

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