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STUDIES OF THE GROWTH AND REGULATION OF BROWN ADIPOSE TISSUE

Ian R. A. Park

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University of Ottawa

Ottawa, Ontario, Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

The objective of this work was to study the growth and regulation of brown adipose tissue (BAT) in the rat. BAT is a mammalian tissue specifically adapted for thermogenesis by uncoupled mitochondrial respiration. BAT is an important organ of energy expenditure in small animals and it increases its thermogenesis and its capacity for thermogenesis in response to trophic stimuli such as cold-acclimation and overfeeding.

The acute regulation of BAT is through the sympathetic nervous system, whose activity is regulated by the hypothalamus. In the first half of the work reported here, I studied the importance of the central nervous system (the hypothalamus) in regulating the growth and function of BAT in response to trophic stimuli. In the first study, I found that, one or two days after surgery, neither ventromedial hypothalamic (VMH) lesions nor lateral hypothalamic (LH) lesions caused activation of thermogenesis in BAT. In the second study, I found that rats having parasagittal knife-cuts between the VMH and the LH (which causes hyperphagia, decreased energy expenditure, and obesity) did not have the normal activation of thermogenesis in BAT when overfed. This result is similar to previous findings in a related type of hypothalamic obesity, the VMH-lesioned rat. In the third study, I found that rats having LH lesions (which causes hypophagia, decreased energy expenditure, and weight loss) had proportionally more BAT than non-lesioned rats. They had a normal response to cold-exposure and an exaggerated diet-induced growth of BAT.

In the second half of the work reported here, I studied the effects of environmental and endocrine factors on the growth and regulation of BAT, with a particular emphasis on thyroxine 5'-deiodinase (T5'D), which converts thyroxine (T_4) to triiodothyronine (T_3), since its activity in BAT greatly increases in response to cold-exposure, and since T5'D in BAT is responsible for a significant proportion of whole-body T_4 conversion in the rat. In the first study, I showed that the activity of T5'D in BAT increased 50-fold within 10 hours of cold-exposure, but that overfeeding, another stimulus causing increased thermogenesis in BAT, had no effect on T5'D activity. In the second study, I confirmed this result by showing that at no time of day did T5'D activity in overfed rats differ from that of normally-fed rats. No daily rhythm of T5'D activity was found for cold-acclimated, overfed, or normal rats. In the third study, using thyroidectomized rats I showed that T_3 was necessary for normal BAT response to acute cold-exposure and to long-term cold-acclimation. I found that circulating, injected T_3 was as effective as injected T_4 in permitting these responses, despite the high activity of T5'D in BAT, showing that the T5'D activity in BAT was not absolutely required for the growth response. In the final study, I investigated the role of the sympathetic innervation in the regulation of the growth of BAT. I first showed that surgical denervation almost completely eliminated noradrenaline content of BAT, and that this reduction persisted for at least 26 days. I next showed that unilateral denervation was equivalent to bilateral denervation, thus validating the use of unilateral denervation as an experimental system (each animal can be used as its own internal control) and demonstrating the unilaterality of the nerve supply to half-pads of BAT. Finally, I

studied, as a time course, the effects of cold-exposure after denervation, both in rats previously warm-acclimated and in animals previously cold-acclimated. This study showed that different aspects of BAT are differently dependent upon sympathetic innervation, and that the changes in time after denervation follow different patterns. Activity of T5'D was most dependent on sympathetic innervation, followed by GDP binding (a measure of the thermogenic state of BAT), levels of the BAT uncoupling protein (a measure of the tissue's thermogenic capacity), and total BAT protein.

DEDICATION

I dedicate this work to the memory of my father.

"You'll never see that you had all of me
You'll never see the poetry you've stirred in me
Of all the stars I've seen that shine so brightly
I've never known, or felt, in myself so rightly..."

- K. Bush

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"No man is an island, entire of itself..."

-J. Donne

In writing a thesis one is struck forcefully with the number, and the breadth, of debts owed, so it gives me pleasure to acknowledge these here.

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My single greatest debt is to my research supervisor, Dr. Jean Himms-Hagen. I don't feel I can express in words all the things she has done for me; indeed, I find I am still discovering in myself things she has taught me. Nor do I expect this influence ever to end. I particularly appreciate the patience she has shown: while she has always given me more than enough rope, she has never quite let me hang myself, tempting though the thought must have been at times. In retrospect, I feel that this patience has been rewarded and that our partnership has been abundantly fruitful.

Finally, I thank my family for their support through this work. Who I am and why I am are rooted so deeply in my family that I cannot separate myself from them. I am proud to present this work as a product of my family as much as of myself, and I hope this thesis repays, in some small way, all the gifts I have been given by my family.

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ABBREVIATIONS USED

ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
BAT	brown adipose tissue
bis-acrylamide	N-methylenebis[acrylamide]
BSA	bovine serum albumin
CAFE	cafeteria-fed animals
CHOW	animals fed chow
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
g	acceleration due to gravity
GDP	guanosine 5'-diphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high-pressure liquid chromatography
IBAT	interscapular brown adipose tissue
i.p.	intraperitoneally
KC	knife-cut (between lateral and ventromedial hypothalamus)
LH	lateral hypothalamus
MH	(ventro)medial hypothalamus
MOPS	3-[N-morphilino]propanesulfonic acid
NL	non-lesioned
NS	not significant
PF	pair-fed
PPO	2,5-diphenyloxazole
PTU	propylthiouracil
RIA	radioimmunoassay
s.c.	subcutaneously
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S.E.M.	standard error of the mean
SNS	sympathetic nervous system
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	N-tris[hyroxymethyl]methyl-2-aminoethane sulfonic acid
Triton X-100	iso-octylphenoxypolyethoxyethanol
TRIS (Trizma)	tris[hyrdoxymethyl]aminomethane
Tween-20	polyoxyethylene sorbitan monolaurate
T_x	thyroidectomized
T_3	3,3',5'-triiodothyronine
T_4	thyroxine
T5'D	thyroxine 5'-deiodinase
UCP	uncoupling protein
VMH	ventromedial hypothalamus

INTRODUCTION

"Man can learn nothing except by going from the known to the unknown."

-C. Bernard

BROWN ADIPOSE TISSUE (BAT), a tissue found only in mammals, is specifically adapted for the production of heat by regulated and reversible uncoupling of mitochondrial respiration. It is primarily involved in adaptation to cold, especially in small and young mammals. BAT responds to cold both by an immediate increase in thermogenesis and by a long-term increase in its size and its capacity for thermogenesis. As well, in small mammals such as rats, it plays a quantitatively important part in energy expenditure even under conditions of minimal thermal stress, and increases its thermogenesis in response to stimuli such as overeating. Therefore, it was suspected that subnormal energy expenditure by BAT might be involved in obesity, a condition which may be described as being caused by inappropriate positive energy balance. Furthermore, under some conditions brown adipose tissue may atrophy, and this can be associated with decreased energy expenditure. More recently, it has been discovered that BAT has a high activity of the enzyme thyroxine 5'-deiodinase (T5'D), an activity which greatly increases in animals exposed to cold. Thus BAT may play a role in the regulation of thyroid hormones.

I studied BAT in the rat for these reasons: to study acclimation to cold, to study energy expenditure in BAT in rats with abnormal energy balance, and to contribute to the emerging understanding of the role of T5'D of BAT. A fourth reason for studying BAT in the rat is to help to establish an understanding of the overall role of BAT in the rat so that it

can be compared to the role of BAT in other animals studied both in our laboratory and by other researchers. In general, I will not make these comparisons here as it is beyond the scope of this thesis.

Much of the research done before I started my work had focused on the mechanism of thermogenesis. When I began, it had been established that thermogenesis in BAT was acutely regulated by the sympathetic efferents innervating the tissue. Less was known about the regulation of growth of BAT in response to different stimuli acting over a longer time, about the regulation of the sympathetic efferents by the central nervous system, or about the regulation of the deiodinase. Therefore, in my work I focused on three aspects of BAT function: the thermogenic state of BAT under different chronic stimuli (as estimated by "GDP binding", described later), the growth of BAT caused by those stimuli, and, later in the work, the regulation of the deiodinase.

In the rest of this introduction I shall describe the nature of BAT through a historical review. I shall then present background information on three effectors of growth and thermogenesis in BAT studied in this work: the hypothalamus, environmental and endocrine factors, and sympathetic innervation. Following a statement of objectives, the introduction is concluded by a rationale for the procedures used.

1. HISTORICAL REVIEW. Brown adipose tissue was first described in 1551 by Gesner. Until the second half of the twentieth century, characterization of BAT had been in the hands of naturalists and a few confused physiologists. Uncertainty of its role is reflected in the wide range of names it was given. These include: interscapular gland, primitive fat organ, adipose gland, oil gland, lipoid gland, fat gland, cholesterol gland, masse hibernal, glandula insularis cervicalis, multilocular adipose tissue, glandular adipose tissue, Winterschlaforgan, and Winterschlafdrus (Johansson 1959); my favorite is "butterfly gland" (Greene 1935). These terms reflect the two main ideas about the tissue: some felt that since it was found particularly in hibernators, it had something to do with response to cold, while others thought it had an endocrine function. Current understanding suggests that both these ideas are correct.

Only by the early 1960s did a clearer understanding emerge. Work from the laboratories of Smith (1961, Smith and Hock 1963, Smith and Roberts 1964) and Mrosovsky (1962, 1964) showed specific changes in the histology, weight, nitrogen content, and oxygen consumption of BAT following cold-adaptation of animals. Smith ascribed to BAT what is still recognized to be its primary function: it is a tissue specifically adapted for thermogenesis.

Work continued through the 1960s and by 1970 enough was known to justify the publication of a book entitled "Brown Adipose Tissue" (Lindberg 1970). However, the most important work up to 1981, the time I began working on my thesis, had been done in the 1970s. By 1981, six important discoveries had been made and synthesized into the models with which I began my work.

First, the mechanism of cellular thermogenesis was elucidated, largely through the work of Nicholls (for reviews, see Nicholls 1979, Lindberg et al. 1981, Nicholls 1983, Nicholls and Locke 1983, 1984, Nicholls et al. 1986). The mitochondria of BAT have a specific proton conductance pathway that permits oxidation of free fatty acids (and other energy substrates), as can be measured by in vitro oxygen uptake, at a rate much greater than in any other tissue, since the rate of respiration in BAT is not limited by the hydrolysis of ATP. Nicholls and coworkers found that the loosely coupled mitochondria could be recoupled by the addition of purine nucleotides, so it was presumed that BAT mitochondria were not permanently uncoupled but that the coupling could be changed as the need for thermogenesis varied.

Despite this understanding, and previous work showing the thermogenic nature of BAT, it was felt by many that BAT could not be quantitatively important in whole-body thermogenesis. This idea was supported by findings that the blood flow to BAT was not particularly impressive, even in cold-exposed animals (Kuroshima et al. 1969). However, it was discovered that the method used by Kuroshima for determining blood flow, which depends on uptake of radioactive rubidium, was invalid for BAT (Foster and Frydman 1978a). In the second important discovery before 1981, in work that represents the watershed in this field, Foster and Frydman used radioactively-labeled microspheres and arterio-venous oxygen gradients to demonstrate that BAT is the major site of the increased tissue oxygen consumption found in rats injected with noradrenaline (Foster and Frydman 1978b). This also confirmed the link between the previously-known increase in oxygen consumption (energy expenditure) in

animals injected with noradrenaline (Himms-Hagen 1970) and thermogenic capacity in BAT. Foster and Frydman then showed that BAT is a significant site of energy expenditure in conscious rats exposed to the cold (Foster and Frydman 1979). This work remains the single most important finding in this field, upon which the validity of all later work rests. An review assessing the quantitative importance of BAT has been recently published (Foster 1986).

Results with the microsphere technique are the most convincing; this method can be considered the "gold standard" in this field. However, the method is technically difficult and expensive and is usually done only to establish the validity of a line of research. Instead of this technique, the method of "GDP binding" is used extensively. In this technique, the degree of binding of purine nucleotides to isolated mitochondria of BAT is measured. This method can be used to estimate the thermogenic state of BAT because the site to which the purine nucleotides bind has been identified as the protein forming the proton conductance pathway described above. This "uncoupling protein" (UCP) was first identified in 1978 by Heaton et al. who used an analogue of ATP, 8-azidoadenosine 5'-triphosphate, as a photoaffinity label for the binding site of purine nucleotides to BAT mitochondria. It was then shown, by means of polyacrylamide gel electrophoresis, that the labeled protein had a molecular weight of about 32000 daltons. This corresponded with a protein of 32000 daltons, earlier demonstrated by polyacrylamide gel electrophoresis (Ricquier and Kader 1976), which was more prevalent in cold-acclimated rats.

In thermogenically-inactive BAT mitochondria, purine nucleotides bind to the UCP, blocking the proton conductance pathway and allowing coupled mitochondrial respiration. In BAT taken from such animals, labeled purine nucleotides have minimal binding to isolated mitochondria. However, when BAT is thermogenically active, increased intracellular free fatty acids displace the purine nucleotides and activate the proton conductance pathway, allowing uncoupled respiration and thermogenesis. Mitochondria isolated from BAT of such thermogenically-active animals show a high degree of binding of labeled purine nucleotides (a "high GDP binding"). See Nicholls et al. (1986) for a historical review of the development of these concepts.

GDP binding is well correlated with the thermogenic state of the tissue. This method, the "silver standard" of this field, was used in virtually all the reports discussed below, and in all the work I present in the Results section.

A related finding occurring before I began work was that the amount of GDP binding to isolated mitochondria does not necessarily correspond to the amount of uncoupling protein present. This was first noted in 1979 (Desautels and Himms-Hagen 1979), when the less-satisfactory method of SDS-PAGE scans were used to quantitate proteins in the 32,000 dalton band. Since I began my work, immunological methods for the quantitation of UCP have been developed (described in more detail later). A host of studies in a variety of animals and under a variety of conditions (including work reported in this thesis; see Results section B4c) have shown that there can be a rapid change in GDP binding without corresponding changes in UCP levels (Desautels 1985, Nedergaard and Cannon 1985, 1987, Swick

and Swick 1986, Gribskov et al. 1986; Trayhurn et al. 1987, Peachey et al. 1988), thus confirming the original finding.

This variation in stoichiometry (moles of GDP bound per mole of UCP) has been interpreted as a "masking" and "unmasking" of purine nucleotide-binding sites on the UCP. In this way, the level of UCP in BAT can be considered an index of thermogenic capacity, and the level of GDP binding an index of the thermogenic state of the tissue. Ideally, both should be measured in the characterization of BAT.

The third important discovery before 1981 used the method of GDP binding. In 1978 Himms-Hagen and Desautels reported that the genetically obese (ob/ob) mouse had defective thermogenesis in BAT, as determined by GDP binding. While it had been known for some time that this mouse is very sensitive to cold (Mayer and Barrnett 1953), this was the first link between defective thermogenesis and obesity through BAT. It lead to the idea that BAT could act as an "energy buffer" which, if deranged, could lead to an inappropriate positive energy balance and, hence, obesity. These results were soon substantiated by determinations of blood flow and oxygen uptake, by the radioactive microsphere method, of BAT in ob/ob mice (Thurlby and Trayhurn 1980).

The fourth important discovery was the link between diet-induced thermogenesis and BAT, made in 1979 by Rothwell and Stock. Diet-induced thermogenesis, under a variety of names such as specific dynamic action, thermic effect of food, luxuskonsumption, and heat increment of feeding, has been described since at least 1902 (Rubner 1902). It can be defined as "the increase in metabolic rate that follows the ingestion of food, as well as changes associated with chronic alterations in the overall level of

energy intake" (Rothwell and Stock 1983). While it is believed by some that this represents only the inevitable release of heat associated with the digestion, absorption, and storage of nutrients, it is widely believed that there is an additional expenditure of energy beyond this, often called adaptive thermogenesis. The work of Rothwell and Stock showed that BAT was an important site of this adaptive thermogenesis in rats induced to overeat. (To induce overeating, rats are fed a varied diet of palatable human foods, the so-called "cafeteria" diet.) This finding was later substantiated by measurements of blood flow and oxygen uptake in BAT by the radioactively-labeled microsphere method (Rothwell and Stock 1981). This work supported and extended the idea of BAT as an "energy buffer" since it showed that BAT could act to dissipate excess energy intake in normal animals, as well as fail to do so in pathological conditions such as genetic obesity.

The fifth important discovery concerned the response of the sympathetic nervous system (SNS) to feeding. It had been assumed that the SNS was more active in fasted than in fed animals. However, in a series of papers Young and Landsberg showed the opposite - that the activity of the SNS, measured as the turnover rate of noradrenaline in BAT and heart, increased with feeding, including sucrose overfeeding and cafeteria feeding (Young and Landsberg 1977a, b, Young et al. 1982). Since BAT is innervated by the sympathetic nervous system, and since noradrenaline was known to increase thermogenesis in BAT, these findings of Young and Landsberg explain the role of BAT in diet-induced thermogenesis when, by the previous belief, feeding should have decreased sympathetic activation and, hence, BAT thermogenesis.

The sixth important discovery made before 1981 (and continuing after I began my work) was the isolation of the UCP and the establishment of immune assays for this protein.

Until December 1982, the method of gel electrophoresis had to be used to estimate the amount of this protein. This was unsatisfactory because of its lack of specificity: many proteins could be in this band, and even a large change in UCP levels could be obscured.

In 1979, Ricquier et al. reported a partial purification of UCP. Since they used GDP affinity chromatography to analyze a Triton X-100 extract of BAT mitochondria, it is possible that the purification was not complete due to contamination by the ADP, ATP translocator. This problem was avoided by the use of hydroxylapatite, introduced by Lin and Klingenberg in 1980. Triton X-100 extracts of BAT mitochondria were separated at room temperature on hydroxylapatite columns; the UCP passes through in the void volume while the ADP, ATP translocator denatures at room temperature in the absence of the stabilizer carboxyatractylate and is thus retained on the column. A slightly modified method (Lin and Klingenberg 1982) is now the standard preparation.

With a reliable method for purification of UCP available, it was then possible to raise antisera to the protein and thus to start using immunological methods for quantitation of UCP in mitochondria and homogenates of BAT. Immunoassays were announced, almost simultaneously, by two groups: an enzyme-linked immunosorbent assay (ELISA) by Cannon et al. (1982) and a radioimmunoassay (RIA) by Lean et al. (1983). Since then, such assays and similar methods, such as Northern blotting, have been developed by several other groups, and the methods are in wide use.

Therefore, when I began my work, our understanding of BAT was the following: brown adipose tissue is a tissue adapted for facultative thermogenesis, activated by the sympathetic nervous system. BAT is active when the animal is subjected to cold or to overfeeding. Subnormal thermogenesis in BAT contributes to obesity in genetically obese animals. BAT produces heat by extremely rapid oxidation of fatty acids in its mitochondria, which can be reversibly uncoupled by a proton conductance pathway; binding of GDP can be used to estimate the thermogenic state of BAT in animals subjected to various regimens.

Progress in our understanding of BAT, gained both before and after I began my work, has been collected into two important books: "Mammalian Thermogenesis" (1983), edited by Girardier and Stock, and "Brown Adipose Tissue" (1986), edited by Trayhurn and Nicholls.

In the time since I began my thesis, workers in the field have tested the implications of this understanding. I have contributed to aspects of this work, as demonstrated in this thesis. Some areas in which research has proceeded include: a) study of BAT thermogenesis in animals undergoing altered energy metabolism (genetic or hypothalamic obesity (Hogan and Himms-Hagen 1980, 1983, Triandafillou and Himms-Hagen 1983, Ashwell et al. 1985, Coscina et al. 1985, Hogan et al. 1985, Eley and Himms-Hagen 1989a, b), genetic and hypothalamic leanness (Triandafillou et al. 1984, Corbett et al. 1985, Kopecky et al. 1986a, Lupien et al. 1986, Park et al. 1986a, 1988, Arase et al. 1987), and lactation and pregnancy (Trayhurn 1985, 1986, 1987, Villarroya et al. 1986a, b, 1987)); b) the investigation of the long-term adaptive responses of BAT, especially of UCP, in cold-adaptation and overfeeding (as contrasted to the acute

effects of these treatments) (Rothwell and Stock 1982a, Ashwell et al. 1983, 1984, 1985, Lean et al. 1983, Trayhurn et al. 1983, 1987, Nedergaard et al. 1984, Falcou et al. 1985, Nedergaard and Cannon 1985, Bukowiecki et al. 1986, Hansen and Knudsen 1986, Géloën et al, 1988); c) investigation of the neural and hormonal regulation of acute responses of BAT, including characterization of α- and β-adrenergic receptors in BAT (and the possible pharmacological modulation of BAT activity) (McMahon and Schimmel 1982, Arch et al. 1984a, b, Ma and Foster 1984, Foster 1985, Rothwell et al. 1985, 1986, Sullivan and Garattini 1985, Harris et al. 1986, Levin and Sullivan 1986, Bray and Cairella 1987, Grassby et al. 1987, Scarpace 1987, Sullivan et al. 1987); d) study of intracellular events coupling adrenergic stimulation and thermogenesis, particularly the roles of cations and inositol phosphates (Girardier and Schneider-Picard 1983, Connolly et al. 1984, Horwitz and Hamilton 1984, Mohell et al. 1984, Schneider-Picard et al. 1985, Girardier and Seydoux 1986, Nedergaard et al. 1986, Schimmel et al. 1986, Nånberg and Putney 1986, Barge et al. 1988); e) isolation and cloning of mRNA for UCP from several species (Falcou et al. 1985, Jacobsson et al. 1985, Ricquier and Bouillaud 1986, Ricquier et al. 1986, Reichling et al. 1987, 1988); f) demonstration, in BAT, of thyroxine 5'-deiodinase (T5'D), the enzyme responsible for the conversion of thyroxine (T_4) to triiodothyronine (T_3) and characterization of its control by the sympathetic nervous system (described in more detail below).

2. THE HYPOTHALAMUS AND CENTRAL REGULATION OF BROWN ADIPOSE TISSUE.

a) Background: The hypothalamus is the region of the diencephalon which regulates many autonomic, homeostatic functions through the autonomic nervous system and through its control of the release of pituitary hormones. Of particular importance to the subject matter of this thesis are its roles in the regulation of fat and carbohydrate metabolism and in thermoregulation; in these ways, the hypothalamus is important in the central regulation of energy balance. Since BAT is an important site of energy expenditure, and therefore important in whole-body metabolism of fat and carbohydrate, it is reasonable to expect that changes in thermogenesis in, and growth of, BAT would reflect changes in the hypothalamus.

Much of our understanding of hypothalamic regulation of homeostasis has come from experiments in which part of the hypothalamus is lesioned or electrically stimulated. The former approach was used in my work. There is an immense literature on the effects of hypothalamic lesions and I cannot present more than a minimal review of a small amount of this, directly dealing with the effects of lesions on BAT.

In 1940, Heatherington showed that lesions of the hypothalamus (made with chromic acid!) caused hyperphagia and obesity in the rat (Heatherington 1940; Heatherington and Ranson 1940). Later work showed that this obesity was caused when the ventromedial region of the hypothalamus (VMH) was damaged, and that it was accompanied by a host of behavioral and physiological changes now termed the "VMH syndrome" (Bray and York 1979). In 1951, Anand and Brobeck showed that lesion of a

region lateral to the VMH, the lateral hypothalamic area (LH), caused a syndrome in some ways opposite to the VMH syndrome; this "LH syndrome" is characterized by hypophagia and rapid weight loss (Powley and Keesey 1970).

In 1969, it was shown by two groups that a knife-cut (KC) dividing the VMH from the LH caused a syndrome similar to, but not identical to, the VMH syndrome (Albert and Storlien 1969; Sclafani and Grossman 1969). This "KC syndrome" includes hyperphagia and obesity, as does the VMH syndrome, but some of the behavioral and physiological changes are different; for example, KC rats tend to be less aggressive than VMH rats; as well, KC rats do not show hyperinsulinemia as do VMH rats (Bray and York 1979, Bray et al. 1982).

It is important to distinguish lesions of the VMH, in which tissue is destroyed, from KC, in which a fiber pathway just lateral to the VMH (the "paraventricular nucleus-hindbrain feeding pathway" (Kirchgessner and Sclafani 1988, Kirchgessner et al. 1988)) is sectioned. We wanted to study KC rats because the effects of this lesion appear to be more specifically related to the pathways involved in the regulation of food intake and body weight than are VMH lesions.

A further advantage of KC over lesions is that they are non-irritative. When lesions are made electrolytically (by passing current through the brain), there is a deposition of metallic ions which act to chronically irritate the hypothalamus, thus complicating interpretation of results (King and Frohman 1985, King et al. 1988). Therefore, when we used hypothalamic lesions, we used non-irritating heat lesions.

Beside lesions and knife-cuts, the second major approach used in studying the hypothalamic regulation of energy expenditure is electrical stimulation of different regions of the hypothalamus. These are described in more detail below. In general, the effects of electrical stimulation are the opposite of those of lesion (or knife-cut).

In my work, I studied the acute effects of VMH and LH lesions on BAT thermogenesis and the long-term effects of KC and LH lesions on growth and thermogenesis of BAT.

b) Acute effects. Stevenson and Montemurro (1962) reported that both LH and VMH lesions had the acute effect of markedly increasing resting metabolic rate. This elevated metabolic rate persisted for at least three days and was particularly high in the first day after lesion. It is reasonable to expect that BAT is a site for at least part of this increased energy expenditure.

This idea is supported by later findings on the changes immediately after brain lesions. Keesey et al. (1984) showed that, over the four days following LH lesion, rats lost more weight than fasted, sham-operated rats. Thus LH-lesioned rats have a higher energy expenditure than do sham-lesioned rats. This means that there is a metabolic component to the acute changes after lesion; the effects are not due to aphagia alone.

Yoshida et al. (1983) showed that the noradrenaline turnover rate, a measure of sympathetic activity, was greater in BAT of LH-lesioned rats (compared to fasted sham-lesioned rats) 18 hours after surgery. Corbett et al. (1983) showed that the increase in oxygen consumption after LH lesion could be attenuated by the β-blocker propranolol. Most recently, Corbett

et al. (1988) showed that immediately after LH lesion, there was an increase in BAT temperature as well as core temperature. Together, these findings suggest that BAT may be a site of increased energy expenditure seen in the period immediately following lesion of the lateral hypothalamus.

Given that, in the long term, VMH lesions cause decreased metabolic rates, it is surprising that the acute effect is to elevate oxygen consumption. Yet the findings of Stevenson and Montemurro (1962) show this to be the case. It is possible that, as for the acute effect of LH lesions, BAT is a site of the increased energy expenditure. This idea is contradicted by the finding that the rate of firing of the nerves innervating BAT is greatly reduced immediately after VMH lesion (Niijima et al. 1984). That the innervation connecting the VMH to BAT is important was demonstrated by Minokoshi et al. (1986); they showed that the increase in temperature of BAT in rats having electrical stimulation of the VMH could be prevented by cutting the nerves.

To help resolve these apparently contradictory findings, I studied BAT thermogenesis one and two days after LH or VMH lesion (Results section A1).

c) Hypothalamic knife-cuts. The KC syndrome is similar to the VMH syndrome. Important aspects include hyperphagia and decreased energy expenditure. Most work has focused upon the former aspect; however, the metabolic changes are significant as well. This is known because obesity can develop even when hyperphagia is prevented by restricted feeding (Han 1968, Bernardis and Goldman 1976). In both syndromes, there are multiple

endocrine disturbances and a disorder of the circadian cycles of activity and eating, with animals eating during the light part of the daily cycle, when normal rats do not. There are behavioral differences between the two syndromes, as well; in particular, VMH lesions render rats irritable and aggressive while KC rats are much less so.

Several effects of lesions or electrical stimulation of the VMH on BAT have been studied. Perkins et al. (1981) showed that the temperature of BAT increased upon electrical stimulation of the VMH. Holt et al. (1987) showed that this temperature response in BAT could be graded, proportional to the stimulus strength, and that the strength of the response was normal in the genetically obese fatty Zucker (fa/fa) rat. Yoshida and Bray (1984) studied noradrenaline turnover in BAT of VMH-lesioned rats and found, surprisingly, an increased turnover in VMH-lesioned rats two weeks after surgery. This greater turnover was also seen in fasted rats (both lesioned and non-lesioned), although the decrease in turnover due to fasting was proportionally greater in non-lesioned rats. Eight weeks after surgery, turnover rates were the same in lesioned and non-lesioned rats when fed; when fasted, VMH-lesioned rats failed to decrease the turnover rate as much as did the non-lesioned animals. These findings seem contradictory to the idea of decreased activity of BAT contributing to the overall lowered energy expenditure in VMH-lesioned rats.

Later work by this group suggested that lesion of the VMH did not affect noradrenaline turnover (Tokunaga et al. 1986) but that electrical stimulation (or injection of glucose into the VMH) did increase the firing rate of the sympathetic efferents innervating BAT, an effect blocked by

chemical destruction of the VMH (Sakaguchi and Bray 1987). This group later studied the daily pattern of firing of the efferents to BAT (Sakaguchi et al. 1988a) and found a shallow circadian cycle of nerve activity, with the minimal activity found at night (at the time of maximal food intake) and the maximum in the middle of the lights-on period; injections of insulin into the VMH caused a dose-dependent suppression of nerve activity which was more pronounced in the middle of the lights-on period.

Perhaps the most convincing study was by Iwai et al. (1987), who showed that electrical stimulation of the VMH caused a great (about 50-fold) increase in blood flow to BAT.

Taken together, studies of electrical stimulation of the VMH, and studies of VMH stimulation by glucose, indicate that these procedures cause the activation of thermogenesis in BAT; insulin injected into the VMH suppressed the firing rate of efferents to BAT. These studies help establish the importance of the VMH in the regulation of BAT thermogenesis. Unfortunately, it is difficult to adapt such protocols to long-term studies. Therefore, in order to study the effects of hypothalamic manipulation on the growth response of BAT, lesions or knife-cuts must be used, as in the work presented in this thesis.

Sakaguchi et al. (1988b) studied the firing rate of sympathetic efferents to BAT after chemical lesion of the VMH. In both the acute phase (30 minutes after lesion) and the long-term phase (7 to 9 days after lesion), the firing rate in lesioned rats was markedly reduced (about half the rate seen in non-lesioned rats). In a similar study, it was found that in rats overfed by tube, VMH-lesioned animals failed to show the 20% increase in firing rate found in non-lesioned overfed rats (Sakaguchi et al.

1988c). Taken together, these results support the idea that VMH lesions decrease sympathetic activity in BAT in both the short term and the long term. They seem to contradict the earlier finding by the same group (Yoshida and Bray 1984) that noradrenaline turnover in BAT of VMH-lesioned rats was actually increased; however, that report is the only one to run counter to the general finding, in many papers, that VMH lesions cause a decrease in sympathetic activity in BAT.

From the above, it is clearly of interest to make direct biochemical studies of BAT in VMH-lesioned rats. Seydoux et al. (1982a) studied GDP binding in female rats subjected to warm-acclimation, cold-acclimation, or food restriction after VMH lesions or no surgery. GDP binding was reduced in both warm- and cold-acclimated, VMH-lesioned rats while it was not changed in food-restricted rats. In our laboratory, Dr. Susan Hogan studied both male and female rats given either VMH lesions or sham operations and maintained at 28°C or exposed to 4°C for 24 hours (Hogan et al. 1982). In female rats she found a reduction in GDP binding in both warm-exposed and cold-exposed rats that had been given a VMH lesion; in male rats, reduced GDP binding was found only in cold-exposed VMH-lesioned rats. The values for GDP binding in the cold-exposed VMH-lesioned rats were not greatly different from the non-lesioned rats.

This study was complemented by a second project in which the effects of VMH lesions on BAT of cafeteria-fed rats was examined (Hogan et al. 1985). In both male and female rats, GDP binding of cafeteria-fed VMH-lesioned rats was markedly lower than that of non-lesioned rats.

From these two studies, it was concluded that VMH-lesioned rats are

capable of responding normally to cold-exposure but had defective diet-induced thermogenesis in BAT.

We wished to extend these findings to rats with hypothalamic knife-cuts (KC rats). Dr. Hogan studied the response of BAT in cold-exposed KC rats (Coscina et al. 1985) and found, as in the VMH-lesioned rat, that there was a normal increase in GDP binding in KC rats exposed to 4°C for 21 hours. My work, reported in Results section A2, was the study of diet-induced thermogenesis in these rats. The objective of this research was to characterize BAT in cafeteria-fed KC rats, to see if the conclusions reached from VMH-lesioned rats could be generalized. This work has been previously published (Coscina et al. 1985).

d) Lateral hypothalamus. Important aspects of the LH syndrome include hypophagia (or aphagia) and rapid weight loss immediately after surgery, followed by a period in which the animal begins to eat and gain weight again, although at a reduced level compared to non-lesioned rats. As cited above, since lesioned animals lose weight more than fasted, non-lesioned animals, there is a metabolic component to the syndrome. As with VMH lesions and KC, most research has focused on the changes in energy intake, with less attention paid to energy expenditure. I wished to determine whether BAT played a role in the altered energy expenditure in the LH syndrome, and to contrast the situation in LH rats to KC rats.

As with VMH-lesioned and KC rats, LH lesions cause multiple endocrine disturbances (different from the other two situations) as well as behavioral changes (the rats become less active, especially in the acute

recovery from surgery). Adipsia and polyuria are serious problems, particularly in the first week after surgery.

The effects of LH lesions on the whole-body energy balance have been carefully studied by Keesey, Corbett, and coworkers (Corbett et al. 1983, 1985, Keesey and Corbett 1984, Keesey et al. 1984). Their principal finding is that the LH rat loses weight and becomes hypophagic initially, then maintains intake and energy expenditure at a level appropriate for its reduced size. They interpret this as a new "set-point" for the LH-lesioned animal.

Things are less clear when the possible role of BAT in altered energy metabolism of LH-lesioned animals is investigated. As with the VMH, studies of the effects of electrical stimulation of the LH have been used in trying to understand the acute regulation of BAT by the central nervous system. Takahashi and Shimazu (1982) found that electrical stimulation of the LH did not increase lipogenesis in BAT. Yoshimatsu et al. (1987) found an increase in the firing rate of sympathetic nerves with electrical stimulation of the LH; in contrast, Holt et al. (1987) found that electrical stimulation of the LH did not change the temperature of BAT.

As with the VMH, for long-term studies of the importance of the LH in the regulation of BAT activity, lesions are a useful preparation. Yoshida et al. (1983) studied noradrenaline turnover in LH-lesioned rats. (The acute effects were described above.) Three weeks after lesion, LH rats had higher turnover rates than did non-lesioned animals when both groups had been fasted for 18 hours. Similarly, Arase et al. (1987) found that both 18 hours and 18 days after LH lesion, the firing rates of sympathetic efferents innervating BAT were significantly greater than controls.

Supporting this is the finding by Saito et al. (1987) that electrical stimulation of the LH did not increase noradrenaline turnover in BAT. (Recall that electrical stimulation generally acts in a manner opposite to that of lesions.) Recently, Sakaguchi et al. (1988d) studied the effect of LH lesion on the firing rate of sympathetic efferents to BAT, finding an increase in firing rate 3, 9, 24 and 72 hours after lesion; the firing rate had returned to control levels by 7 days after lesion, which seems to contradict the finding of Arase et al. (1987).

From the above, it is possible to postulate a role for the LH in the acute regulation of thermogenesis in BAT. It is more difficult to extrapolate this to the long-term growth of BAT, but, since the whole-animal effects are well-characterized, and given the quantitatively important role of BAT in energy expenditure (at least in small animals), it seems plausible that thermogenesis in BAT, accompanied by growth of BAT, would occur in LH-lesioned rats. Most of the studies cited above support this concept; others do not. In an attempt to better resolve this problem, I studied BAT in LH rats, as reported in Results section A3.

3. ENVIRONMENTAL AND ENDOCRINE EFFECTS ON THE GROWTH AND FUNCTION OF BROWN ADIPOSE TISSUE.

A wide variety of environmental and endocrine factors are known to influence thermogenesis and growth in brown adipose tissue. Background information for those areas of particular importance in this thesis is given below.

a) Thyroxine 5'-deiodinase. Largely through the work of Silva and Larsen, the existence of this enzyme in BAT has recently been established (Leonard et al. 1983; Silva and Larsen 1983). This enzyme catalyzes the peripheral conversion of the thyroid hormone thyroxine (T_4) to the more active hormone triiodothyronine (T_3). In 1985, Silva and Larsen showed that T5'D of BAT was an isozyme distinct from the form found in most tissues (type I); BAT T5'D is similar to that found in brain and has been described as type II. As such, it has kinetic properties different from the form found in liver and other tissues (for example, it has a comparatively low K_M compared to the isozyme found in liver and kidney) (Leonard et al. 1983, Silva et al. 1987); it is reasonable to expect that the regulation of its activity in BAT is also different. Because of this idea, because it has been found that, under certain conditions, BAT T5'D can deliver T_3 to the serum (Silva and Matthews 1984, Silva et al. 1984, Fernandez et al. 1987) in amounts that could be quantitatively important as a source of T_3 for the whole body (30-50% of whole-body T_3 production in rats kept at 23°C) (Silva and Larsen 1985, Van Doorn et al. 1985), and since thyroid hormones have long been associated with the regulation of metabolic rate, energy balance, and body temperature, it is clearly of interest to further

characterize this enzyme in BAT. This was the major objective of the work presented in the second half of this thesis (Results section B).

b) Circadian cycles. Rats have daily cycles of feeding and activity, which could be expected to affect BAT. There is a variety of daily cycles in the levels of some hormones (such as corticosterone, which can be altered by meal-feeding (Moberg et al. 1975), and thyroid hormones, in both cold-exposed and cold-acclimated rats (Kuhn et al. (1983)) but not in others (meal-feeding does not alter the daily rhythm of growth hormone). Since cafeteria-fed animals are given fresh food at the same time each day, this entrainment of the cycle of corticosterone levels (or other hormone levels) could affect the function of BAT. This is important because a number of studies suggest that corticosterone can suppress thermogenesis and sympathetic activity in BAT (Rothwell and Stock 1986, Marchington et al. 1986) since adrenalectomy reverses the suppression, particularly in older rats, where administration of corticosterone then restores the suppression (Fukushima et al. 1985, Rothwell and Stock 1984a, 1988, Rothwell et al. 1984a).

Daily cycles in brown adipose tissue itself have also been studied. Goubern and Portet (1981) studied the circadian variation in activity of lipoprotein lipase in BAT in warm- and cold-acclimated rats; they found a large increase in specific activity of the lipase in cold-acclimated rats just before lights-out, with a small increase in warm-acclimated rats during the period of darkness. Circadian cycles of lipogenesis in BAT have also been found (Goubern and Portet 1986, Thompson and Grigor 1987), the highest rates occurring in the dark phase.

The importance of the activity of the sympathetic nervous system in the regulation of BAT metabolism has already been mentioned. Circadian cycles in SNS activity have been studied (Fukushima et al. 1985, Yoshida et al. 1987, Sakaguchi et al. 1988a, Yen et al. 1988). In one elegant study, Yen et al. used the accumulation of dopamine in BAT (of mice) following inhibition of dopamine β-hydroxylase by 1-cyclohexyl-2-mercapto-imidazole as an index of tissue noradrenaline turnover. They found a marked circadian variation of SNS activity in BAT, with the basal "daytime" activity rising during the "nighttime" period to a maximum, just before lights-on, that was four-fold greater than the baseline. Sympathetic activity fell sharply at the beginning of the "daytime" period.

Rothwell et al. (1983) studied the circadian cycles of several hormones and of GDP binding in chow- and cafeteria-fed rats. GDP binding was relatively constant in chow-fed rats, but in cafeteria-fed rats binding peaked with lights-on. Like Kuhn et al. (1983), they also showed daily rhythms of thyroid hormones in both kinds of animals.

I was interested in studying the circadian pattern of T5'D activity for two reasons. First, given the circadian variation in a variety of indices of BAT function cited above, it seemed plausible that a circadian cycle in T5'D activity might be found, especially since this enzyme's activity is known to be regulated by the sympathetic nervous system (Leonard et al. 1983, Silva and Larsen 1983), whose activity does vary with a circadian pattern. Second, the results of the first study reported in this part of the thesis (Results section B1) showed that cafeteria-feeding did not increase T5'D activity; I wanted to see whether this was the case throughout the circadian period.

c) BAT in thyroidectomized rats. Although sympathetic innervation is thought to be largely responsible for the mediation of growth of BAT (Barnard et al. 1980), at the time I began my work it was felt that chronic noradrenaline injections did not cause the characteristic changes of mitochondrial polypeptide composition usually seen in cold-acclimation (Desautels and Himms-Hagen 1979). However, more recent work indicates that injections can produce both an increase in the levels of mRNA for the uncoupling protein (Bouillaud et al. 1984, Ricquier et al. 1984, 1986) and of the UCP itself (determined by scans of electrophoretic gels) (Mory et al. 1984, Young et al. 1984), as expected from our knowledge of the importance of the sympathetic nervous system in mediating various aspects of the adaptation of BAT to thermogenic stimuli.

Nevertheless, there is evidence that other factors beside noradrenaline are important in the mediation of the adaptive changes in BAT. For example, from work presented in this thesis, it is known that the activity of T5'D in BAT is highly dependent upon its sympathetic innervation (Results section B4) but that manipulations that increase sympathetic activity in BAT, such as cafeteria-feeding, fail to increase the activity of this enzyme (Results sections B1 and B2). Therefore, it is well worth investigating the importance of other factors in mediating acute and adaptive responses in BAT. In particular, the role of thyroid hormones is of particular interest because of their well-known role in the regulation of basal metabolism and energy expenditure, because of the presence of T5'D in BAT, and because of the effects it has in modulating the thermogenic response of BAT to catecholamines (Garcia-Sainz and Fain 1980, Seydoux et al. 1982b, Sundin et al. 1984, Woodward and Saggerson 1986).

Using hypophysectomized rats maintained on T_4 and corticosterone, Fellenz et al. (1982) showed that neither glucocorticoids nor pituitary hormones were the mediators of the change of polypeptide composition and that these hormones were required only in maintenance levels. Thyroid hormones seemed a good candidate as a mediator, given the increase in their levels in cold-acclimated animals. However, Triandafillou et al. (1982) studied BAT in thyroidectomized (T_x) rats and concluded that T_4 had only a permissive role in the cold-induced changes in BAT. T_x rats exposed to cold for 15 hours did not have the usual increase in GDP binding; T_x rats injected with T_4 had the normal response to cold-exposure. T_x rats, treated with low maintenance doses of T_4, could be acclimated to cold and had normal changes in BAT, including altered polypeptide composition of BAT mitochondria.

There were two problems with this study, as it turned out. First, the polypeptide composition of the mitochondria was determined by the method of electrophoresis described earlier in this Introduction. The insensitivity and non-specificity of this technique have been noted. More seriously, with the discovery of T5'D activity in BAT, it was possible that even small amounts of T_4 could be sufficient to permit normal changes in BAT in cold-exposed and cold-acclimated T_x rats. due to the high concentrations of T_3 that could be produced in BAT by the deiodinase. Therefore, the conclusion that thyroid hormones play only a permissive role in the growth of BAT could be wrong.

To investigate this possibility, I studied both the acute and long-term responses to cold in thyroidectomized rats supplemented with either T_3 or T_4, as further explained in Results section B3.

4. ROLE OF SYMPATHETIC INNERVATION.

The last section of the thesis deals with a series of experiments in which I studied the role of the sympathetic innervation in the regulation of the acute and adaptive responses to the cold.

a) Rationale. Denervation is a technique widely used in physiological research. By denervating a structure, one can study in vivo the role of the innervation in the function of that structure while all other factors (such as hormones and substrates) remain unaltered. As well, since many structures are paired, it is often possible to perform unilateral denervation, thus leaving the contralateral structure as an internal control. These two aspects made unilateral denervation of the interscapular depot a desirable system for the study of the role of the sympathetic innervation in the regulation of the acute and adaptive responses of BAT to cold and to cafeteria-feeding.

The advantages of denervation have attracted other workers as well. This method has been used in four kinds of study.

The first involves the study of acute changes in BAT (often, changes in temperature are measured). Electrical stimulation of regions of the brain are often the stimuli used (see Introduction Section 2). The effects are observed first with innervation intact and compared to the effects when tissue is denervated. A similar design was employed by Shibata and Nagasaka (1982, 1984).

In the second type of study, BAT is denervated to reduce or eliminate its contribution to whole-body metabolism. This was done by Dulloo and

Miller (1984), who studied energy balance in rats having either denervation or extirpation of BAT.

In the third type of study, BAT is denervated to isolate it from the sympathetic nervous system in animals which are then manipulated in some way to change hormone or substrate levels. Changes in BAT, independent of the sympathetic nervous system, can then be studied. With this sort of system, aspects of BAT metabolism (cyclic AMP levels and adenylate cyclase activity, lipolysis and oxygen consumption in isolated cells) have been studied (Forn et al. 1970; Knight 1974; Granneman et al. 1985, Granneman 1988, Granneman and MacKenzie 1988). Studies of the effects of manipulation of hormone levels (adrenocorticoids and insulin) have also been made (Rothwell and Stock 1984a; Bartness et al. 1986), including the effects of injection of corticotropin releasing factor into the paraventricular nucleus (LeFeuvre et al. 1987). Likewise, the effect of altered fuel substrates, as in cafeteria-feeding, can be studied in isolation from the SNS (Granneman and Campbell 1984).

In the fourth kind of study, into which my work falls, the role of sympathetic innervation in the adaptive and metabolic changes in BAT of animals subjected to thermogenic stimuli (cold-acclimation or cafeteria-feeding) is studied. (Often the thermogenic state of the BAT as measured by GDP binding is also evaluated.) A historical review of this type of study is presented below. There are several technical points which must be discussed first, however.

b) Technical considerations. The interscapular depot of BAT is innervated by five paired sympathetic nerves emerging from between the intercostal muscles near the spine and running to the ipsilateral half-pad. There is also evidence that there is a sixth pair which enter along the blood vessels and presumably innervate them (Foster et al. 1982, Néchad 1986, Girardier and Seydoux 1986).

There are two ways of performing a sympathectomy: local or systemic. Local sympathectomy can be done by surgical denervation (axotomy). Systemic sympathectomy can be performed either chemically (administration of guanethidine, 6-hydroxydopamine, or similar agents) or immunologically (administration of antibodies to nerve growth factor). Clearly, for the type of study I wished to do, only surgical denervation was appropriate. It is completely specific, simple, and highly reproducible in my hands. Its action is rapid, with noradrenaline depleted within 24 hours (Sidman et al. 1962). Unlike chemical sympathectomy, which does not produce long-lasting denervation (Seydoux et al. 1981), surgical denervation persists for the duration of the experiment (Results section B4b).

It has been suggested that there are two sympathetic nerve supplies to BAT, one to the blood vessels and the other to "intrinsic ganglia" and thence to brown adipocytes (Derry et al. 1969). In the study by Derry and coworkers, fluorescent histochemistry was used to observe changes in the location of catecholamines in surgically- or immunologically-sympathectomized BAT. Denervation abolished fluorescence around blood vessels but not in other areas of BAT. Because of this, it was suggested that surgical denervation would not cause denervation of brown adipocytes.

At the time, this idea was supported by others (Barde et al. 1975, Thureson-Klein et al. 1976a, 1976b).

There are several reasons for distrusting these findings. The method is notably non-specific for catecholamines. It cannot be quantified (Derry et al. 1969). It is insensitive because, as Girardier (1983, page 85) points out, while Derry and Daniel (1970) could not find catecholamines in the BAT of newborn rats, Schneider-Picard and Girardier (1982) showed that electrical stimulation of the nerves produced a maximal response at birth. Since Derry et al. (1969) studied animals four weeks after surgical denervation, it is possible that there had been enough regrowth of nerves to permit fluorescence. More likely, something else was causing the fluorescence. Finally, it seems strange that cutting the nerves would not abolish the innervation of the brown adipocytes, even if there are ganglia between the terminal fibers and the axons entering the tissue, since those ganglia themselves would have to be stimulated somehow, presumably by the nerves entering the tissue.

Therefore, in my work I have assumed (along with other researchers) that surgical denervation does indeed eliminate the sympathetic innervation of the brown adipocytes. Since this procedure results in such a marked (but incomplete) reduction in noradrenaline content of the tissue (about 1-5% of the original content remains) (Sidman et al. 1962; Foster et al. 1982; Results section B4a), it seems more likely to me that the innervation to the blood vessels, not the brown adipocytes, is left intact by surgical denervation.

Finally, that the design of unilateral denervation experiments be correct, the innervation of the two sides of the interscapular pad must be

separate. This had always been assumed on anatomical grounds. Despite this, Seydoux et al. (1977) reported cross-innervation. Eight weeks after unilateral surgical denervation, they incubated tissue in the false neurotransmitter α-methyl-noradrenaline, then performed fluorescent histochemistry. They found depletion of "nerve fiber content" on both the denervated and contralateral sides.

The problems with the histological fluorescence method have been discussed. After eight weeks, it is more than likely that regrowth of innervation had occurred (see also Results section B4a). Seydoux et al. do not state their control for estimating reduction of content in the side contralateral to the denervation, nor do they validate their method of quantitation.

In later work, this group used horseradish peroxidase to map the innervation of the interscapular depot (Seydoux et al. 1984, Girardier and Seydoux 1986). They found that peroxidase injected into one side of the interscapular depot labelled ganglia in both the ipsilateral and the contralateral sympathetic chains. However, as they point out (Girardier and Seydoux 1986), this method is not specific for sympathetic nerves.

Foster et al. (1982) studied the question of cross-innervation. They studied blood flow, noradrenaline content, and dopamine β-hydroxylase activity in unilaterally-denervated BAT and clearly showed the independence of the two sides. This result is somewhat more convincing that the work of Seydoux and coworkers because both noradrenaline and dopamine β-hydroxylase are specific to sympathetic nerves. I therefore assumed that unilateral denervation, with the intact side as an internal control, was valid. To rule out the possibility that there actually is cross-

innervation, this assumption was further tested as part of my study (Results sections B4a and B4b).

c) Historical review. Surgical denervation of BAT was performed as long ago as 1934 by Hausberger but, after a brief flurry of activity, was ignored for twenty years. In 1954, Sidman and Fawcett studied the histological and metabolic effects of surgical denervation of BAT in mice. Like Hausberger (1934), they found a "transient deposition of glycogen and a lasting increase of lipid in denervated brown fat".

Nevertheless, it was only after the thermogenic nature of BAT was understood that sense could be made of the effects of denervation. Hull and Segall (1965) showed that stimulation of the cervical sympathetic nerves caused an increase in temperature of the ipsilateral side of cervical BAT in rabbits. They were then able to correctly interpret the retention of lipid in denervated tissue, even when the animals were fasted 48-72 hours, as being due to lack of the sympathetic stimulation which normally mobilizes the lipid.

Steiner et al. (1970) showed that conversion of labeled glucose into lipid in cold-acclimated rats was reduced to half by denervation, while it was much less affected in animals reared at 23°C, reflecting the relative sympathetic activity at the two temperatures. Interestingly, lipid synthesis in denervated BAT of cold-acclimated rats was still 10 times greater than in warm-acclimated rats, suggesting that factors other than sympathetic innervation affect metabolism in brown adipose tissue.

Since previous work had not characterized the effects of denervation on BAT growth and thermogenesis, I studied these aspects both in cold-

acclimated and cafeteria-fed rats, measuring BAT protein, DNA, cytochrome oxidase, and GDP binding (Himms-Hagen and Park 1984). I found that denervation reduced, but did not eliminate, the changes in BAT caused by these thermogenic stimuli. Rothwell and Stock (1984b) reported a similar study soon afterward and found results that suggested that changes in BAT in response to cold or to diet could be completely prevented by denervation. To resolve this dilemma, I studied the effect of denervation as a time-course over two weeks. I studied the effect of cold-acclimation after unilateral denervation and I studied the effect of unilateral denervation in rats which had been previously cold-acclimated for two weeks (Results section B4c). As a further control, for the possible effects of cross-innervation (not considered likely), or for possible paracrine growth factors released by the intact side, the effects of bilateral denervation were compared to those of unilateral denervation or sham surgery in both situations described above. As well as tissue protein and GDP binding, the activity of T5'D and specific UCP were measured in these studies.

A study similar to the second part of this one, in which changes in unilaterally-denervated BAT of previously warm- or cold-adapted mice were determined, has been reported by Desautels et al. (1986). Since mice were used by that group, that study complements mine. It is further considered in the Discussion.

5. STATEMENT OF OBJECTIVES.

The overall objective of this work was to study thermogenesis and growth in brown adipose tissue in rats subjected to a variety of conditions. In each study, conditions were chosen because it was thought that the "strategy" of energy expenditure would be different than that seen in normal rats, and that this would be reflected in altered thermogenesis in BAT as well as an altered growth response.

Two major approaches were used. In the first, rats were subjected to manipulations of the hypothalamus known to cause abnormalities in whole-body energy metabolism. Here, the principal objective was to determine the contribution of BAT to the altered energy expenditure, to determine any changes in response to two stimuli known to affect thermogenesis and growth of BAT (exposure to cold and overfeeding), and to contribute to the understanding of the central regulation of energy metabolism.

Three studies were made under this approach. In the first, the acute response to two types of hypothalamic lesion, one thought to chronically activate BAT (lesion of the lateral hypothalamus) and the other thought to chronically inhibit BAT (lesions of the ventromedial hypothalamus) was studied. The objective was to determine whether these lesions had the same effects immediately after the lesions, and to determine whether any effects were specific to the type of lesion.

In the second study, I studied the effects on thermogenesis and growth of BAT in rats having parasagittal knife-cuts dividing the lateral hypothalamus from the ventromedial hypothalamus. This lesion causes obesity because of hyperphagia and reduced energy expenditure. The objective of this study was to evaluate the involvement of BAT to the

altered energy expenditure of these rats and to study changes in the growth of BAT in these rats when challenged with the chronic stimulus of overfeeding.

In the third study, I studied rats having lesions of the lateral hypothalamus, which cause hypophagia and increased energy expenditure. Here again, the objective was to evaluate the involvement of BAT thermogenesis to the increased energy expenditure, to study the ability of the rats to respond to an acute stimulus of cold-exposure, and to study the growth response of BAT to a chronic stimulus of overfeeding, since the response of BAT to these stimuli could be altered by the hypothalamic lesion.

The second major approach was to use environmental and endocrinological (including neuroendocrinological) manipulations to study BAT. As under the first approach, the overall objective was to study how these manipulations affected thermogenesis and growth in BAT. In addition, a major objective was to characterize the regulation in BAT of the activity of thyroxine 5'-deiodinase (T5'D), the enzyme converting thyroxine (T_4) to triiodothyronine (T_3). Four studies were performed.

In the first study, the objective was to characterize the effects of acute and long-term cold-exposure, and of overfeeding, on the activity of T5'D. These stimuli were chosen since it was known that they cause activation of thermogenesis and growth of BAT.

The second study followed from the finding in the first study that overfeeding did not cause an increase of activity of T5'D. The objective of the second study was to determine whether there was any circadian variation of T5'D activity, since the result of the first experiment may

have been an artifact of the time of day that the experiment was performed. It was thought that, since a variety of factors known to affect BAT vary with a circadian cycle, and that other indices of BAT function also show a circadian variation, the activity of T5'D might also. As in the first experiment, the stimuli of cold-acclimation and overfeeding were used to elicit response in BAT.

The objective of the third study was to determine whether T_3 produced in situ in BAT by the T5'D present there was required for the cold-induced acute activation of thermogenesis or for the long-term growth of BAT in rats being acclimated to cold. While it had been shown previously that circulating T_4 was sufficient for these responses, the presence of the T5'D could invalidate this finding. Therefore, thyroidectomized rats injected with T_3, T_4, or vehicle were studied after acute or long-term cold-exposure.

The objective of the fourth study was to assess the importance of the sympathetic innervation of BAT in mediating the acute and long-term responses of BAT to cold, including its importance in maintaining the cold-adapted state. In the first section of this work, the objective was to demonstrate the validity of surgical denervation as a system; noradrenaline content was studied at various times after denervation. The objective of the second section of the study was to show the equivalence of unilateral and bilateral denervation (to permit the use of the former as a system in the last part of the experiment, so that animals could be used as their own internal controls) and to characterize the effects of denervation on BAT. The objective of the last part was to study, as a time course, the changes in the most important indices of the function of BAT (total

protein, GDP binding, T5'D activity, and UCP level), and the importance of the innervation in mediating these changes, in rats exposed to cold either after or before denervation surgery. This was done by, in the first part of the study, studying the effects of cold-exposure I, 3, 6 or 12 days after unilateral denervation of BAT. The importance of the sympathetic innervation in maintaining the cold-adapted state was studied in the second part of this study, in which rats previously cold-acclimated for two weeks were subjected to unilateral denervation of BAT, then returned to the cold for 1, 3, 7 or 14 days.

6. RATIONALE FOR PROCEDURES. The overall objective of this work was to characterize the acute thermogenic response and the chronic adaptive response of BAT in rats subjected to a variety of regimens. From this objective come the rationale for the methods by which I studied BAT and for the experimental designs used.

In the experiments presented in this thesis, a number of standard procedures were used. Not all of these procedures were used during all experiments; with time, other methods became available and so I used different combinations of procedures at different times. As the Results sections are arranged chronologically, one can see how the methods used progressed in time. I now briefly describe the methods used and why they were used.

a) Body weights were routinely measured to ensure that animals were grouped on the basis of equal weights and growth rates at the start of each experiment. Changes in body weight were determined to characterize the over-all effects of the experimental manipulations.

b) Wet weight of gonadal white adipose tissue was used as a measure of the adiposity of the rats. With the body weight change, this measurement can be used to characterize the effect on body composition of the experimental manipulation.

c) Body temperature at death was used as a measure of the overall thermogenic state of the rat.

d) Food intake determinations were done in some studies to help assess the effect of experimental manipulations on energy intake and macronutrient selection.

e) Serum levels of T_3 and T_4 were measured in some experiments. These hormones are important in understanding whole-body metabolic rate (and hence energy expenditure). They are also important in interpreting the importance of thyroxine 5'-deiodinase. In the experiment with thyroidectomized rats, serum levels of thyroid hormones were useful in assessing the replacement therapy.

f) Wet weight of brown adipose tissue was determined as an estimate of tissue size. It should be noted that this is an unreliable indicator of the importance of BAT in a particular animal because it can be increased for a number of reasons. In cold-acclimated rats, there is a great increase in protein content while in cafeteria-fed rats there is a great increase in stored lipid. In these two cases, the BAT may have similar wet weights (2-3 times the weight of BAT in normal rats) yet the state of the tissue is very different in these two cases. Thus these wet weights must be interpreted carefully.

g) Total protein content of BAT is a much better index of the effective metabolic mass of tissue than is wet weight. and was therefore determined in all studies.

h) Total DNA content of BAT was measured in earlier experiments; an increase in treated rats compared to control rats indicates hyperplasia. In my hands, cold-acclimation consistently increased total DNA content while cafeteria-feeding did not. This measurement was not made in later experiments because the method we were using required too much of the homogenate.

i) Total activity of cytochrome oxidase of BAT was measured in some earlier experiments as an estimate of mitochondrial mass. In my hands, the specific activity of this enzyme (per mg homogenate protein) was nearly constant in rats treated under various experimental regimens, so total protein was judged to be an adequate estimate of this index of tissue size. Therefore this activity was not measured in later studies. This also spared homogenate for other assays.

j) GDP binding was measured as an estimate of the thermogenic state of BAT, as previously explained. (It should be noted that since, in living cells, adenine nucleotides are much more abundant than guanine nucleotides, ADP, not GDP, is probably the physiological ligand. GDP is used because of its somewhat greater binding specificity and because it is not translocated into the mitochondria.)

k) Thyroxine 5'-deiodinase was measured in later experiments when the technique became available in our experiment. Its importance and meaning were described in more detail above.

l) Specific uncoupling protein (UCP), the molar amount of UCP expressed per mass of mitochondrial protein, was determined in later experiments. It is the most sensitive index yet devised for the thermogenic capacity of BAT and has replaced the method of gel scans, as described above. It complements the findings with GDP binding in that the former is a measure of how thermogenic a particular sample of BAT can be (i.e., it is a measure of the adaptive response) while the latter is an estimate of how thermogenic a sample of tissue is (i.e., it is a measure of the acute response).

Results were expressed as nmol UCP per mg mitochondrial protein to facilitate calculation of ratios of GDP binding (which are expressed as pmol GDP bound per mg mitochondrial protein) to UCP concentration. It is this ratio that changes during "masking" and "unmasking" of GDP-binding sites. The molar concentration of UCP was calculated by assuming a molecular weight of 32 kDa.

m) Final considerations. It is important to note that in measurements involving homogenates of BAT (total protein, total DNA, total cytochrome oxidase activity, total deiodinase activity), other cells, not just brown adipocytes, are measured. This in no way deters from the value of the observations, since BAT is a complex tissue which requires the support of the other cell types. It is only to be expected that an increase in the number and size of brown adipocytes would be accompanied by an increase in the amount of blood vessels, nerves, interstitial cells, and extracellular matrix. It should also be noted that these supporting tissues have few

mitochondria compared to the brown adipocytes, so isolated mitochondria from BAT are almost all from brown adipocytes.

Not all the measurements described above are of equal importance. The most important ones, upon which the major conclusions rest, are GDP binding, total BAT protein, and specific UCP level. These may be thought of as estimates of thermogenic state (acute response), active metabolic mass, and thermogenic capacity (adaptive response). We consider the activity of thyroxine 5'-deiodinase to be of great importance and to be a significant index of BAT function, but this is not as well established as the other indices and its importance is still a matter of dispute.

MATERIALS AND METHODS

"Check if you can disconnect the effect
And I'll go after the cause."

-P. Gabriel

1. ANIMALS

a) Types. Rats and rabbits were used for the work reported in this thesis. Almost all experimental work was done with male Holtzman rats, of weight ranges as reported in the Results section for each experiment, which were obtained from Charles River Breeding Laboratories, Montreal. (Female Holtzman rats were used in the study of hypothalamic knife-cuts and were obtained from Canadian Breeding Laboratories, St. Constant, Quebec, and were shipped to Ottawa after surgery in Toronto.) Male and female white New Zealand rabbits, used in the development of antisera to the uncoupling protein, were obtained from local sources ("Laboratory Animal Farm").

b) Standard holding conditions. Animals were kept at 26°C except as noted. (An electrical space heater was used to ensure a constant room temperature.) Fluorescent lights were on from 07h00 to 19h00.

On arrival from the supplier, rats were first placed in large plastic group cages (3 to 5 rats per cage), with chow (Purina rat chow 5012) and water ad lib., for 3 to 7 days. After this recovery period, they were placed in individual hanging wire-mesh cages with chow and water (and, for some rats, cafeteria food, as described below) for the rest of the experimental periods. (In the study of thyroidectomized rats (Results section B3), all animals were kept in individual plastic cages after the initial recovery

period and thyroidectomized rats were given 0.125% calcium gluconate to drink instead of water.)

Rabbits were kept in large individual metal cages in the rabbit colony of the Animal Care Services.

c) Experimental procedures. Three main variations to the standard holding conditions were used, cold-exposure, cafeteria-feeding, and pair-feeding.

i) Cold-exposure. Animals were subjected to cold-exposure for hours to days, depending on the experiment (see Results sections below) by placing them, in their original cages, in a rack kept in a cold-room maintained at 4°C. Feeding and water were as described above. Lighting was on from 07h00 to 19h00, but it was provided by a small portable fluorescent source and so the intensity was lower than for the rats kept in the warm-room.

ii) Cafeteria-feeding. In some experiments (see Results sections below) animals were fed a palatable diet of human food rotated on a four-day schedule. The details of the foods given vary for each experiment, but, in general, foods given were similar in all experiments. An example of a schedule for a cafeteria diet is given in Appendix 1. In general, a daily menu consisted of a piece of a sandwich, a cookie, some breakfast cereal or snack food, and candy or a piece of a chocolate bar.

Animals were fed daily just before lights-out, and uneaten food from the previous day was removed at that time. To control for this daily disturbance of the cafeteria-fed rats, chow was put into the cages of

control rats at the same time as the cafeteria-feeding was done. (Cafeteria-fed rats also had chow put into their cages.)

iii) Pair-feeding. In two experiments, pair-feeding was used. In this procedure, control animals were paired to treated animals on the basis of pre-treatment body weights and growth rates.

In the first experiment where this method was used (Results section A2), the amount of chow fed to rats having knife-cuts dividing the lateral hypothalamus from the ventromedial hypothalamus (which causes the rats to become hyperphagic) was restricted to the amount eaten by the paired, non-lesioned rats the previous day. In the second experiment (Results section A3), non-lesioned rats were restricted to the amount eaten the previous day by the paired rats having lesions of the lateral hypothalamus (which causes hypophagia).

In pair-feeding, daily chow intakes were determined (as described in Materials and Methods section 1d below) and the appropriate amount of food, plus 10% extra to account for spillage, was weighed and presented to the pair-fed rats just before lights-out.

d) Food intakes. Twenty-four hour food intakes were determined by weighing the food to be given to the rats (test meal), removing the rat's previous supply of food, placing the weighed food in the rat's cage, then weighing the food left by the rat after 24 hours. Rats were fed the test meal within four hours before lights-out. Paper towel was placed beneath each cage to collect fragments of uneaten food that fell out of the wire-mesh cages. Where necessary, wet left-over food was dried in an oven

(55°C). Correction was made for cafeteria food which had dried in the animal room by leaving a piece of the same food in an empty cage.

From the weight of food left the energy and composition (fat, carbohydrate, protein) were calculated from published analyses. For rat chow, values supplied by the manufacturer (Purina) were used (22.5% protein, 4.5% fat, with the balance as carbohydrate). For cafeteria menus, values from Pennington and Church (1980) were used.

e) Body weights. Except on days of preparations, when body weights were determined just before decapitation, body weights were determined within four hours of lights-out, usually in the hour before lights-out.

f) Body lengths. After light ether anaesthesia, naso-anal lengths were determined by stretching the rat full-length on a ruler.

2. **SURGERY.** Two types of surgery were used. Brain surgery was performed by Drs. Don Coscina and John Chambers of the Clarke Institute, Toronto. Denervation surgery was performed by me.

a) Brain surgery. Rats were anaesthetized with sodium pentobarbital (Nembutal, 35 mg/kg i.p.). The heads of the rats were shaved and the crania were opened with a dental drill. A Kopf stereotaxic apparatus was used to direct the instruments used in making the lesions.

Two types of surgery were performed. Bilateral radio-frequency heat lesions of the lateral hypothalamus or the ventromedial hypothalamus (see Results sections A1 and A3) were made with a Radionics RFG-4 radio-

frequency generator and thermistor probe (0.75 mm diameter). The sham lesions were made by lowering the probe but not turning on the power. Knife-cuts dividing the lateral hypothalamus from the ventromedial region (Results section A2) were made with a retractable wire knife. The sham operations were performed by lowering the knife-guide but not the wire knife.

Lesions of the ventromedial hypothalamus were made at 55°C for one minute for each hemisphere at stereotaxic coordinates 5.5 mm anterior to the interaural line, 0.5 mm lateral to the mid-sagittal sinus, 8.6-9.0 mm below the dura, with the heads held flat between the lambda and bregma sutures. This procedure was used in the study of the acute effects of hypothalamic lesions (Results section A1).

Small lesions of the lateral hypothalamus were made at 50°C for one minute for each hemisphere at stereotaxic coordinates 6.0-6.2 mm anterior to the interaural line, 1.8 mm lateral to the mid-sagittal sinus, 8.0-8.2 mm below dura with the heads flat. This procedure was used for both the acute and long-term studies of lesions of the lateral hypothalamus (Results sections A1 and A3).

Hypothalamic knife-cuts (to separate the ventromedial hypothalamus from the lateral hypothalamus) were made to be 3x3 mm in area, extending 5.0-8.0 mm rostral to the interaural line, 0.7-0.8 mm lateral to the midline and 3 mm dorsoventral from the bottom of the thalamus to the base of the brain (see Results section A2).

After surgery, the skin around the incision was closed with two to four 9 mm "Autoclip" (Totco) wound clips.

b) Denervation of interscapular BAT. Rats were anaesthetized either with ether or with halothane. Hair was cut off the back of the rat over an area of about 2x3 cm centered on the midline at the crest of the scapulae. A midline longitudinal incision of 1.5-2 cm was made. By retracting the edges of the incision the interscapular depot of BAT was exposed. A small incision of the deep fascia at the anterior edge of the depot was then made, 2-5 mm lateral to the midline. By blunt dissection, one side of the BAT pad was lifted from the posterior chest wall. By abducting the scapula, the five nerves innervating the BAT could be seen running from the chest wall to the inferior aspect of the pad on that side. The nerves were then cut, at two places (near the chest wall and near the BAT) where possible. In most cases, all five nerves were cut. In some of the earlier experiments (Results Section B4a) only the first four nerves were cut when the fifth nerve was too distant to reach easily. In the later experiments (Results Sections B4b and B4c) all five nerves were cut in all cases. After nerve section, the pad was replaced to its normal position and the incision was closed with non-dissolving 3-0 silk suture (Ethicon cuticular 684G).

For most experiments, unilateral surgical denervations were performed as described above. The contralateral side was left undisturbed. In bilateral denervations, both sides were denervated as above. In sham operations, rats were anaesthetized only.

Rarely, the surgery was irritating to the rat, which scratched itself near the stitches. Such rats were treated daily with a spray antiseptic powder. Usually, rats stopped scratching within three days of treatment.

3. BRAIN HISTOLOGY. At the end of the studies of rats having hypothalamic lesions or knife-cuts, placement of the lesions or knife-cuts was determined for each rat.

After decapitation, the brains of the rats were removed and stored in 10% formalin before being shipped to Toronto for histological examination. Brains were then frozen and coronal cryostat sections made through the sites of lesion or knife-cut. Nissl staining with cresyl violet was performed at the Clarke Institute by Dr. John Chambers.

4. INSTRUMENTS. Major instruments used are listed in Appendix 2.

5. REAGENTS. Reagents used and suppliers are listed in Appendix 3.

6. STANDARD PREPARATION. A standard preparation was used in all experiments. (Not all the procedures listed below were done in all experiments. For the exact measurements made in each experiment, see the appropriate Results section.)

a) Collection of tissue. Preparations were begun between 07h00 and 08h30, usually at about 08h00. Rats, in their wire cages, were removed from their holding room to a nearby preparation room. They were weighed and then killed by decapitation immediately thereafter. Blood was collected at once, without anticoagulant, and placed in glass centrifuge tubes kept at room temperature. Within 30 seconds core (rectal) body temperature was determined with a BAT-8 digital thermometer equipped with a thermistor probe. The interscapular BAT depot was then removed and placed in ice-

cold isolation medium (see below). Gonadal white adipose tissue was then removed from the carcass.

Usually eight or twelve rats were killed on each preparation day. After all animals had been killed, BAT was cleaned of adhering white fat and muscle and weighed. Gonadal white fat was also weighed. Blood was kept at room temperature for 30-60 minutes, then centrifuged at 16,000 g at 4°C for 15 minutes. Serum thus obtained was transferred to 1.5 mL plastic Eppendorf centrifuge tubes and centrifuged in a benchtop centrifuge at 4°C for 15 minutes. This separated serum from fat, particularly in serum from rats which had been cafeteria-fed. The infranatant serum was then recovered and frozen in liquid nitrogen. Frozen serum was then kept at -20°C until assayed for thyroid hormones.

Brown adipose tissue was minced and then homogenized in about 5 mL ice-cold isolation medium (250 mM sucrose, 0.2 mM EDTA, 1 mM HEPES, pH 7.2) in a Kontes #23 glass homogenizer, with a teflon pestle, kept ice-cold during the homogenization. The homogenate was then made to 10 mL with isolation medium and small samples taken for later analyses. (When samples were taken for estimation of thyroxine 5'-deiodinase (see Materials and Methods section 10 below), they were placed in a plastic Eppendorf microcentrifuge tube and frozen at once in liquid nitrogen.)

b) Isolation of mitochondria. Homogenates of BAT remaining after samples were taken were then made up to 42 mL with isolation medium. Mitochondria were isolated and washed three times as described by Slinde et al. (1975). (When twelve preparations were being made, the resuspension of the first low-speed pellet was usually omitted to save time.) The final

mitochondrial pellet was resuspended in 100-1000 µL isolation medium to give a final protein concentration of about 10 mg/mL. Usually 250 µL of isolation medium was used to resuspend mitochondria from warm-acclimated rats while 500 µL was used for those from cold-acclimated rats. Mitochondrial suspensions were kept ice-cold until assayed for GDP binding, done immediately after the isolation was completed. Some of the mitochondrial suspension was saved for estimation of protein concentration; in later experiments, aliquots were also saved for estimation of uncoupling protein.

c) Estimation of GDP binding. The amount of GDP binding to isolated mitochondria of BAT ("GDP binding") was estimated by the method of Desautels et al. (1978). 20 µL mitochondria (about 200 µg protein) were incubated for one minute at room temperature (about 21° C) in Eppendorf microcentrifuge tubes, in 275 µL incubation medium (1 mM EDTA, 10 mM choline chloride, 20 mM TES, 100 mM sucrose, 5 µM rotenone, 100 µM atractyloside, pH 7.1; 1000 µM ADP was present in half the assay tubes to allow correction for non-specific binding of GDP; ^{14}C-sucrose (about 2000 Bq per assay (about 54 nCi/assay)) having specific activity 20.7 GBq/mmol (= 560 mCi/mmol) was used to estimate GDP trapped in the pellet but not bound). Then 5 µL of 10 µM ^{3}H-GDP (about 7500 Bq per assay (about 0.20 µCi/assay) having specific activity 418 GBq/mmol (= 11.3 Ci/mmol)) plus carrier was added, and the suspension was again mixed and incubated for a further 30 seconds. The tubes were then centrifuged for 2 minutes in a benchtop centrifuge. Assays were done in quadruplicate except for those reported in Results Section B4c, which were done in triplicate.

After centrifugation, the supernatant incubation medium was aspirated and the tips of the tubes, containing the mitochondrial pellets, were cut into scintillation vials. The pellets were dissolved overnight in NCS tissue solubilizer (1 mL); pellets in NCS were incubated at 55°C to ensure complete solution. The next day, samples were cooled, then 10 mL scintillation cocktail (PPO in toluene, 6.5 g/L) and 50 µL of a 10% aqueous solution of ascorbic acid were added to each vial, which was then counted with a dual-label program.

7. **ESTIMATION OF PROTEIN.** Almost all protein estimations were done with the modified Lowry assay described below, but occasionally the Coomassie blue ("Bio-Rad") was used as noted in the Results sections. Samples were measured in triplicate. Bovine serum albumin (BSA) was used as the standard for all assays.

a) Modified Lowry assay. The protein assay of Lowry et al. (1951) as modified by Schacterle and Pollack (1973) was used. Protein in samples was first precipitated in 5 mL ice-cold trichloroacetic acid (12.5%). The precipitated protein was pelleted by centrifugation (4000 g for 15 minutes) after the samples had stood for at least 30 minutes. (Typically, centrifugation was done the day after precipitation.) Supernatant trichloroacetic acid was removed and the protein pellet dissolved in 1 mL 0.5 M sodium hydroxide with the tubes heated in a 55°C water bath. The procedure of Schacterle and Pollack was followed from this point.

For some determinations done in the presence of interfering detergents (during the isolation of uncoupling protein) the above procedure was done

except that samples were not first precipitated and the copper and sodium hydroxide solutions were made to 1% with SDS. To keep the SDS dissolved, the solutions were kept at 30°C and the 10 minute incubation (copper reagent) was done at the same temperature.

b) Coomassie blue ("Bio-Rad") assay. This assay is based on the work of Bradford (1976). It was performed essentially as described in the manual accompanying the kit (Bio-Rad); modifications of the volumes used were made as required.

8. ESTIMATION OF DNA. Content of DNA in BAT was estimated from concentrations found in homogenates by the diphenylamine method of Burton (1956). DNA in samples was hydrolyzed in 2 mL 5% trichloroacetic acid heated to 90°C for 10 minutes. After cooling, 1 mL 2.25 M perchloric acid was added to each tube and the resulting suspension was filtered under vacuum through Millipore type SS filters (pore size 3.0 µm), to remove precipitated protein and other materials. 2 mL of the filtered sample was mixed with 2 mL of 4% diphenylamine (dissolved in glacial acetic acid) and with 100 µL of 16 mg% acetaldehyde. This was incubated at 30°C for 15 hours and absorbence at 595 and 700 nm was determined. Purified DNA from calf thymus, whose concentration had been verified by ultraviolet spectrophotometry, was used as the standard. DNA contents of samples were determined in triplicate.

9. ESTIMATION OF CYTOCHROME OXIDASE. Total tissue activity of cytochrome oxidase was estimated by a polarographic method, as described

by Behrens and Himms-Hagen (1977). Tissue homogenates were homogenized in 0.1 M potassium phosphate buffer, pH 6.6, with Lubrol WX (3 µg Lubrol per 10 µg protein) and these homogenates were frozen overnight.

The next day, the assay was performed by suspending a volume of the Lubrol homogenate in a solution of cytochrome c (0.2 mM reduced cytochrome c plus 20 mM ascorbic acid in the potassium phosphate buffer) at 37°C; to ensure oxygen saturation before the beginning of the assay, the cytochrome c solution had been vigorously stirred in the chamber for three minutes before incubation. Oxygen consumption of these samples was measured polarographically with a Clarke electrode. The total activity of cytochrome oxidase was then calculated. The concentration of reduced cytochrome c present in the reagent solution was verified spectrophotometrically, with the molar extinction coefficients for oxidized and reduced cytochrome c reported by Yonetani (1967) used for calculation. Samples were tested in triplicate; routinely, different amounts of protein from the same sample were tested to ensure that the assay varied linearly with protein assayed.

10. ESTIMATION OF THYROXINE 5'-DEIODINASE. This assay is based on the methods developed in the laboratory of Larsen (Visser et al. 1982, Leonard et al. 1983, Silva and Larsen 1983) as modified in our laboratory by Dr. Jan Kopecky (Kopecky et al. 1986b). The assay is based on the release of free iodide from thyroxine (T_4) as it forms triiodothyronine (T_3), as catalyzed by the enzyme thyroxine 5'-deiodinase (T5'D). The reaction can be determined by the release of radioactive iodide from T_4 labeled in the outer ring.

Radioactive T_4 was first purified by paper electrophoresis (50 mM ammonium acetate buffer, pH 7.1) at 600 V for 10 minutes. After the electrophoresis, T_4 was extracted in a 99:1 mixture of methanol and ammonium hydroxide; the extract was then concentrated under nitrogen.

Samples of homogenate (which had been frozen in liquid nitrogen as described in Materials and Methods section 6a above) were thawed on ice and then incubated for 30 minutes in the assay mixture (10 mM dithiothreitol (prepared in the sucrose-HEPES-EDTA mitochondrial isolation buffer described above), 2.57 nM carrier T_4 (dissolved in 50 mM sodium hydroxide plus 0.1% BSA, then diluted to the final concentration), 0.15 nM labeled T_4 (high specific activity reagent: 1000 Bq/assay (= 27 nCi/assay)), and 1 mM propylthiouracil (PTU) as an inhibitor of the type I enzyme, all in a buffer of 10 mM potassium phosphate plus 1 mM EDTA, pH 7.0). For warm-acclimated rats, 30 µL homogenate (30-150 µg protein) was used; for cold-acclimated rats, the homogenate was usually diluted 5- or 10-fold before the assay. The incubation was done at 37°C in a shaking water bath. The reaction was stopped by the addition of 50 µL thyroid hormone-free human serum (which binds T_3 and T_4): after that, all protein was precipitated by addition of 300 µL 12.5% trichloroacetic acid. The protein was pelleted by a two-minute centrifugation in an Eppendorf benchtop centrifuge. Then 500 µL of the supernatant was applied to a short column of cation exchange resin. This was washed 3 times with 1 mL acetic acid (10% glacial acetic acid in water) and the supernatant and washings, containing the freed radioactive iodide, were counted in a gamma counter. Assays were done in triplicate for each sample.

11. ESTIMATION OF SERUM THYROID HORMONES. Two methods were used for the estimation of serum levels of the thyroid hormones T_3 and T_4. The particular method used for each study is mentioned in the respective Results section. Assays were done in triplicate, for all samples, for both methods.

a) Amerlex kits. In the earlier studies, thyroid hormone levels were measured with kits sold by Amersham and developed to determine levels of thyroid hormones in human serum. These kits are liquid-phase systems, with the antibody bound to solid polymer particles. Serum levels of thyroid hormones are determined by competition for these antibodies against radioactively-labeled T_3 or T_4. These kits were used exactly as directed in the instruction manuals accompanying the kits.

b) Method of Silva and Larsen. More sensitive radioimmunoassays for thyroid hormones in rat serum were made possible by the generous gift of antisera for T_3 and for T_4 from the laboratory of Dr. P.R. Larsen. The procedures used were based on those used in his laboratory (Larsen 1972, Larsen et al. 1973, Larsen 1976). The assays were done in liquid phase, with competition between thyroid hormone in serum and added labeled hormone. Here, however, the antibodies are not bound to beads; the free hormone was separated from that bound to antibodies by use of a dextran-charcoal mixture.

Hormone-free rat serum was prepared by adding activated charcoal (2 g/100 mL) and stirring this overnight; the suspension was then centrifuged at 48,000 g for 30 minutes. Radioactive hormone had been

added to indicate removal of hormone; when necessary, the charcoal treatment was repeated. This hormone-free serum was then used to prepare series of standards for T_3 and for T_4. (Concentrated solutions of T_3 and T_4 were first made in 50 mM sodium hydroxide plus 0.1% BSA. Hormone concentrations were verified spectrophotometrically before the solution was diluted to the final series of concentrations in the hormone-free serum.)

For assay of T_4, samples were first diluted 20-fold in glycine-acetate buffer (GAB) (200 mM glycine, 200 mM sodium acetate, pH 8.6). 100 µL aliquots were added to 900 µL volumes of the assay buffer (GAB plus 2% sodium salicylate and 20 mg% BSA) which contained 120 Bq (= 3.2 nCi) high-specific activity ^{125}I-T_4 per assay and an appropriate dilution of antiserum. After an overnight incubation at 4°C, 1 mL of the dextran-charcoal suspension (0.125 g dextran and 1.25 g activated charcoal in 200 mL ice-cold GAB, diluted 1/16 in GAB just before use) was added. After a 45 minute incubation, tubes were centrifuged for 20 minutes at 1500 g. The supernatant was decanted and counted in the gamma counter.

The assay of T_3 was similar but there were some differences. 25 µL of undiluted rat serum were added to 875 µL of assay buffer (1.14% sodium salicylate plus 0.1% BSA in GAB (200 mM glycine plus 125 mM sodium acetate, pH 8.6)) containing an appropriate dilution of antiserum. This was incubated at 4°C for 48 hours. Only then was tracer radioactive T_3 added (100 µL of the high-specific activity reagent, 33 Bq (= 0.9 nCi) per assay. This was incubated for 48 hours at 4°C before addition of the dextran-charcoal mixture and centrifugation as described above.

12. ESTIMATION OF NORADRENALINE. To assess the importance of the sympathetic nervous system for growth and maintenance of growth in brown adipose tissue, surgical denervations were performed (Results section B4a). To evaluate the effectiveness of the denervation, noradrenaline levels in brown adipose tissue were determined by reverse-phase high-pressure liquid chromatography (HPCL) with electrochemical detection. This method was developed for our laboratory by Dr. Gloria Zaror-Behrens, who standardized all conditions used here (Himms-Hagen et al. 1986).

BAT frozen on dry ice was homogenized in 3.85 mL 1% perchloric acid (PCA) plus 150 µL 1000 ng/mL 3,4-dihydroxybenzylamine (DHBA) in 0.1 M PCA (DHBA is used as an internal control for recovery of catecholamines) for 10 seconds with a Polytron at its maximum setting. Standards were prepared by addition of 150 µL or 15 µL 600 ng/mL noradrenaline in 0.1 M PCA to 150 µL DHBA in 0.1 M PCA, with the balance to 4.00 mL made up with 1% PCA. (The standards containing the lesser amount of noradrenaline were used as standards for denervated half-pads.)

Homogenates were then centrifuged at 3000 g for 30 minutes at 4°C. Fat was removed from the supernatant by filtration through glass wool; the filtrate was then quickly frozen and kept at -80°C until recovery of catecholamines as described below.

Catecholamines from samples were purified for chromatography by adsorption onto, and desorption from, alumina. PCA extracts were melted and sodium metabisulfite was added to give a final concentration of 0.1%. Samples were alkalinized by addition of 1.3 mL TRIS-EDTA buffer (2 M TRIS, 5.2% EDTA, pH 8.6) and mixed. 100 mg washed alumina was added and the tubes were mixed vigorously for 5 minutes. The alumina, with

adsorbed catecholamines, was washed three times by first sedimenting it with a benchtop centrifuge and then, after aspiration of the bulk of the supernatant, resuspending it in doubly-distilled water. After the last washing, as much of the supernatant was aspirated as possible. The alumina was then acidified by addition of 0.5 mL 0.2 M PCA, thereby desorbing the catecholamines. The tubes were mixed and the alumina sedimented as above. The supernatant was collected and centrifuged a final time in Eppendorf microcentrifuge tubes. This supernatant was collected and stored frozen at -80°C until chromatography. Standards were prepared in exactly the same way.

Noradrenaline content of 50 µL aliquots of samples were estimated in triplicate by analysis by high-performance liquid chromatography (HPLC). Samples were analyzed by reverse-phase chromatography, with a mobile phase of 150 mM chloroacetic acid, 2 mM EDTA, 6 mg% sodium octyl sulfate, pH 3.1. A C_{18} column with mesh size of 5 microns and volume of 4 x 150 mm was used; chromatography was performed at room temperature (21°C) with a flow rate of 1.5 mL/minute. Retention times were determined by amperometric electrochemical detection. Since Dr. Zaror-Behrens had shown that there was a linear relationship between the amount of catecholamine assayed and the height of the peak, this method (analysis of peak-heights) was used to calculate the content of noradrenaline in each sample.

13. RADIOIMMUNOASSAY OF UNCOUPLING PROTEIN. I developed a radioimmunoassay (RIA) for rat uncoupling protein (UCP). This assay was based largely on the method developed by Lean et al. (1983), although

some procedures were modified from other sources, particularly Cannon et al. (1982). There were three steps in developing this assay: purification of the UCP, raising and testing of the antiserum, and determination and optimization of the conditions for the RIA.

a) Purification of uncoupling protein. Purification of UCP was performed by a procedure based on the method of Lin and Klingenberg (1982), with modifications as noted below.

Rats were cold-acclimated two to three weeks before preparation of UCP. They were killed and mitochondria prepared as described in Materials and Methods sections 6a and 6b above. Mitochondria were incubated for 30 minutes at 0°C in a modified "standard medium" (20 mM MOPS, 0.16 mM EDTA, and 50 mM sodium sulfate (not 20 mM as in the published procedure), pH 6.7) containing 3.2% Lubrol WX. The suspension was centrifuged at 100000 g for 30 minutes at 4°C, then the supernatant medium was removed, along with as much of the sedimented Lubrol WX as could be removed without disturbing the pellet. The pellet (as well as the remaining pelleted Lubrol WX) was resuspended in ice-cold sucrose medium (300 mM sucrose, 10 mM TRIS, 2 mM EDTA, pH 7.2) and centrifuged again as described above. Remaining pelleted Lubrol WX was again removed with the supernatant, and the mitochondrial membrane pellet was resuspended in sucrose medium and again centrifuged. This final washed pellet was then stored overnight at -80°C.

The next day, the pellet was suspended in ice-cold standard medium containing 5% Triton X-100. After a 30 minute incubation at 0°C, the suspension was centrifuged as above and the supernatant separated at room

temperature (about 20°C) on a column of hydroxylapatite that had been equilibrated with standard medium. The pass-through fractions, containing the UCP (in Triton X-100 micelles) was pooled. (The peak fractions were determined by detection of Triton X-100 by ultraviolet spectrophotometry.) The purification procedure was stopped at this point; unlike the published procedure, pressure dialysis followed by centrifugation on a sucrose density gradient was not performed.

Determination of protein concentration was done with the modified Lowry assay in the presence of 1% SDS; determination of Triton X-100 (which was critically important for the RIA, as explained below) was done spectrophotometrically by determination of absorption at 290 nm, which was compared to the absorption of a series of standard solutions of Triton.

In an original pilot-scale purification, binding of tritiated GDP to column fractions was done essentially as described in Lean et al. (1983) to demonstrate that the pass-through fractions did indeed bind GDP. (The tritiated GDP was incubated 10 minutes at room temperature with 25 µL fractions from the hydroxylapatite column, after which bound GDP was separated from free GDP by chromatography (Sephadex G50).) However, this procedure was not used in the large-scale preparations because it was too time-consuming.

Uncoupling protein prepared by this procedure was homogeneous as demonstrated by SDS-PAGE done in a 8-20% gradient gel (Figure 1). The homogeneity of the preparation was further demonstrated (indirectly) by the finding that the antiserum raised against it (section b, below) had the same specificity as antisera (against hamster uncoupling protein) prepared independently by Dr. Jan Kopecky in Prague, and that the anti-hamster

FIGURE 1. METHODS: SDS-PAGE OF PURIFICATION OF UNCOUPLING PROTEIN.

Uncoupling protein was purified as described in the text. Electrophoresis was based on the method of Laemmli (1970). Samples (10 µg) from the various steps of the purification were denatured in lysis medium (2.3% SDS, 10% glycerol, 5% mercaptoethanol, 125 mM TRIS, pH 6.8) for three minutes at 100°C. Electrophoresis was performed in an 8%-20% SDS-polyacrylamide gel in a discontinuous system (upper buffer (stacking gel):125 mM TRIS, 0.2% SDS, pH 6.8); lower buffer (separating gel (400 mM TRIS, 0.1% SDS, pH 8.8); running buffer 25 mM TRIS, 200 mM glycine, 0.1% SDS, pH 8.3) at 4°C (with cooling) at 100 volts for 20 hours. The gel was then fixed in 5:5:1 methanol:water:acetic acid, stained with Coomassie blue, and destained (65:25:8 water:methanol:acetic acid).

Samples analyzed (left to right): 1. molecular weight standards: 2. purified UCP; 3. supernatant of Triton extract; 4. pellet of Triton extract; 5. pellet from second wash in sucrose medium; 6. supernatant from that wash; 7. pellet from first wash in sucrose medium; 8. supernatant from that wash: 9. pellet from incubation in Lubrol medium; 10. supernatant from Lubrol incubation; 11. Lubrol suspension at the beginning of incubation; 12. isolated BAT mitochondria; 13. homogenate of brown adipose tissue.

antiserum reacted specifically with this rat UCP preparation. (It has been shown in a number of laboratories that there is general cross-reactivity between antisera and UCP preparations from different species.)

b) Immunization of rabbits and testing of antiserum. Pre-immune serum was collected from rabbits before they were immunized. Initially, rabbits were inoculated for three consecutive days: on the first, UCP was prepared in Freund's complete adjuvant; on the second and third days, it was prepared in Freund's incomplete adjuvant. On each day, rabbits were injected (s.c.) in four different locations with 50 µg UCP in 2 mL adjuvant.

Rabbits were bled weekly and the antibody titer was assessed from antiserum dilution series with preimmune serum used as the control (see Figure 2). The antibody titer was maximal by six weeks; rabbits were then boosted by s.c. injection (in four sites) of 50 µg UCP in 2 mL Freund's incomplete adjuvant. However, this did not increase the titers as expected. Three months after the initial injections, rabbits were again boosted with 100 µg UCP suspended in 2 mL Freund's incomplete adjuvant injected s.c. in four sites. After three weeks, the titer had not increased and it was decided that further boosters would serve no purpose. The rabbits were then killed by an overdose of Innovar-Vet (0.2 mL/kg) and bled completely. The blood was collected without anticoagulant and centrifuged at 16000 g for 20 minutes and the serum thus obtained was divided into 5 mL volumes and frozen. Except for current stock, which was mixed 1:1 with glycerol and kept at -20°C, all serum was stored at -80°C.

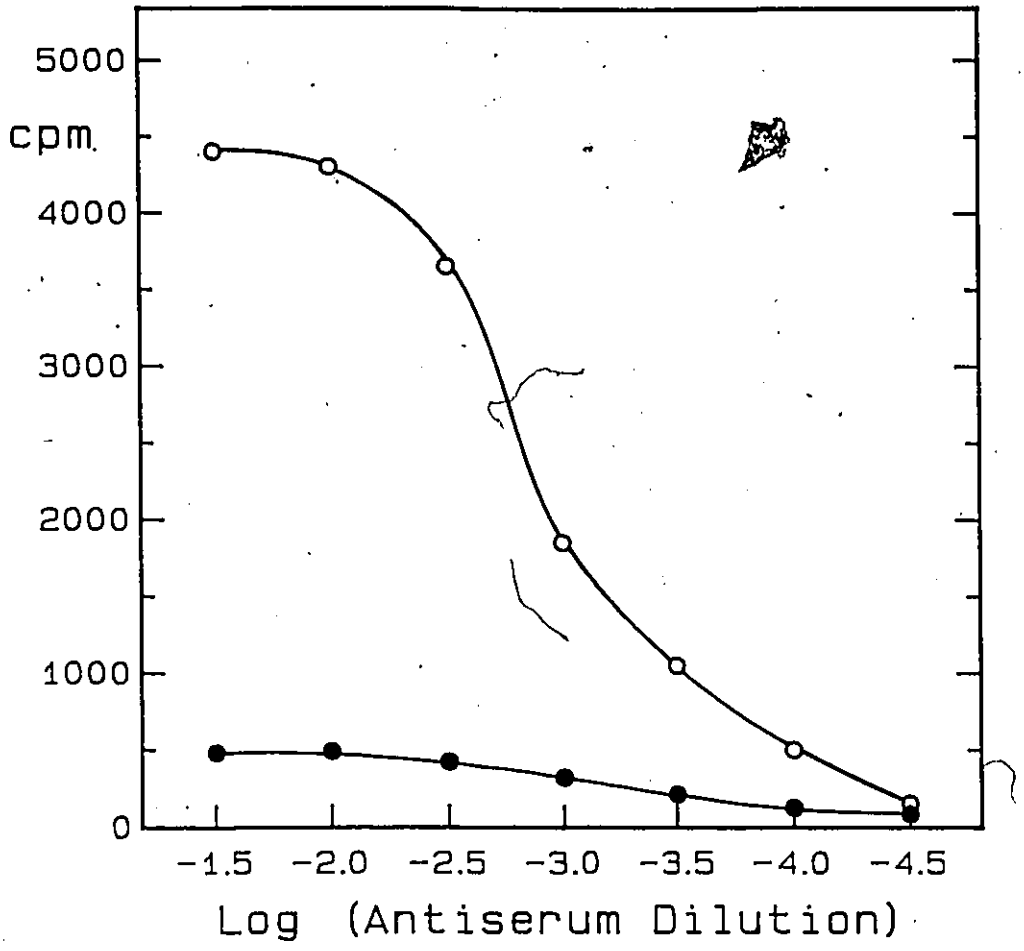

FIGURE 2. METHODS: ANTIBODY DILUTION CURVE FOR IMMUNE AND PREIMMUNE SERA.

Microtiter plates were coated with UCP, then washed with RIA buffer, as described in Materials and Methods section 13b. Serial dilutions of preimmune serum (solid circles) or immune serum (empty circles) were incubated overnight at 4°C. Wells were then washed and incubated with ^{125}I-protein A as described in section 13c.

The specificity of the serum was verified in two ways. First, as was mentioned above, Dr. Kopecky tested this antiserum, in comparison with antisera prepared in his laboratory, by performing Western blots of mitochondria from BAT and of purified UCP; the same specificity was observed when blots were developed with his antisera as with mine.

Second, the antiserum was tested for specificity by making Western blots of homogenates of various tissues (BAT, white adipose tissue, heart, liver, kidney, skeletal muscle, and brain). It was shown that the antibody reacted only to BAT homogenate and to purified UCP (Figure 3).

c) Radioimmunoassay of uncoupling protein. After some considerable difficulty, conditions for the radioimmunoassay of UCP were established. Considerable time was spent in trying to reproduce the procedure used by Lean et al., but it was found that the use of "Bio-beads" confounded the results. They are intended to remove excess detergent; in my hands, they were equally successful in removing the UCP. Therefore, their use was abandoned. Instead, the concentration of Triton X-100 present in the UCP preparation was carefully determined (spectrophotometrically). Then, in preparing standard curves and samples for assay, it was ensured that exactly the same final concentration of Triton X-100 was present in all wells. Thus, although the presence of detergent interferes with the binding of the antibodies, this interference was the same for all standards and samples. However, due to this, the dilution of antiserum used was lower than that stated in reports published by other laboratories.

The preparation of the samples to be assayed was also done differently

FIGURE 3. METHODS: WESTERN BLOT OF HOMOGENATES OF VARIOUS RAT TISSUES DEMONSTRATING SPECIFICITY OF ANTISERUM TO UNCOUPLING PROTEIN.

Electrophoresis of homogenates of BAT, WAT, heart, liver, kidney, muscle, and brain (30 µg), and purified UCP (3 µg) were performed as described in the legend to Figure 1. A Western blot was made of the gel based on the method of Towbin et al. (1979). After electrophoresis, the gel was incubated in blotting buffer (150 mM glycine, 20 mM TRIS, 0.02% SDS, 20% methanol, pH 8.3) for 30 minutes. It was transferred to nitrocellulose (0.2-0.4 µm pore size, Bio-Rad) at 4°C at 7 V/cm for 3 hours. The nitrocellulose was frozen overnight. The next day, it was incubated with shaking in PBS-Tween buffer (PBS as described in text plus 0.05% Tween-20) for 30 minutes at room temperature. It was then incubated with shaking for 2 hours at room temperature in PBS-Tween buffer containing antiserum at a final dilution of 1/100. The blot was then washed for two hours in PBS-Tween with three changes of washing buffer. Bound antibodies were detected after a 1-hour wash in radioiodinated protein A diluted to 20000 Bq/mL (0.54 µCi/mL). The blot was washed for 10 hours in PBS-Tween buffer with several changes of wash buffer in that time. After drying in air, the blot was exposed to X-ray film in a cassette with intensifying screens for 24 hours at -80°C. After development, the autoradiograph was photographed and then used to localize the bands on the blot, which were then cut out and counted in a gamma counter.

Except for the BAT homogenate (two samples analyzed) and the purified UCP, which both had counts more than 15 times the background, no other band had more than 1.5 times the background counts. Visible bands on the autoradiograph were found for only the BAT homogenates and the purified UCP.

from the procedure published by Lean et al.; rather, it was done exactly as described by York (1985, personal communication).

d) Standard procedure. 96-well polyvinylchloride plates were coated overnight at 4°C with 50 µL UCP diluted to 20 µg/mL. The protein was then recovered for re-use and the plates were washed three times with RIA buffer (phosphate-buffered saline (PBS)(6.7 mM potassium phosphate, 2.7 mM potassium chloride, 8% sodium chloride) plus 1% RIA-grade BSA and 0.1% sodium azide, pH 7.4).

Standards were diluted in PBS to give final protein content of 0, 5, 20, 40, 65, 125 and 250 ng; all wells were also made to a final concentration of 0.01% in Triton X-100. Samples were made to 1 mg/mL with PBS and solubilized in Triton X-100 at a final concentration of 0.5% for 30 minutes at room temperature (about 20°C). They were then diluted 5-fold and centrifuged for 1 minute in a benchtop centrifuge. The samples were diluted 1:10 in PBS (thus giving a final concentration of 0.01% in Triton X-100). Two different volumes were then assayed to act as an internal control. (For samples from cold-acclimated rats, smaller volumes were assayed than for samples from warm-acclimated rats.) Antiserum was diluted in PBS to give a final dilution of 1:1000. The total volume in each well was 50 µL.

The plates, containing antiserum and standard or sample, were then incubated overnight at 4°C. The next day, the solutions were aspirated from the wells and the plates were washed three times with RIA buffer. ^{125}I-protein A (specific activity 1.59 MBq/µg (=43.1 µCi/µg)) was diluted in PBS and each well was incubated with 2000 Bq (=54 nCi) in 50 µL for 2

hours at room temperature. The protein A was then aspirated and the plates were washed three times with RIA buffer. Finally, the wells were cut out and counted in a gamma counter.

From this, standard curves were calculated (an example is given in Figure 4) and UCP content of the samples was determined. All samples and standards were done in triplicate; standards were run on every plate. To ensure reliability of the determinations, they were repeated as necessary; this included changing the volumes assayed so that the samples assayed were within the linear portion of the standard curve. In general, results were not accepted unless determinations of two different volumes of the same sample were in reasonable agreement (differing by less than 10%).

14. COMPUTER PROGRAMS. I wrote computer programs to simplify calculation of results of many of the assays described above, including food intakes and assays of GDP binding, protein, DNA, cytochrome oxidase, and thyroxine 5'-deiodinase. (Program listings are not presented here.)

15. STATISTICAL ANALYSIS. Except for the analysis of the effects of knife-cuts and of lateral hypothalamic lesions (Results sections A2 and A3), which were analyzed at the Clarke Institute, all results were analyzed at the University of Ottawa. The statistical package $SPSS_X$ was used. One-way ANOVAs, with Duncan, Student-Newman-Keuls, and Scheffé post-hoc tests, and two-way ANOVAs were performed as appropriate. Student t-tests were also performed as needed. $\alpha = 0.05$ was taken as the critical level of significance for all tests.

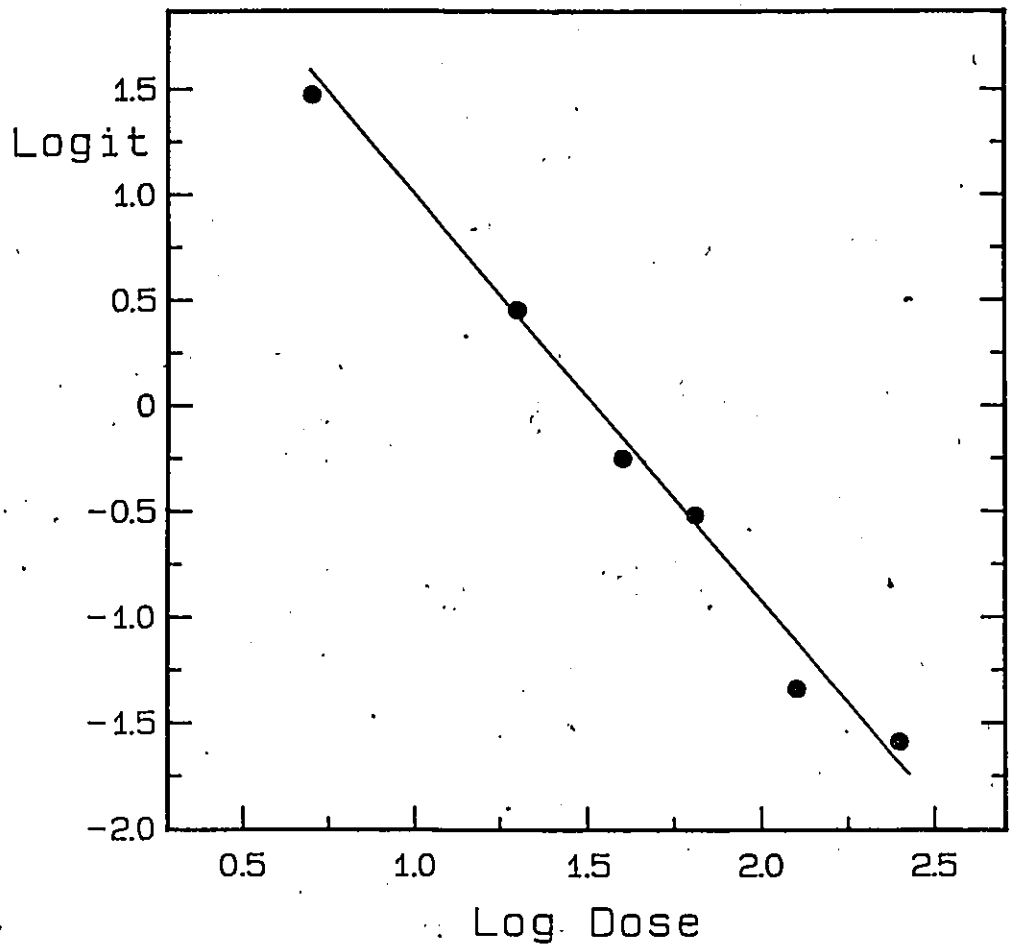

FIGURE 4. METHODS: STANDARD CURVE FOR THE RADIOIMMUNOASSAY OF UNCOUPLING PROTEIN.

This standard curve was prepared as described in section 13c of the Materials and Methods. Logit, a linear transformation of the data (Chard, 1982), was used so that a least-squares analysis could be used to interpolate the results for the samples. In all cases, $r < -0.97$ for the results reported in this thesis.

RESULTS

"There was a line
There was a formula
Sharp as a knife
Facts cut a hole in us"
-D. Byrne

PART A. HYPOTHALAMIC CONTROL OF BROWN ADIPOSE TISSUE

Section A1. The acute effects of hypothalamic lesions on the function of brown adipose tissue.

Objective: I studied the acute effect of LH and VMH lesions on thermogenesis in BAT (estimated by GDP binding) to answer two questions: a) what importance, if any, does BAT thermogenesis have in the acute effects of these hypothalamic lesions; b) is the effect of brain lesion specific or nonspecific.

Design: The effects of lesions of the ventromedial hypothalamus were compared to those occurring with lesions of the lateral hypothalamus, with sham-operated rats and non-lesioned rats serving as controls. Two experiments were performed. In the first experiment, rats weighing 280 to 305 g were grouped for equal mean body weights and growth rates and subjected to one of six treatments: fed non-lesioned (NL), fasted NL, bilateral lesion of the lateral hypothalamus (LH), sham LH, bilateral lesion of the ventromedial hypothalamus (VMH), or sham VMH. Animals in the last four groups also were fasted for the duration of the experiment. Two days (36 to 48 h) after surgery, rats were killed and the standard preparation of BAT was made.

In the second experiment, rats weighing 270 to 290 g were treated as above except that there was no fasted NL group. Animals were killed and the standard preparation was made 20-23 h after the operations were performed.

After both experiments, brains were recovered and analyzed histologically as described in section 3 of the Materials and Methods.

The results were analyzed by one-way Analysis of Variance (ANOVA), with $\alpha = 0.05$ for a two-tailed test. Post-hoc tests were by the method of Duncan.

Results: Both LH and VMH lesions were bilaterally symmetrical and were centered from the antero-posterior level of the VMH to the caudal boundaries of the paraventricular nucleus. Dorso-ventrally, both lesions were centered at the dorsal edge of the VMH; medio-laterally, LH lesions were centered halfway between the fornix and the medial edge of the zona incerta while VMH lesions were centered at the lateral edge of the VMH. An example of an acute LH lesion is shown in Figure 5 and one of an acute VMH lesion is given in Figure 6; the lesions are further described there.

The most important result of this experiment is the lack of acute effect of any lesion (or sham-lesion) on GDP binding, either after two days or one day (Figure 7). At both times, there were no significant differences between groups for all other indices measured except body temperature: LH-lesioned rats were hyperthermic compared to sham-lesioned controls both after one day and two days (Figure 8). Results for other indices are presented in Tables 1 and 2.

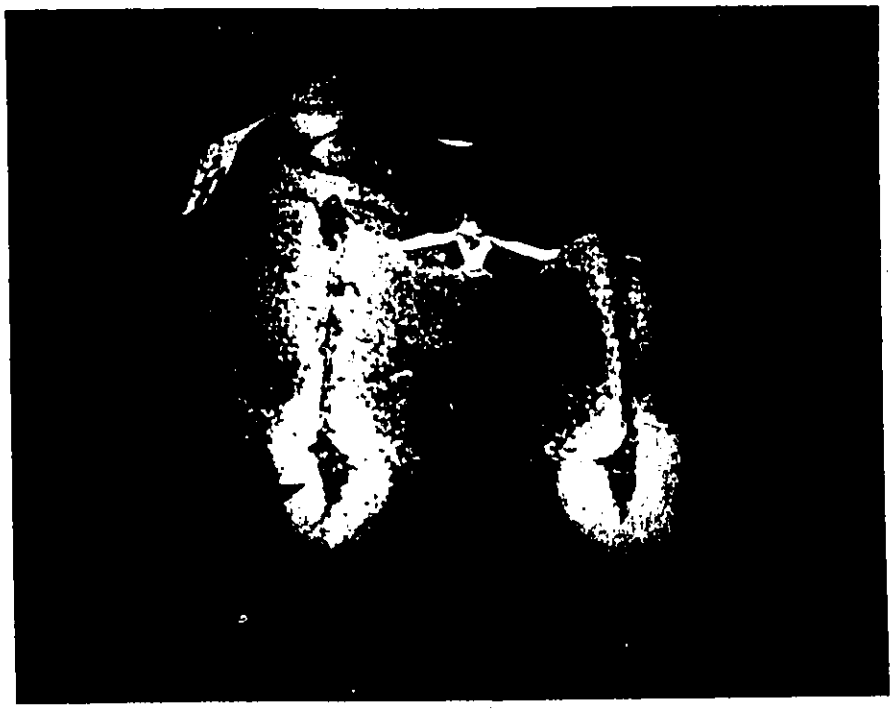

FIGURE 5. STUDY OF THE ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: PHOTOMICROGRAPH OF AN ACUTE LESION OF THE LATERAL HYPOTHALAMUS.

Bilateral lesions of the lateral hypothalamus shown in this photomicrograph were produced as described in section 2a of the Materials and Methods; the position of the lesion on left side of the micrograph is indicated by the arrowhead. Note the large size of the edematous area surrounding the lesion and contrast this to the size of long-term LH lesions (Figure 10). Compare the medio-lateral position of the lesions to the VMH lesions shown in Figure 6. In some animals, LH lesions encroached on the zona incerta and the basal aspects of the thalamus.

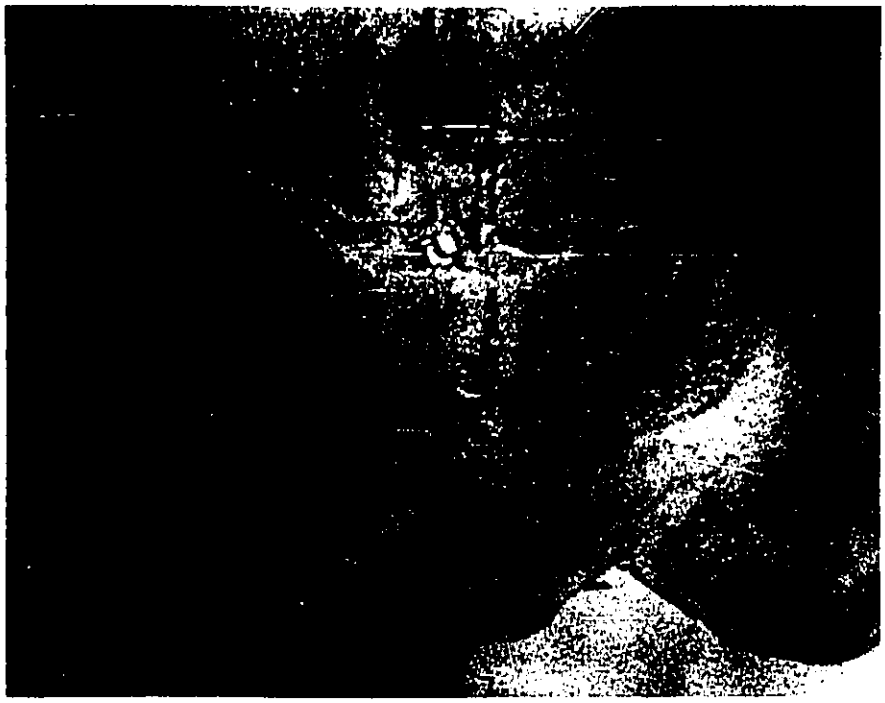

FIGURE 6. STUDY OF THE ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: PHOTOMICROGRAPH OF AN ACUTE LESION OF THE VENTROMEDIAL HYPOTHALAMUS.

Bilateral lesions of the ventromedial hypothalamus were made as described in section 2a of the Materials and Methods: the lesion on the left side of the micrograph is indicated by the arrowhead. As with the acute LH lesions (Figure 5), the lesions were markedly larger than are seen in long-term VMH lesions. While VMH lesions were generally confined to the hypothalamus, both LH and VMH lesions commonly bilaterally overlapped for 0.2-0.4 mm in the fornix and surrounding tissue.

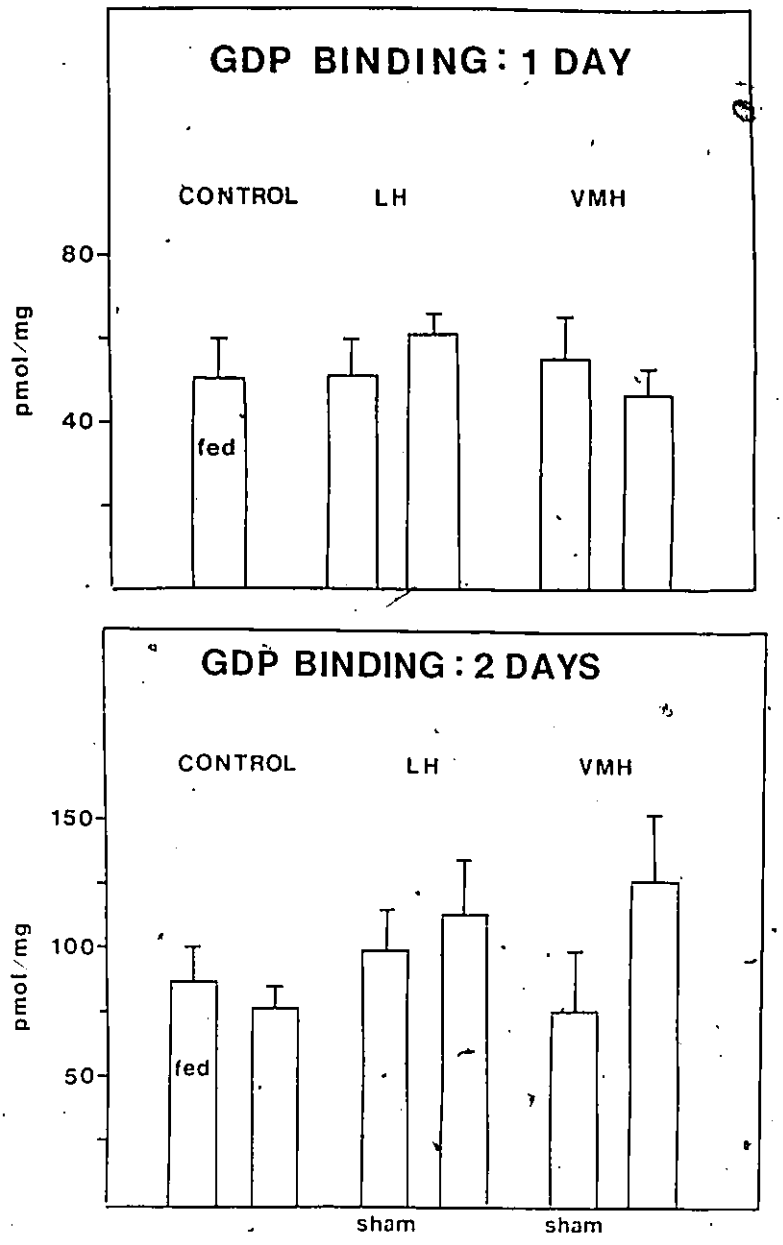

FIGURE 7. STUDY OF ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: GDP BINDING.

Rats were studied 1 or 2 days after receiving one of the following treatments: i) unoperated (**Control**), either fed or fasted (only the 2-day study had a fasted control), ii) lesioned, either of the lateral hypothalamus (**LH**) or of the ventromedial hypothalamus (**VMH**), or iii) sham lesioned (**Sham LH** and **Sham VMH**).

There was no significant effect of any treatment at either time. Values shown are means; error bars represent S.E.M. for n values as given in Tables 1 and 2.

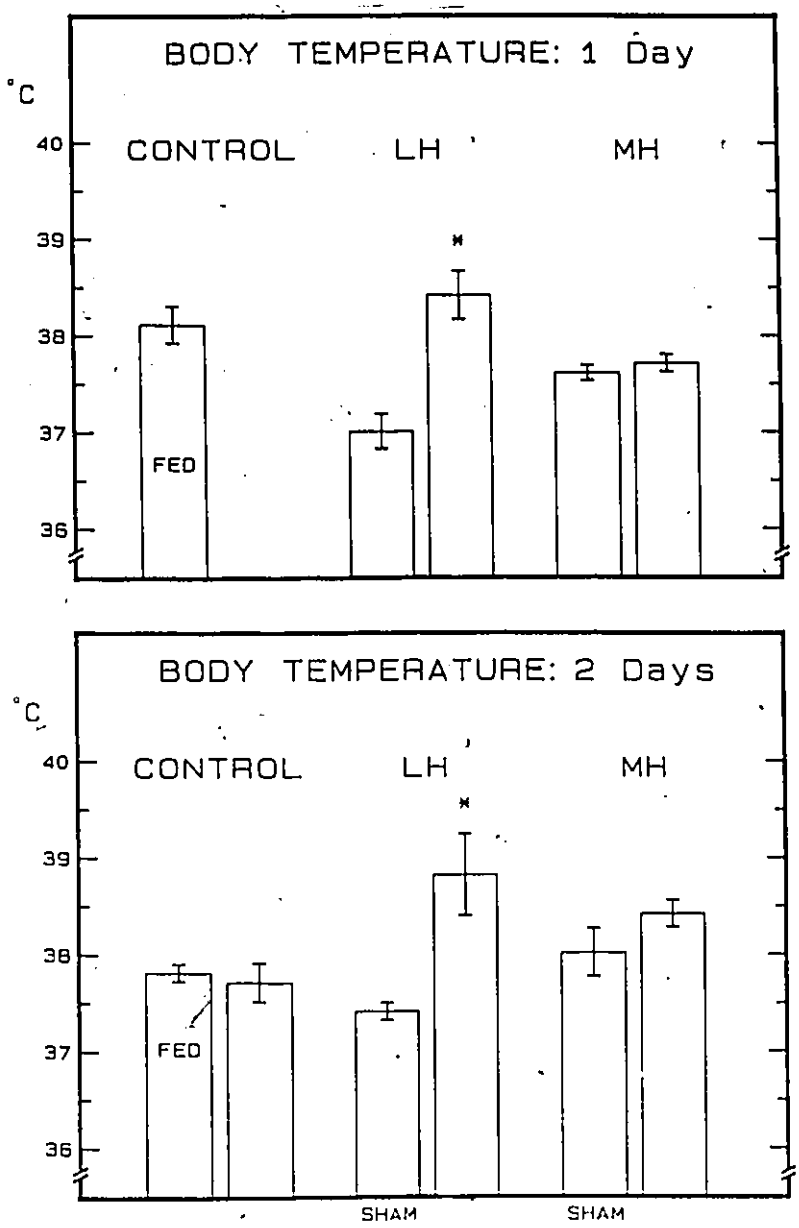

FIGURE 8. STUDY OF THE ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: BODY TEMPERATURES.

Rats were treated as described in the legends to Figure 7.

There was no effect of any treatment except in LH-lesioned rats, which were hyperthermic (compares to sham-operated controls) at both times. Values shown are means; error bars represent S.E.M. for n values as given in Tables 1 and 2.

	CONTROL		LESIONED			
	Fed (n=5)	Fasted (n=5)	Sham LH (n=5)	LH (n=6)	Sham VMH (n=5)	VMH (n=6)
Body weights (g)						
Initial	303 ± 3.9	308 ± 3.9	292 ± 4.3	291 ± 5.7	291 ± 2.3	289 ± 2.8
Final	312 ± 6.5[*]	262 ± 2.7[§]	257 ± 2.2[§]	250 ± 4.8[§]	259 ± 1.4[§]	252 ± 2.1[§]
Change	10 ± 2.8[*]	-46 ± 3.7[§]	-35 ± 1.7[¶]	-41 ± 2.2[§]	-32 ± 1.2[¶]	-37 ± 2.7[¶]
Epididymal fat						
Wet weight (g)	3.0 ± 0.07[*]	2.3 ± 0.14[§]	1.9 ± 0.12[¶]	2.1 ± 0.13[§¶]	2.2 ± 0.18[§¶]	2.0 ± 0.09[§¶]
Brown adipose tissue						
Wet weight (mg)	403 ± 20.1[*]	328 ± 20.6[§]	288 ± 23.2[§¶]	234 ± 16.0[¶]	325 ± 17.2[§]	232 ± 15.9[¶]
Protein (mg)	24.9 ± 1.28[*]	14.6 ± 1.05[§]	19.5 ± 1.53[§]	19.1 ± 1.25[§]	18.6 ± 0.97[§]	18.1 ± 1.86[§]
Protein/wet weight (mg/g)	62.3 ± 3.98[*]	44.8 ± 2.76[§]	68.2 ± 4.32[*]	78.5 ± 3.83[¶]	57.8 ± 3.95[*]	79.7 ± 8.76[¶]

TABLE 1. STUDY OF THE ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: 2 DAYS AFTER SURGERY.

Results for unoperated (**Control**) rats, either fed or fasted, and for rats receiving lesions (**Lesioned**), either of the lateral hypothalamus (**LH**) or of the ventromedial hypothalamus (**VMH**), or sham lesions (**Sham LH** and **Sham VMH**). Determinations were made 2 days after operations; lesioned rats were fasted during this time. Results are means ± S.E.M. for n values as shown in the Table.

One-way ANOVA was performed. Within each line, means with different superscripts are significantly different ($p < 0.05$ or smaller). Duncan's post-hoc test was used.

	CONTROL		LESIONED		
	Control (n=8)	Sham LH (n=8)	LH (n=10)	Sham VMH (n=8)	VMH (n=10)
Body weights (g)					
Initial	N.D.	276 ± 1.9	276 ± 4.0	276 ± 1.7	274 ± 2.0
Final	299 ± 5.2*	242 ± 2.7§	239 ± 3.6§	243 ± 1.6§	240 ± 1.7§
Change	N.D.	-35 ± 1.1*§	-38 ± 1.7*	-33 ± 1.4§	-34 ± 1.4*§
Epididymal fat wet weight (g)	2.1 ± 0.07	1.9 ± 0.14	1.9 ± 0.12	1.8 ± 0.05	2.0 ± 0.11
Brown adipose tissue					
Wet weight (mg)	419 ± 21.7*	372 ± 20.8*§	348 ± 13.8*§	360 ± 16.1§	344 ± 18.4§
Protein (mg)	21.2 ± 1.71	18.6 ± 1.43	20.8 ± 1.63	19.6 ± 1.57	18.3 ± 1.17
Protein/wet weight (mg/g)	51.8 ± 5.18	51.4 ± 5.65	61.6 ± 6.54	55.7 ± 6.24	55.8 ± 6.24

TABLE 2. STUDY OF THE ACUTE EFFECTS OF HYPOTHALAMIC LESIONS: 1 DAY AFTER SURGERY.

Results for unoperated (**Control**) rats and for rats receiving lesions, either of the lateral hypothalamus (**LH**) or of the ventromedial hypothalamus (**VMH**), or sham lesions (**Sham LH** and **Sham VMH**). Determinations were made 1 day after operations; all lesioned rats were fasted during this time. Results are means ± S.E.M. (N.D., not determined) for n values as given in the Table.

One-way ANOVA was performed. Within each line, means with different superscripts are significantly different ($p < 0.05$ or smaller). Duncan's post-hoc test was used.

There may have been some selective loss of lipid in BAT of both LH-lesioned and VMH-lesioned rats after two days, as suggested by the ratio of protein per total wet weight of BAT (Table 1). This difference was not seen after one day (Table 2) and is not due to differences in protein content (Tables 1 and 2). There was an unexpectedly low amount of protein in non-lesioned rats that had been fasted two days (Table 1).

Conclusion: These results suggest that there is no acute effect of either type of hypothalamic lesion on the functional status of BAT and that acute changes in body weight in LH rats can be explained by the effect of fasting and fever alone. The similarity of these results to the febrile animal is considered in the Discussion.

Section A2. The effects of obesity-causing parasagittal hypothalamic knife-cuts on diet-induced thermogenesis in brown adipose tissue.

Objective: The objective of this experiment was to study the long-term effects of parasagittal knife-cuts (KC) of the hypothalamus on diet-induced thermogenesis in BAT. Previous work in our laboratory by Dr. Susan Hogan had shown that lesions of the ventromedial hypothalamus of rats caused different patterns of response in BAT to cold-exposure compared to overfeeding: such rats were able to respond normally to cold stress (Hogan et al. 1982) but were unable to respond normally to overfeeding (Hogan et al. 1985). She continued this work by studying cold-induced thermogenesis in BAT in a second, similar preparation, the KC rat. My work, described in this section, completed this study. These results have been published previously (Coscina et al. 1985).

Design: Sixty female rats were sent to the Clarke Institute, Toronto from Canadian Breeding Laboratories (St. Constant, Que.) and kept in individual wire cages at 22°C with lights on 08h00-20h00 for one week before surgery. Bilateral parasagittal hypothalamic knife-cuts (36 rats) or sham lesions (24 rats) were made as described above in section 2a of the Materials and Methods; surgery was performed at the Clarke Institute by Dr. Donald Coscina and Dr. John Chambers. After three days for recovery from surgery, rats were shipped by car to Ottawa, where they were kept under the standard holding conditions. Three days after their arrival, they were subdivided into groups of 12 (three groups for the KC rats and two for the non-lesioned controls) based on similar body weights.

Two groups (one KC, one control) were fed chow ad lib. (the CHOW groups). Two other groups (one KC, one control) were fed a palatable "cafeteria" diet (the CAFE groups). The fifth (KC) group was pair-fed to the non-lesioned CHOW group. (For details on cafeteria-feeding and pair-feeding, see Materials and Methods.) Food intakes were measured twice and energy and macronutrient composition were estimated from food tables (Pennington and Church 1980).

Three weeks after the start of the diets, all rats were lightly anaesthetized with ether and naso-anal lengths were measured.

After 28 to 36 days of the feeding regimens, each of the groups was subdivided into two subgroups of six, with one subgroup fasted for 36 hours and the other fed normally. Rats were killed by decapitation and the standard preparation made over eight consecutive days. The BAT of some of the KC rats was so fatty that it was impossible to homogenize ice-cold; such samples were warmed to 30°C before homogenization. When this was necessary, all samples from that day's preparation were similarly treated.

After the experiment, brains were recovered and analyzed histologically as described in section 3 of the Materials and Methods.

Serum thyroid hormone levels were estimated with Amerlex kits from Amersham.

Statistical analysis was by 2-, 3-, or 4-way ANOVA or t-test; two-tailed distributions with $\alpha = 0.05$ were used. $p < 0.05$ or smaller for all differences noted to be statistically significant.

Results: Histology showed that knife-cuts were similar in size and location to those previously published (Chambers 1983; Clavier, Chambers and Coscina 1983). Cuts descended ventrally from the bottom of the thalamus to the base of the brain along a sagittal plane parallel to and medial to the fornix. Cuts ranged from 2.5-3.0 mm in antero-posterior length centered at the anterior to middle region of the ventromedial nuclei. Most cuts were bilaterally symmetrical, although occasional asymmetries were seen; such asymmetries appeared unrelated to the degree of overeating or magnitude of obesity finally seen. An example of a knife-cut is shown in Figure 9.

Results for body weights are given in Table 3. Even at the start of the diet regimens the KC rats were significantly heavier that the controls and these differences increased by the end of the experiment. Analysis of variance (ANOVA) showed that CAFE treatment also caused a significantly greater weight gain. Fasting for 36 h caused a significant decrease in body weight.

Results for body weight changes, body lengths, and the Lee Index of obesity are given in Table 4. Naso-anal lengths were greater in KC rats than in controls. CAFE treatment alone did not modify length, but it did increase length in KC rats compared to chow-fed and pair-fed KC rats. Both KC and CAFE treatments caused obesity as determined by the Lee index.

Results of the analysis of food intake are given in Table 5. KC rats ate more total energy than control rats, as did CAFE-treated rats. ANOVA showed interaction between KC and CAFE treatments.

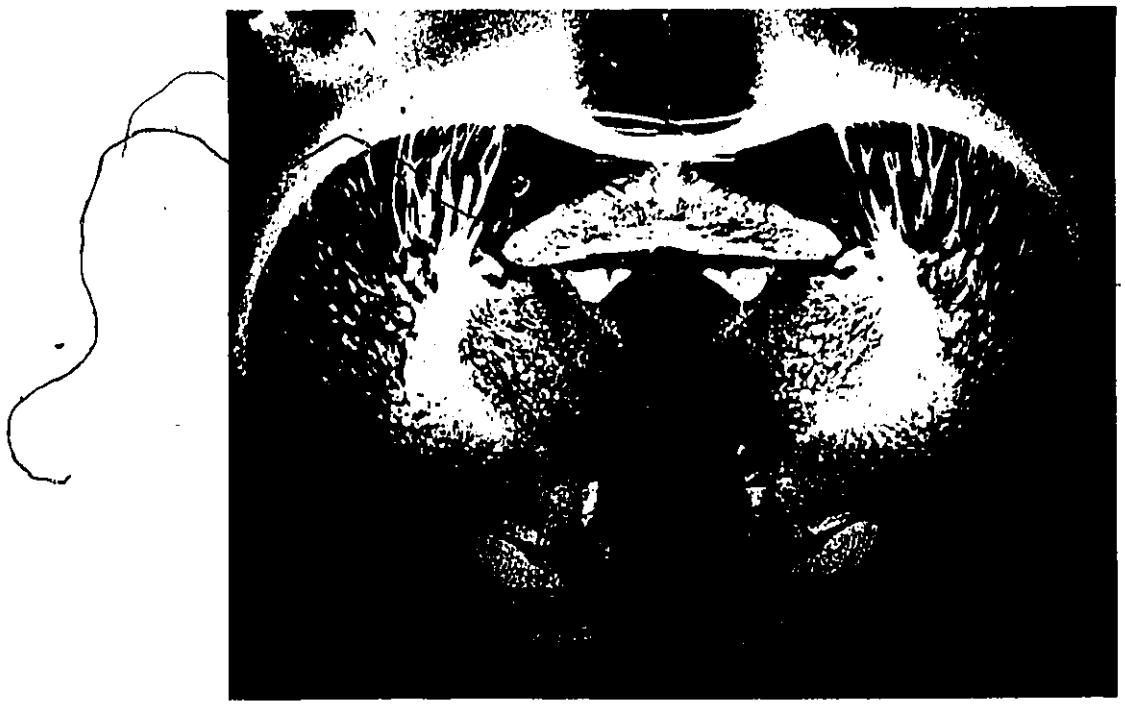

FIGURE 9. STUDY OF HYPOTHALAMIC KNIFE-CUTS: PHOTOMICROGRAPH OF A PARASAGITTAL KNIFE-CUT OF THE HYPOTHALAMUS.

Bilateral parasagittal knife-cuts of the hypothalamus, separating the lateral hypothalamus from the ventromedial hypothalamus, were made as described in section 2a of the Materials and Methods. The location of the knife-cut on the right side of the photomicrograph is indicated by the line of arrowheads.

TREATMENT	Chow	Pair-fed	Cafe
		Body weight (g)	
		At lesion	
NL	205 ± 2.6	--	--
KC	209 ± 1.9	--	--
		At start of diets	
NL	213 ± 2.3	--	--
KC	263 ± 3.5	--	--
		At start of fasting	
NL	267 ± 4.1	--	275 ± 5.6
KC	381 ± 10.8	300 ± 2.3	455 ± 8.9

TABLE 3. STUDY OF HYPOTHALAMIC KNIFE-CUTS: BODY WEIGHTS.

Results for sham-operated (**NL**, non-lesioned) rats, fed either **Chow** or a cafeteria diet (**Cafe**), and for rats receiving knife-cuts (**KC**) between the lateral and ventromedial hypothalamus, fed either **Chow**, a cafeteria diet (**Cafe**), or pair-fed to chow-fed controls (**Pair-fed**). Results given are means ± S.E.M. Blanks in the Table (--) indicate that there are no animals in those particular categories. n = 24 (NL rats) or n = 36 (KC rats) for the first two time periods shown; thereafter, n = 12 for all five groups.

Analysis by ANOVA showed a significant main effect of cafeteria-feeding on body weight. At sacrifice, ANOVA indicates that KC rats were heavier than all NL rats and CAFE rats were heavier than chow-fed rats.

TREATMENT	Chow	Pair-fed	Cafe
		Body weight change (g)	
		During diet period	
NL	55 ± 3.3	--	63 ± 4.0
KC	118 ± 11.0	48 ± 3.6	180 ± 6.2
		During fasting period	
- fed ad lib.			
NL	0.7 ± 1.4	--	1.8 ± 2.1
KC	7.0 ± 1.6	2.8 ± 1.4	6.2 ± 2.0
- fasted			
NL	-22.0 ± 1.0	--	-11.0 ± 1.3
KC	-34.0 ± 3.8	-18.0 ± 3.2	-21.0 ± 2.7
		Naso-anal lengths (mm)	
NL	206 ± 1.0	--	205 ± 1.3
KC	214 ± 2.0	208 ± 1.5	219 ± 1.1
		Lee Obesity index x 10^3	
NL	304 ± 1.5	--	308 ± 2.5
KC	329 ± 4.4	311 ± 1.9	337 ± 2.7

TABLE 4. STUDY OF HYPOTHALAMIC KNIFE-CUTS: BODY WEIGHT CHANGES, NASO-ANAL LENGTHS, AND LEE INDICES.

Rats were treated and statistical analyses were performed as described in the legend to Table 3. The Lee Index of obesity is the cube root of body weight (in grams) divided by body length (in cm). n = 12 for all groups except for body weight changes during fasting period, where n = 6.

Analysis by ANOVA showed a significant main effect of KC on naso-anal length. Both KC and CAFE had significant main effects on the Lee index, indicating obesity was produced by these two treatments. Rats fasted 36 hours before sacrifice weighed less than corresponding fed rats (see the body weight change), although there was no significant difference in the wet weight of their gonadal white adipose tissue (see Table 7).

TIME	CONTROL		KNIFE-CUT	
	Chow	Cafe	Chow	Cafe
		Total energy intake (kcal/d)		
Period 1	70.0 ± 1.9	95.7 ± 4.2	129.2 ± 7.7	175.5 ± 7.5
Period 2	71.3 ± 2.9	81.6 ± 3.0	106.9 ± 7.2	161.4 ± 5.5
		Macronutrient intake (g)		
		Carbohydrate		
Period 1	10.9 ± 0.3	11.2 ± 0.6	20.2 ± 1.2	20.9 ± 1.0
Period 2	11.0 ± 0.4	9.9 ± 0.3	16.6 ± 1.1	19.8 ± 0.8
		Fat		
Period 1	0.9 ± 0.02	4.5 ± 0.19	1.7 ± 0.10	8.0 ± 0.32
Period 2	0.9 ± 0.04	3.7 ± 0.27	1.4 ± 0.09	7.4 ± 0.31
		Protein		
Period 1	4.6 ± 0.1	2.7 ± 0.1	8.5 ± 0.5	5.0 ± 0.3
Period 2	4.7 ± 0.2	2.4 ± 0.1	7.0 ± 0.5	3.9 ± 0.2

TABLE 5. STUDY OF HYPOTHALAMIC KNIFE-CUTS: FOOD INTAKES.

Rats were treated and statistical analyses were performed as described in the legend to Table 3. Food intakes were determined, as described in Materials and Methods section 1d, on two occasions (**Period** 1 and **Period** 2). Data for knife-cut rats pair-fed chow are not listed since they were the same as control rats fed chow (column 1). Results given are means ± S.E.M.; n = 12 for all four groups.

Results of ANOVA showed that KC rats ate more total calories than did controls and CAFE rats ate more than chow-fed rats; for both, increases in consumption were seen for fat and carbohydrates (except for carbohydrate intake by CAFE rats, which was not different from chow-fed rats); protein intake by CAFE rats was lower than in chow-fed rats.

KC rats ate more carbohydrate, fat, and protein than did controls; rats fed CAFE diets ate more fat but less protein than did CHOW-fed rats. As with the total energy intake, there was a significant interaction of KC with CAFE to produce greater intakes of the three macronutrients.

Indices of BAT function are presented in Table 6. The total protein content of BAT was not changed by KC or by fasting, but it was increased by CAFE-treatment compared to CHOW-feeding. ANOVA showed an interaction between CAFE-feeding and fasting for protein content of BAT.

DNA content of BAT was actually reduced in KC rats but increased by CAFE-treatment. The results for the activity of cytochrome oxidase were similar: KC rats were not different from controls, but CAFE-treatment caused a significant increase in activity.

Results for the principal index of thermogenic activity in BAT, the specific binding of GDP to isolated mitochondria, are also given in Table 6. Three-way Analysis of Variance showed that binding was reduced in KC rats and increased by CAFE-treatment and by fasting. When results were broken into the subgroups, as is shown in Table 6, it can be seen that the increase in GDP binding normally seen in CAFE-fed rats was reduced (in fasted) or abolished (in fed) KC rats. Therefore, as in rats having lesions of the VMH, KC rats have impaired thermogenic response to overfeeding.

The results for tissue wet weights and body temperatures are given in Table 7. Wet weight of BAT was significantly increased by KC and CAFE-treatments. Fasting for 36 h reduced BAT wet weight compared to fed rats. ANOVA also showed a triple interaction among KC, CAFE, and fasting conditions. Body temperatures were significantly lower in KC rats and in fasted rats.

TREATMENT	Chow	Pair-fed	Cafe
		Protein (mg)	
Fed ad lib			
NL	22.6 ± 3.8	--	21.9 ± 2.7
KC	20.3 ± 2.2	30.0 ± 3.0	28.8 ± 3.2
Fasted			
NL	16.4 ± 1.2	--	29.0 ± 2.2
KC	18.1 ± 2.2	22.9 ± 2.9	31.3 ± 3.1
		DNA (µg)	
Fed ad lib.			
NL	801 ± 36.4	--	1034 ± 138.6
KC	676 ± 29.9	801 ± 48.7	842 ± 76.5
Fasted			
NL	819 ± 38.8	--	833 ± 25.9
KC	717 ± 59.7	820 ± 91.4	856 ± 39.0
		Cytochrome oxidase (µg atom O_2/min)	
Fed ad lib.			
NL	120 ± 22.9	--	169 ± 20.6
KC	61.1 ± 14.5	104 ± 23.0	117 ± 34.3
Fasted			
NL	49.7 ± 12.0	--	159 ± 28.6
KC	62.3 ± 16.1	87.6 ± 14.4	148 ± 24.7
		GDP Binding (pmol/mg)	
Fed ad lib.			
NL	70 ± 15.3	--	146 ± 9.4
KC	51 ± 9.2	48 ± 5.0	54 ± 7.0
Fasted			
NL	69 ± 10.6	--	172 ± 25.9
KC	71 ± 20.5	66 ± 12.4	118 ± 29.2

TABLE 6. STUDY OF HYPOTHALAMIC KNIFE-CUTS: BROWN ADIPOSE TISSUE.

Results for animals treated as described in the legend to Table 3. Results given are means ± S.E.M. n = 6 for all groups.

Results were analyzed by ANOVA. Only CAFE feeding, not KC nor fasting, had a main effect on BAT protein content. DNA content was decreased by KC and increased by CAFE. Cytochrome oxidase activity was significantly increased by cafeteria-feeding. GDP binding was reduced by KC but it was increased both by cafeteria-feeding and by a fast of 36 hours.

TREATMENT	Chow	Pair-fed	Cafe
	Brown adipose tissue wet weight (mg)		
Fed ad lib.			
NL	515 ± 82	--	974 ± 95
KC	1620 ± 150	954 ± 104	1748 ± 193
Fasted			
NL	317 ± 19	--	453 ± 45
KC	947 ± 177	484 ± 86	1775 ± 229
	Gonadal white adipose tissue wet weight (g)		
Fed ad lib.			
NL	4.2 ± 0.8	--	8.8 ± 1.1
KC	14.3 ± 1.4	7.8 ± 1.1	22.9 ± 2.4
Fasted			
NL	2.6 ± 0.6	--	7.0 ± 1.0
KC	12.4 ± 1.3	7.1 ± 0.7	22.4 ± 1.0
	Body temperature (°C)		
Fed ad lib.			
NL	38.1 ± 0.30	--	38.4 ± 0.26
KC	37.8 ± 0.20	37.7 ± 0.24	38.1 ± 0.24
Fasted			
NL	37.8 ± 0.32	--	38.1 ± 0.22
KC	37.2 ± 0.10	37.3 ± 0.13	37.8 ± 0.24

TABLE 7. STUDY OF HYPOTHALAMIC KNIFE-CUTS: TISSUE WET WEIGHTS AND BODY TEMPERATURES.

Results for animals treated as described in the legend to Table 3. Results given are means ± S.E.M with n = 6.

Results were analyzed by ANOVA which showed main effects of KC and CAFE on the wet weight of brown adipose tissue; fasting before sacrifice significantly reduced this measure. It also showed that KC treatment and fasting significantly decreased body temperature.

Results for serum levels of thyroid hormones are given in Table 8. Serum T_3 levels were lower in KC rats and elevated in CAFE-treated NL rats; however, KC rats failed to increase their serum T_3 levels in response to CAFE treatment while non-lesioned rats did. ANOVA indicated that the interaction between KC and CAFE was lower than expected from main effects alone. Serum T_4 levels were not different in KC versus control rats; T_4 levels were slightly elevated in CAFE-treated rats.

Pair-feeding KC rats to non-lesioned, CHOW-fed controls largely prevented the excess increase of body weight seen in ad lib.-fed KC rats (Tables 3 and 4). ANOVA showed that pair-feeding reduced all indices of body mass and tissue mass, compared to ad lib.-fed KC rats. However, save for naso-anal length, pair-fed KC rats had increased body- and tissue-mass indices compared to non-lesioned controls. Likewise, indices of functional capacity of BAT (DNA content, and activity of cytochrome oxidase), body temperature, and thyroid hormone levels (see Tables 6-8) were between those found for KC rats and for ad lib.-fed controls, while not differing significantly (statistically) from either of these groups. GDP binding of pair-fed KC rats was almost identical to that of chow-fed KC rats, both fed and fasted (Table 6). The protein content of BAT was higher than in either KC or control chow-fed rats (Table 6).

Conclusion: These results suggest that the KC rat, like the rat made obese by lesions of the ventromedial hypothalamus, cannot activate the thermogenesis in BAT normally caused by overfeeding.

TREATMENT	Chow	Pair-fed	Cafe
		Serum T_3 (ng/dL)	
Fed ad lib.			
NL	17.8 ± 2.1	--	39.8 ± 6.3
KC	10.8 ± 3.1	13.5 ± 5.0	10.3 ± 1.4
Fasted			
NL	14.7 ± 5.0	--	43.7 ± 12.6
KC	10.2 ± 2.2	7.6 ± 3.1	19.8 ± 2.3
		Serum T_4 (ng/mL)	
Fed ad lib.			
NL	21.8 ± 2.8	--	28.5 ± 4.1
KC	23.7 ± 3.3	23.6 ± 5.5	19.6 ± 2.2
Fasted			
NL	20.9 ± 3.3	--	35.1 ± 6.0
KC	22.4 ± 4.6	16.6 ± 4.1	32.2 ± 6.6

TABLE 8. STUDY OF HYPOTHALAMIC KNIFE-CUTS: SERUM THYROID HORMONE LEVELS.

Results for animals treated as described in the legend to Table 3. Results given are means ± S.E.M with n = 6.

Results were analyzed by ANOVA which showed that serum T_3 levels were decreased by KC and increased by CAFE. Serum T_4 levels were not changed by KC and increased by CAFE.

Section A3. The effects of lesions of the lateral hypothalamus on brown adipose tissue.

Objective: The objective of this study was to determine the effects of bilateral lesion of the lateral hypothalamus (LH lesion) on growth of, and thermogenesis in, BAT. Since LH lesions cause increases in body temperature and weight loss, it seemed reasonable to predict an increased growth and thermogenesis in BAT of such rats.

Design: To separate metabolic effects of the lesion from the effects of lessened food intake, typical of the LH syndrome, and to contrast the effects of cold-acclimation from those of overfeeding, I studied the effects of both cold-acclimation and cafeteria-feeding; as an additional control, non-lesioned rats were pair-fed to LH rats during the lesioned rats' recovery from surgery.

Ninety-six male rats, initially weighing 140 to 160 g, were used. After 12-14 days under standard holding conditions, rats were divided into three groups of 32 based on equal body weights and growth rates. One group was given bilateral heat lesions of the lateral hypothalamus (LH group), as described in the Materials and Methods (section 2a), while the other two groups were anaesthetized without receiving surgery. Surgery, performed by Dr. Donald Coscina of the Clarke Institute, was done between 06h00 and 10h00 over a three-day period.

For two weeks after surgery (Phase 1), LH rats and one group of non-lesioned (NL) controls were fed chow ad lib., while the third group (also non-lesioned) was pair-fed to the LH group. Food intakes for all animals

were measured throughout this phase. To ensure sufficient hydration, all LH-lesioned rats received 4 mL physiological saline i.p. for the first three days after surgery; six rats required continued treatment for four more days.

After Phase 1, each of the three groups was subdivided into two subgroups of 16, based on equal body weights and growth rates. One of the sub-groups was fed chow ad lib. while the other was given a "cafeteria" diet. During this phase (Phase 2), which continued for the next 15-23 days, food intakes were determined four times.

After Phase 2, each sub-group was again equally subdivided based on body weights and growth rates. One of these secondary sub-groups was kept at 26°C while the other was exposed to cold (4°C) for 15 h. At 08h00 the next day, rats were killed by decapitation and a standard preparation made, as described in the Materials and Methods.

After the experiment, brains were recovered and analyzed histologically as described in section 3 of the Materials and Methods.

Statistical analysis was by 1-, 2-, or 3-way ANOVA. Corrections for repeated measurements were made as necessary. Between-group differences were tested post-hoc by Duncan Multiple Range tests.

Results: Sizes of almost all lesions were "small" (0.4-0.6 mm diameter) or "moderate" (one-third of LH tissue, medio-laterally, destroyed). The centers of the lesions were consistently in the dorso-lateral LH near the basal border of the thalamus; bilateral damage to the zona incerta was common. Damage was always bilateral, although sometimes there was some leftward or rightward lateral deviation due to slight errors in original

determination of the midline. Antero-posterior placement of the lesions was consistently between the caudal aspects of the paraventricular nucleus and the middle to caudal aspects of the ventromedial nuclei. The sizes of the lesions had no clear effect on any of the results reported below. An example of a large LH bilateral lesion (several weeks after surgery) is shown in Figure 10.

Results for the first week after surgery (Phase 1) are given in Table 9. LH rats and pair-fed (PF) rats lost weight during this time, but the LH rats lost significantly more. Their food efficiency was also significantly lower than the PF rats ($p < 0.001$), being actually less than zero during this time. Thus the LH lesions were judged successful.

Results for body weights are presented in Table 10 and results for weight gains and food intakes are given in Table 11. By the end of Phase 1, LH-lesioned rats weighed much less than normal rats (Table 10). PF rats had body weights that were similar to the LH-lesioned rats but they still weighed significantly more than the LH rats (Table 10). The rate of weight gain of the LH-lesioned rats was not significantly different from the PF rats but these two groups were significantly different from the normal rats (Table 11).

During Phase 2, chow-fed LH-lesioned rats had the same food intake and growth rate as normal rats (Table 11); PF rats gained more weight than the other two groups (Table 11) and had caught up to a normal body weight by the end of Phase 2 (Table 10).

Cafeteria-feeding caused an increase in energy intake in all groups (Table 11) but this did not affect body weight gain in either the normal or previously PF rats. Cafeteria-fed LH-lesioned rats gained 30% less weight

FIGURE 10. STUDY OF LATERAL HYPOTHALAMIC LESIONS: PHOTOMICROGRAPH OF A LONG-TERM BILATERAL LESION OF THE LATERAL HYPOTHALAMUS.

Bilateral lesions of the lateral hypothalamus were made as described in section 2a of the Materials and Methods. The lesion on the left side of the photomicrograph is indicated by the arrowhead. Rats were killed five to six weeks after surgery, and this is reflected in the well-defined regions of damage. Note that the edema that occurs acutely after lesion (Figure 5) is now absent. The lesions shown here are large; in most other animals, smaller lesions were produced.

	Normal	LH-lesioned	Pair-fed
Weight gain (g)	52.7 ± 1.6*	-8.2 ± 4.9§	-2.3 ± 3.0¶
Energy intake (kcal)	607 ± 7.7*	301 ± 18.8§	301 ± 18.8§
Food efficiency (g gain/100 kcal eaten)	8.8 ± 0.25*	-6.6 ± 0.24§	1.4 ± 0.14¶

TABLE 9. STUDY OF LATERAL HYPOTHALAMIC LESIONS: INITIAL WEIGHT CHANGES, FOOD INTAKE, AND FOOD EFFICIENCY (DAYS 1-7 AFTER LESION).

For this Table and those following (Tables 10-14), the following was done. Rats were given LH lesions (**LH-lesioned**), or **pair-fed** to lesioned rats during phase 1, or fed chow ad lib. (**Normal**). Values shown are means ± S.E.M. for n = 32 for all groups.

Within each line, results of one-way ANOVA (Duncan's Multiple Range Test used as a post-hoc test) are shown, with significant differences ($p < 0.001$) indicated by differing superscripts. (If no superscripts are shown, there was no significant difference across groups.)

DIET	GROUP		
	Normal	LH-lesioned	Pair-fed
Initial body weights(g)			
Chow	149 ± 1.5	147 ± 1.1	147 ± 1.2
Body weight at lesioning (g)			
Chow	253 ± 1.7*	242 ± 1.0§	246 ± 1.3§
Body weight at end Phase 1 (end of pair-feeding) (g)			
Chow	329 ± 2.9*	278 ± 4.5§	289 ± 8.1§
Body weight at end Phase 2 (end of chow or cafe period) (g)			
Chow	421 ± 6.1*	361 ± 7.9§	406 ± 4.6*
Cafe	431 ± 5.6*	337 ± 12.1§	399 ± 6.8¶

TABLE 10. STUDY OF LATERAL HYPOTHALAMIC LESIONS: BODY WEIGHTS.

Rats were treated and statistical analyses were performed as described in the legend to Table 9. Values shown are means ± S.E.M for n = 32 for all means except for those from the end of phase 2, where n = 16.

Normal rats were significantly heavier than LH-lesioned rats and pair-fed rats at the time of lesioning because treatments were staggered by a day for logistical reasons.

Two-way ANOVA for values at the end of phase 2 showed a significant group effect ($p < 0.001$) and no diet effect.

	DIET	GROUP		
		Normal	LH-lesioned	Pair-fed
		Weight gain (g/day)		
Phase 0		8.0 ± 0.16	8.3 ± 0.14	7.9 ± 0.14
Phase 1		6.9 ± 0.28*	2.9 ± 0.35§	3.7 ± 0.26§
Phase 2	Chow	5.0 ± 0.23*	4.6 ± 0.35*	6.4 ± 0.29§
	Cafe	5.6 ± 0.26*	3.3 ± 0.45§	6.0 ± 0.37*
		Energy intake (kcal/day)		
Phase 2				
Meal 1	Chow	94.9 ± 2.7*	88.1 ± 2.0*	104.1 ± 3.3§
	Cafe	122.7 ± 4.2*	110.8 ± 3.8§	119.9 ± 1.9*
Meal 2	Chow	83.6 ± 3.0*	74.1 ± 2.6§	85.5 ± 2.1*
	Cafe	107.2 ± 2.4	99.9 ± 4.6	106.8 ± 2.3
Meal 3	Chow	73.3 ± 1.9*	85.8 ± 3.0§	100.3 ± 2.6¶
	Cafe	124.6 ± 4.8*	101.0 ± 5.7§	123.2 ± 3.5*
Meal 4	Chow	80.2 ± 1.8	74.6 ± 2.5	80.5 ± 3.1
	Cafe	106.9 ± 4.3	95.0 ± 4.0	104.7 ± 4.4

TABLE 11. STUDY OF LATERAL HYPOTHALAMIC LESIONS: BODY WEIGHT GAINS AND FOOD INTAKES.

Rats were treated and statistical analyses were performed as described in the legend to Table 9. p-values were less than 0.05 or smaller for all significant differences. Values shown are means ± S.E.M for n = 32 for phases 0 and 1, and n = 16 for phase 2.

Two-way ANOVA for energy intake results for phase 2 showed significant main effects of diet and meal ($p < 0.001$).

than the chow-fed LH-lesioned rats despite an average energy intake 26% greater than the chow-fed rats (Table 11). The energy intakes of the three cafeteria-fed groups was similar, yet the LH-lesioned rats gained 40% less than normal rats and 45% less than the previously PF rats (Table 11). Thus by the end of Phase 2, cafeteria-fed LH-lesioned rats weighed 22% less than cafeteria-fed normal, rats while the previously PF rats had a deficit of only 7% (Table 10). Likewise, chow-fed LH-lesioned rats weighed 14% less than chow-fed control rats, while the previously PF rats had a deficit of only 4% (Table 10).

Wet weights of gonadal white adipose tissue are presented in Table 12. LH lesions significantly reduced the size of gonadal white adipose tissue (used here as an index of body fat content), even when calculated in proportion to reduced body size. This difference was also found in cafeteria-fed rats. Wet weights of white fat from normal and previously PF rats did not differ from one another.

Body temperatures are presented in Table 13. Cafeteria-feeding had a significant effect on body temperature across all groups. Body temperatures were maintained in cold-exposed rats; the effect of cafeteria-feeding was still seen in cold-exposed rats.

Findings for brown adipose tissue are given in Table 14. The wet weight of BAT of chow-fed LH-lesioned rats was lower than that of the normal rats, probably due to lower fat content in the tissue, since the total protein content was the same as in normal animals. BAT protein content, expressed per tissue wet weight, was actually elevated in chow-fed LH-lesioned rats. Thus these animals seemed to maintain a lower-than-

DIET	AMBIENT TEMPERATURE	GROUP		
		Normal	LH-lesioned	Pair-fed
		Total wet weight (g)		
Chow	Warm	5.2 ± 0.44*	3.5 ± 0.41§	4.9 ± 0.28*
	Cold	4.7 ± 0.25*	3.3 ± 0.27§	4.9 ± 0.15*
Cafe	Warm	7.3 ± 0.52*	4.3 ± 0.49§	6.7 ± 0.42*
	Cold	8.2 ± 0.56*	4.9 ± 0.78§	7.8 ± 0.49*
		Wet weight per metabolic body mass ($g/kg^{0.75}$)		
Chow	Warm	9.9 ± 0.71*	7.5 ± 0.74§	9.7 ± 0.52*
	Cold	8.9 ± 0.44*	7.1 ± 0.46§	9.6 ± 0.31*
Cafe	Warm	13.6 ± 0.89*	9.5 ± 0.84§	13.2 ± 0.79*
	Cold	15.3 ± 0.92*	10.8 ± 1.32§	15.7 ± 1.06*

TABLE 12. STUDY OF LATERAL HYPOTHALAMIC LESIONS: WEIGHT OF GONADAL WHITE ADIPOSE TISSUE AT THE END OF PHASE 2.

Rats were treated and statistical analyses were performed as described in the legend to Figure 9. $p < 0.05$ or smaller for all one-way ANOVA comparisons. Values shown are means ± S.E.M for $n = 8$.

For both total wet weight and wet weight per metabolic body mass, two-way ANOVA showed main effects of group and of diet ($p < 0.0001$) with no interaction.

DIET	AMBIENT TEMPERATURE	GROUP		
		Normal	LH-lesioned	Pair-fed
Chow	Warm	37.2 ± 0.17	37.4 ± 0.19	37.7 ± 0.12
	Cold	37.5 ± 0.12	37.7 ± 0.11	37.7 ± 0.10
Cafe	Warm	38.3 ± 0.17	38.7 ± 0.13	38.4 ± 0.14
	Cold	38.0 ± 0.14	38.1 ± 0.11	38.3 ± 0.17

TABLE 13. STUDY OF LATERAL HYPOTHALAMIC LESIONS: BODY TEMPERATURES AT THE END OF PHASE 2.

Rats were treated and statistical analyses were performed as described in the legend of Table 9. Values shown are means ± S.E.M. for n = 8 for all means.

There were no significant differences for comparisons made by one-way ANOVA. Two-way ANOVA showed a significant main effect of diet ($p < 0.0001$) and no main effect of group; a significant interaction ($p < 0.002$) was found between ambient temperature and diet.

DIET	AMBIENT TEMPERATURE	GROUP		
		Normal	LH-lesioned	Pair-fed
		Wet weight (mg)		
Chow	Warm	692 ± 28.2	503 ± 74.3	724 ± 28.9
	Cold	532 ± 37.6	392 ± 56.8	493 ± 28.3
Cafe	Warm	1304 ± 56.1	1162 ± 76.3	1138 ± 44.8
	Cold	1054 ± 62.8	822 ± 108.9	966 ± 83.9
		Total protein (mg)		
Chow	Warm	21.3 ± 2.34	25.5 ± 2.75	21.9 ± 3.04
	Cold	42.0 ± 2.74*	32.3 ± 2.17§	34.7 ± 2.22§
Cafe	Warm	25.2 ± 3.05*	37.3 ± 4.18§	27.3 ± 2.54*
	Cold	50.1 ± 5.70	44.6 ± 4.28	37.6 ± 3.30
		Protein concentration (mg/g)		
Chow	Warm	30.9 ± 3.21	45.4 ± 6.66	32.0 ± 6.72
	Cold	80.7 ± 6.32	75.0 ± 6.16	70.2 ± 1.83
Cafe	Warm	19.2 ± 1.90*	32.4 ± 4.27§	24.0 ± 2.00*
	Cold	48.4 ± 5.27	59.6 ± 8.27	41.2 ± 5.09

TABLE 14. STUDY OF LATERAL HYPOTHALAMIC LESIONS: BROWN ADIPOSE TISSUE.

Rats were treated as described in the legend to Table 9. n = 8 for all means; $p < 0.05$ or smaller for all one-way ANOVA comparisons.

Results of two-way ANOVA are summarized below (NS = not significant):

FACTOR ANALYZED	AMBIENT TEMPERATURE	MAIN EFFECT GROUP	MAIN EFFECT DIET
Wet weight	Warm	NS	$p < 0.0001$
	Cold	NS	$p < 0.0001$
Total protein	Warm	$p < 0.05$	$p < 0.01$
	Cold	$p < 0.05$	$p < 0.01$
Protein concentration	Warm	$p < 0.02$	$p < 0.01$
	Cold	NS	$p < 0.0001$

normal tissue concentration of lipid, even when the total amount was elevated, as in the cafeteria-fed LH-lesioned rats.

The cafeteria treatment almost doubled the wet weight of BAT, but since there was only a 25% increase in total protein content, the increase was due mostly to stored fat. Cold-exposure consistently decreased BAT wet weight, likely due to mobilization of lipid, since total protein content and protein concentrations increased due to this treatment.

The increased protein concentration in BAT of LH-lesioned rats can be expressed in terms of metabolic mass ($BW^{.75}$, where BW = body weight) and this is presented graphically in Figure 11. When so expressed, BAT protein was present in greater amounts than normal in LH-lesioned rats, especially in those that had been cafeteria-fed. In response to cafeteria-feeding, BAT grew 51% in LH-lesioned rats as opposed to 15-25% in normal or previously PF rats. Thus, expressed per metabolic body mass, cafeteria-fed LH-lesioned rats had 53-77% more metabolically-active BAT than did normal or previously PF rats. However, growth of BAT (increase in protein content) due to the 15 h cold-exposure, which occurred in all groups, was smaller in LH-lesioned groups than in other groups.

GDP-binding to mitochondria is presented in Figure 12. GDP binding tended to be higher in LH-lesioned rats across groups. It increased due to cafeteria-feeding in all warm-exposed rats. Cold-exposure caused a greater increase in GDP binding, in all animals, than did cafeteria-feeding, a result found in other studies as well. In cold-exposed, cafeteria-fed rats, binding was higher in LH-lesioned rats than in the other two groups.

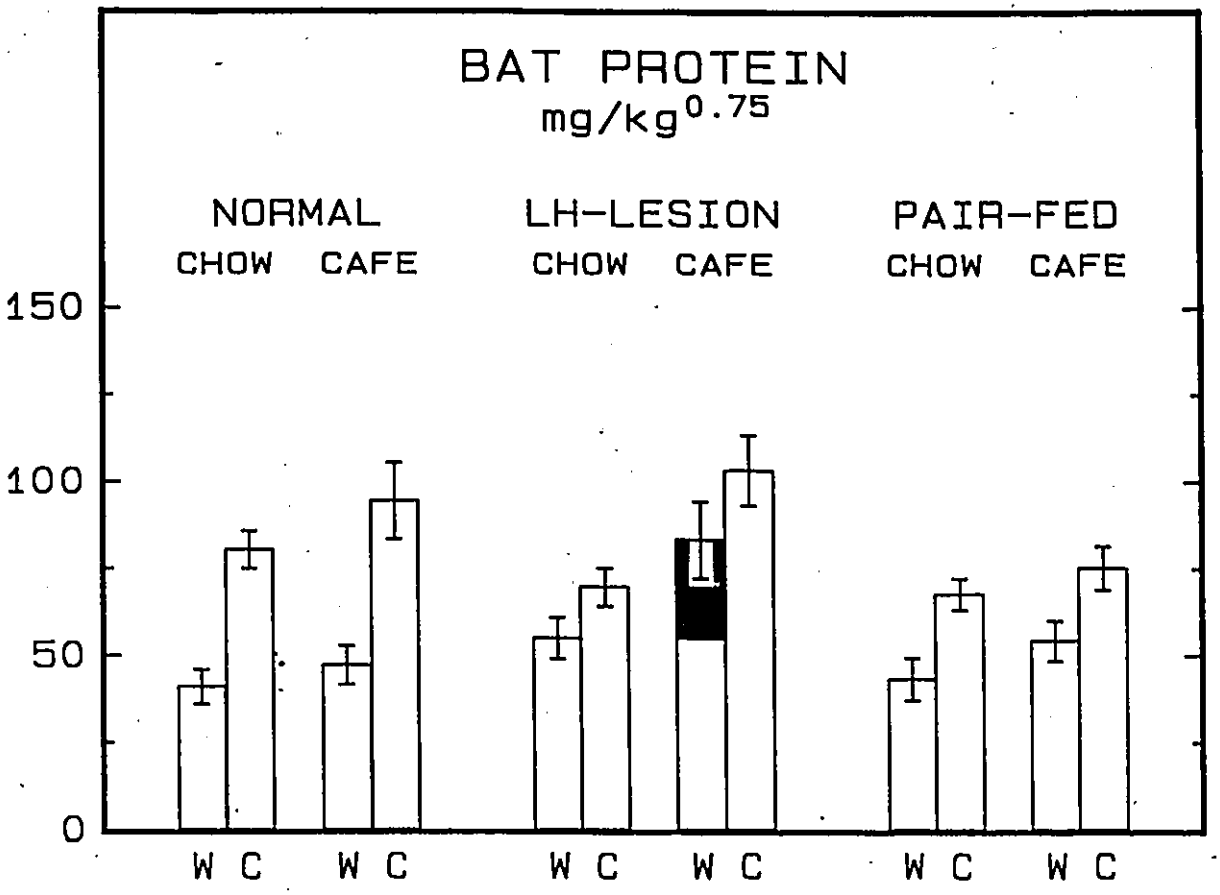

FIGURE 11. STUDY OF LATERAL HYPOTHALAMIC LESIONS: PROTEIN.

Rats were treated and statistical analyses were performed as described in the legend to Table 9. Here, protein content of brown adipose tissue is expressed in terms of body metabolic size ($BW^{.75}$, where BW is the body weight; i.e., the Kleiber relationship). Values shown are means; error bars represent S.E.M. for n = 8.

For rats kept in the warm (26°C, symbol **W**), one-way ANOVA showed no differences between chow-fed groups but a significantly greater protein content in the LH-lesioned **CAFE** group than in **CAFE**-fed nonlesioned or pair-fed groups (indicated by darkened portion of bar). Two-way ANOVA by diet and surgery treatments for **W** rats showed significant effects of both of these treatments.

For groups in the cold (4°C, symbol **C**), one-way ANOVA showed no significant differences between groups for any diet; two-way ANOVA across diet groups shows a significant diet effect and no group effect. $p < 0.01$ or smaller for all significant effects.

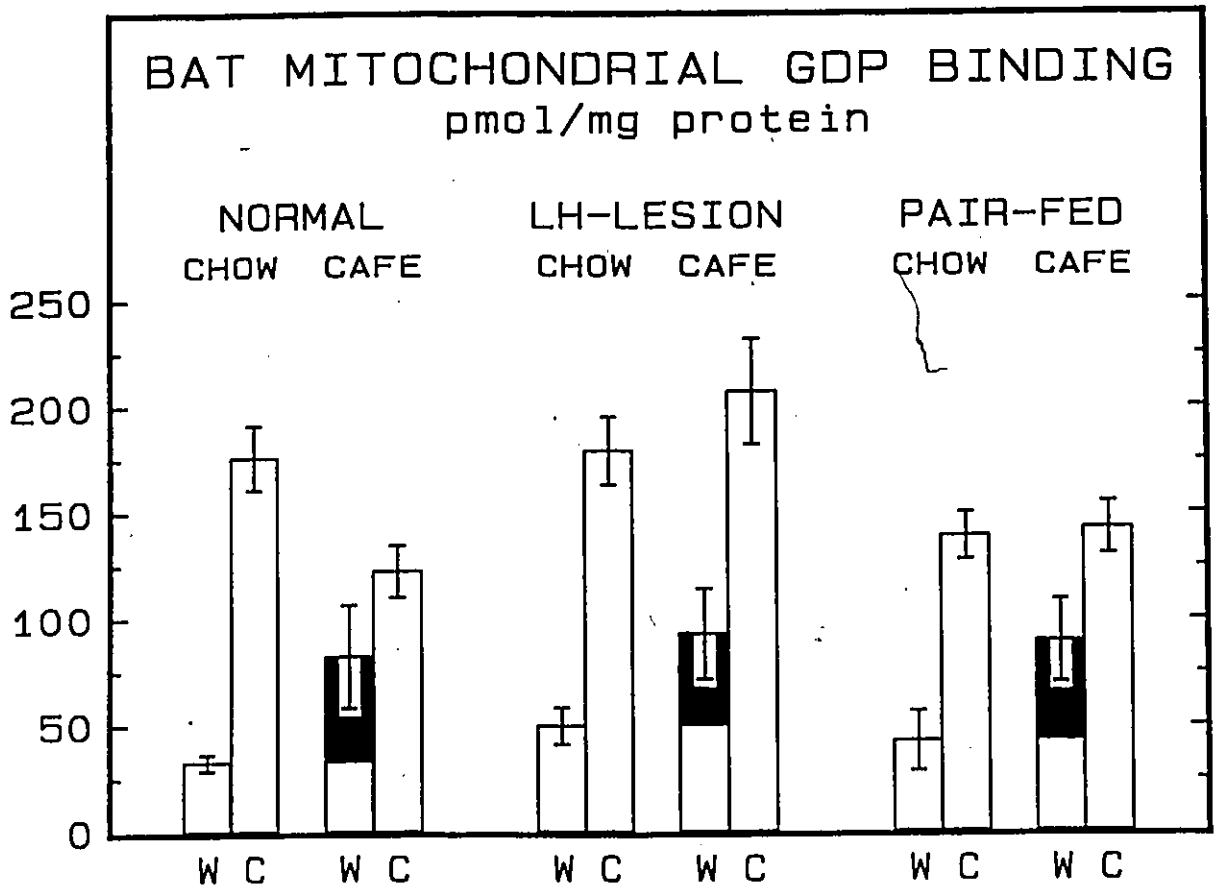

FIGURE 12. STUDY OF LATERAL HYPOTHALAMIC LESIONS: GDP BINDING.

Rats were treated and statistical analyses were performed as described in the legend to Table 9. Values shown are means; error bars represent S.E.M. for n = 8.

For animals kept in the warm (26°C. symbol **W**), ANOVA shows no significant differences between chow-fed groups or between **CAFE**-fed groups; two-way ANOVA by diet and surgery showed no group effect but a significant diet effect.

For animals kept in the cold (4°C, symbol **C**), ANOVA showed no significant differences between chow-fed groups and a significantly greater value for **CAFE**-fed LH-lesioned rats than for nonlesioned or pair-fed rats. Two-way ANOVA of **C** rats across diet groups showed no significant diet effect but a significant effect of surgery.

$p < 0.01$ or smaller for all significant effects.

Conclusion: These findings suggest that the LH-lesioned rat has more BAT per body weight than do normal or previously pair-fed rats. Rats having LH-lesions are capable of the normal cold-induced increase in non-shivering thermogenesis in BAT, and demonstrate an exaggerated diet-induced growth of BAT. These changes occur in spite of the usual effects of a reduced food intake, which include atrophy of BAT and suppression of thermogenesis in BAT.

PART B. THYROXINE 5'-DEIODINASE IN BROWN ADIPOSE TISSUE: CONTROL AND FUNCTION.

Section 1. Effects of cold and of diet on thyroxine 5'-deiodinase activity in brown adipose tissue of rats.

Objective: The objective of this work was to characterize the effects of cold and of diet on thyroxine 5'-deiodinase (T5'D) activity in brown adipose tissue of rats.

Design: This work was done in conjunction with Dr. Jan Kopecky of the Czechoslovakian Academy of Science. We studied the time course of changes in T5'D activity in rats exposed to cold, a stimulus known to stimulate activity of this enzyme. To determine the relationship between elevations in T5'D activity and in GDP binding, we also studied T5'D in rats fed a cafeteria diet, known to increase GDP binding.

In the first experiment, changes in T5'D activity in cold-exposed rats were studied as a time-course. Rats weighing 290 to 340 g, which had been previously kept under standard holding conditions, were exposed to cold (4°C) for 0, 2, 10, or 24 hours or for 3 or 10 days. Rats were then killed and their BAT was removed and cleaned as described in the Materials and Methods. BAT was homogenized in ice-cold mitochondrial isolation medium, then aliquots were frozen and stored at -80°C until assayed for activity of T5'D.

In the second experiment, rats were fed either chow, or chow plus cafeteria food, for 12 days. They were then killed and homogenates prepared for assay of GDP binding and T5'D activity as described above.

Results: The time course of changes in body weight, wet weight of BAT, and total protein in BAT are presented in Table 15. Body weights were not affected by treatment (except in rats exposed to cold for 2 hours, probably due to diuresis). The wet weight of BAT did not significantly increase over initial levels until 10 days in the cold; in contrast, total protein was increased within 10 hours of cold-exposure.

The effect of cold-exposure on T5'D activity (both specific activity and total activity) is shown in Figure 13. The increase in activity after 2 hours of cold-exposure was statistically significant. Nevertheless, it is after 10 hours that the increase is most striking. The specific and total activities of T5'D then decreased from the peak at 10 hours but remained greatly elevated (some 50-fold) over the initial values seen in the warm-acclimated rats.

The effects of cafeteria-feeding on T5'D activity and other indices of BAT function are presented in Table 16. The BAT of cafeteria-fed rats had greater wet weight and protein content than that of chow-fed rats. As expected, cafeteria-feeding greatly increased the level of GDP binding; however, it had no significant effect on the total activity of T5'D, thus demonstrating a dissociation between these two indices, which do rise in concert in cold-exposure and cold-acclimation. Indeed, when expressed as specific activity, there was a significant decrease in the cafeteria-fed rats, due to growth of the tissue (an increase in total protein) without a corresponding increase in total activity of T5'D.

Conclusions: Exposure to cold caused an extremely great increase in activity of T5'D even within 10 hours. Cafeteria-feeding did not affect the

TIME AT 4°C	BODY WEIGHT (g)	BROWN ADIPOSE TISSUE	
		Weight (mg)	Protein (mg)
0 h	321.3 ± 4.8	386 ± 22.5	34.9 ± 2.45
2 h	291.5 ± 7.4*	393 ± 22.5	41.3 ± 3.30
10 h	332.3 ± 7.4	337 ± 33.5	50.7 ± 3.55*
24 h	326.8 ± 9.3	411 ± 28.0	69.5 ± 8.40*
3 d	318.8 ± 9.2	449 ± 36.0	64.9 ± 4.35*
10 d	306.0 ± 12.2	859 ± 43.5*	119.9 ± 10.3*

TABLE 15. STUDY OF THYROXINE 5'-DEIODINASE: TIME-COURSE OF CHANGES IN BROWN ADIPOSE TISSUE IN RATS EXPOSED TO COLD.

Rats were placed in the cold (4°C) for the times indicated. They were then killed and BAT was isolated, cleaned, and prepared as usual. Values shown are means ± S.E.M. for $n = 4$. Asterisks indicate values significantly different from values at 0 hours of cold-exposure (Student's t-test); $p < 0.05$ or smaller for all significant differences.

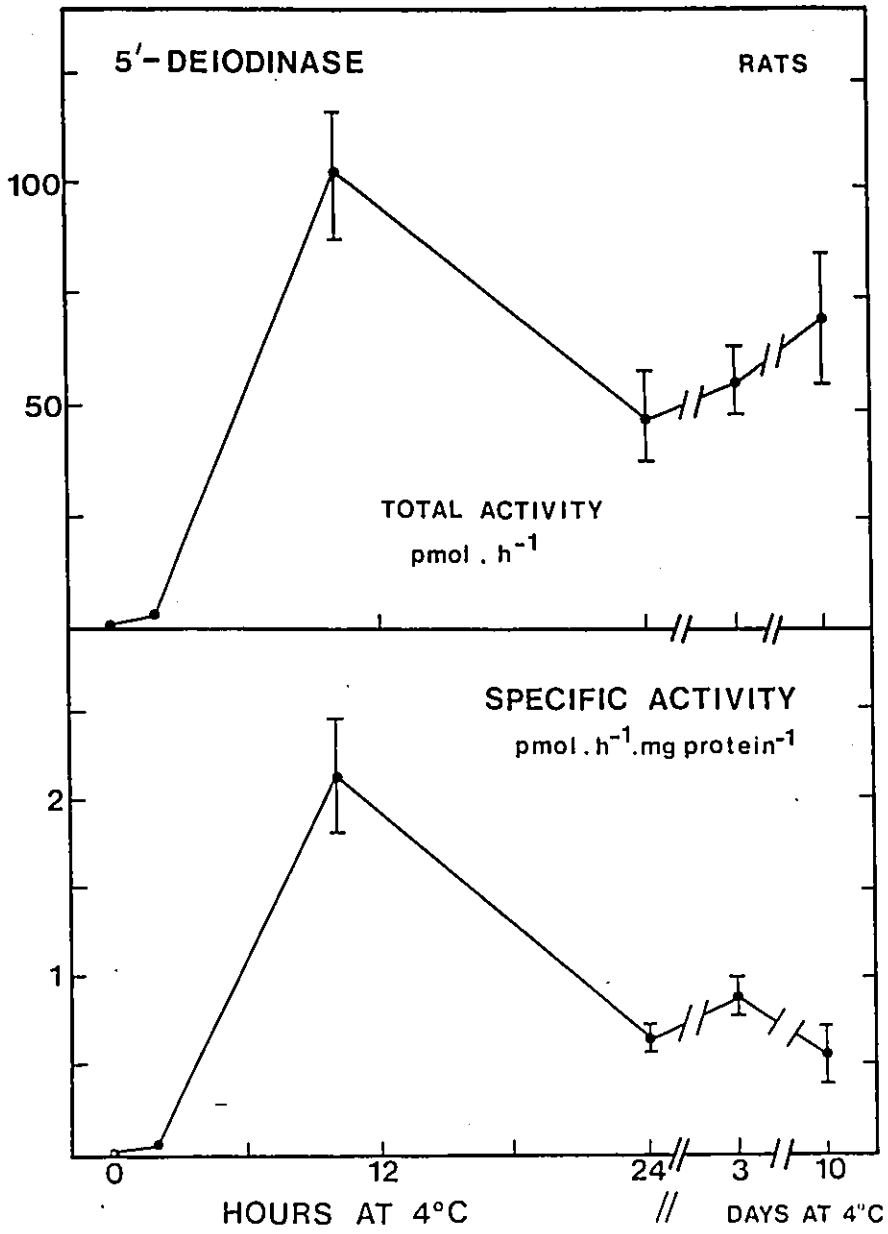

FIGURE 13. STUDY OF THYROXINE 5'-DEIODINASE: TIME-COURSE OF ENZYME ACTIVITY IN RATS EXPOSED TO COLD.

Thyroxine 5'-deiodinase activity (both specific and total) in brown adipose tissues of rats during acute and long-term exposure to cold (4°C). Error bars for initial values (0 h and 2 h of cold-exposure) are too small to show. All later values are significantly greater (Student's t-test) than the initial values ($p < 0.01$ or smaller).

	CHOW-FED	CAFETERIA-FED
Body weight (g)	268 ± 4.3	232 ± 7.7*
Gonadal white adipose tissue (g)	2.23 ± 0.25	3.19 ± 0.41
Brown adipose tissue		
Wet weight (mg)	363 ± 28.0	751 ± 39.1*
Protein (mg)	16.4 ± 0.62	26.1 ± 1.10*
T_4 5'-deiodinase:		
Specific activity (fmol/mg/h)	79 ± 5.4	38 ± 9.0*
Total activity (pmol/h)	1.29 ± 0.089	0.97 ± 0.219
GDP binding (pmol/mg)	43 ± 5.7	208 ± 37.0*

TABLE 16. STUDY OF THYROXINE 5'-DEIODINASE: EFFECTS OF A CAFETERIA DIET ON BODY WEIGHT, BROWN AND WHITE ADIPOSE TISSUE.

Rats, initially having the same mean body weight, were fed either chow, or a cafeteria diet plus chow, for 12 days. They were then killed and BAT prepared as described in the text. Values represent means ± S.E.M. with n = 4. An asterisk indicates a significant effect of cafeteria-feeding (Student's t-test). $p < 0.01$ or smaller for all significant differences.

total tissue activity of this enzyme. This demonstrates that the regulation of this enzyme is not identical with the regulation of BAT thermogenesis (as indicated by GDP binding).

Section 2. Circadian changes in thyroxine 5'-deiodinase activity and GDP binding in warm-acclimated, cold-acclimated, and cafeteria-fed rats.

Objective: The objective of this experiment was to determine whether there is any circadian variation of the activity of thyroxine 5'-deiodinase (T5'D) in BAT of warm-acclimated, cold-acclimated, and cafeteria-fed rats, and to correlate this with changes in GDP binding. From the studies cited in the Introduction showing daily cycles in other aspects of BAT, it was expected that a circadian cycle in T5'D activity might be found. A second objective of this study was to determine whether the finding from Results section B1, that cafeteria-feeding did not cause the activation of T5'D, was due to an artifact of the time of day, or was generally true.

Design: Seventy-two rats weighing 190 to 220 g were divided into three groups of 24 based on equal body weights and growth rates. Rats were then subjected for two weeks to one of three treatments: a) standard holding conditions; b) exposure to cold (4°C); c) cafeteria-feeding. Lighting was on a 12L:12D pattern, with lights-on at 07h00 and lights-off at 19h00. Cafeteria food was changed daily and was offered just before lights-out.

Each of the three groups was subdivided into four groups of six based on body weights and growth rates. At four different times of day (01h30, 07h30, 13h30, and 19h30) rats from three subgroups (two of each treatment type) were killed by decapitation and standard preparations were made. Statistical analyses were as described in the legends to the Tables and Figures below.

Results: Results for body weights of rats in the three treatment groups at the four times studied are given in Table 17. There were no significant differences in body weights for any groups at the beginning of treatments. There was a significant effect of time of day on final body weights (and, perforce, on change of body weight during the experimental period), with the maxima for all three treatment groups at 01h30 and minima at 13h30. This is likely related to the rats' circadian pattern of eating, with rats heaviest in the middle of the dark period (after feeding) and lightest at 13h30, in the middle of the "daylight" period of fasting.

Body temperatures and tissue wet weights (for white adipose tissue and brown adipose tissue) are shown in Table 18. As expected, treatment had significant effects on body temperature, WAT wet weight, and BAT wet weight, as documented numerous times in previously-reported studies. More interestingly, these results show that there is no circadian variation in the size of WAT stores but there was such a rhythm in both body temperature and wet weight of BAT. For all three treatment groups, body temperature was minimal at 13h30 and maximal at 01h30. This is probably due to the amount of activity performed by the rats, which is correspondingly minimal and maximal at those times. Note also that the range of variation is the greatest for the cafeteria-fed rats and least for the cold-acclimated rats. A statistically significant variation in the wet weight of BAT was also found but the amount of variation within any one treatment group was small and difficult to interpret.

Results for total protein content of BAT are presented graphically in Figure 14. Not surprisingly, there was a significant effect of treatment on

TREATMENT	Time of day 07h30	13h30	19h30	01h30
		Body weight (g)		
		At the start of treatment		
WA	200.5 ± 4.6	206.2 ± 4.4	197.2 ± 5.0	209.2 ± 4.1
CAFE	194.7 ± 6.5	204.3 ± 4.6	205.8 ± 3.6	206.8 ± 4.2
CA	200.7 ± 7.2	204.2 ± 3.1	204.2 ± 5.7	206.7 ± 3.6
		Final		
WA	311.5 ± 8.4*	308.8 ± 6.7*	315.2 ± 7.1*	346.0 ± 7.7§
CAFE	289.0 ± 7.0*	290.0 ± 6.6*	295.8 ± 9.0*	311.0 ± 8.4*
CA	281.5 ± 10.6*§	268.7 ± 4.7*	287.0 ± 7.7*§	296.8 ± 5.7§
		Change		
WA	111.0 ± 7.6*	102.7 ± 6.0*	118.0 ± 5.1*	136.8 ± 5.3§
CAFE	94.3 ± 1.0*	85.7 ± 2.9*	90.0 ± 5.8*	104.2 ± 9.0*
CA	80.8 ± 9.6*§	64.5 ± 3.5*	82.8 ± 3.9§	89.8 ± 5.3§

TABLE 17. CIRCADIAN STUDY: BODY WEIGHTS.

For two weeks, rats were kept under standard holding conditions (**WA**, warm-acclimated), cafeteria-fed (**CAFE**), or exposed to 4°C (**CA**, cold-acclimated), then killed at 07h30, 13h30, 19h30, or 01h30. Values shown are means ± S.E.M. for n = 6.

Results were analyzed by one-way ANOVA (Duncan's post-hoc test). Across any line, means not differing significantly from one another are indicated by the same symbol. (If no groups differed from any others, no symbols are shown.)

Results were also analyzed by two-way ANOVA for main effects of treatment and of time studied. No effects were found for body weights at the beginning of treatments. There were significant effects of both treatment and time for both final body weight and change of body weight over the experiment.

	Time of day			
	07h30	13h30	19h30	01h30
TREATMENT	BODY TEMPERATURE (°C)			
WA	37.5 ± 0.17*§	36.9 ± 0.26*	37.8 ± 0.24§	38.1 ± 0.16§
CAFE	37.6 ± 0.31*§	37.4 ± 0.24*	38.3 ± 0.15§	38.7 ± 0.19§
CA	37.2 ± 0.18	37.1 ± 0.30	37.4 ± 0.39	37.8 ± 0.17
	TISSUE WET WEIGHTS:			
	Gonadal white adipose tissue (g)			
WA	2.6 ± 0.16	3.2 ± 0.10	3.0 ± 0.22	3.0 ± 0.24
CAFE	4.1 ± 0.34	4.3 ± 0.22	3.5 ± 0.32	4.5 ± 0.35
CA	2.0 ± 0.16	2.0 ± 0.13	1.8 ± 0.07	1.7 ± 0.15
	Brown adipose tissue (mg)			
WA	458 ± 24.2	430 ± 19.2	483 ± 37.2	539 ± 28.4
CAFE	863 ± 63.6*	990 ± 48.1*§	929 ± 40.7*§	1042 ± 111.9§
CA	972 ± 11.0*§	844 ± 38.6*	968 ± 45.2*§	1022 ± 44.3§

TABLE 18. CIRCADIAN STUDY: BODY TEMPERATURES AND TISSUE WET WEIGHTS.

Rats were treated and statistical analyses were performed as described in the legend to Table 17.

Results of one-way ANOVA are presented as described in the legend to Table 17. Results were analyzed by two-way ANOVA for main effects of treatment and of time studied. Significant effects of treatment and of time of day were found for all indices here reported except for wet weight of white adipose tissue, for which there was no significant effect of time of day.

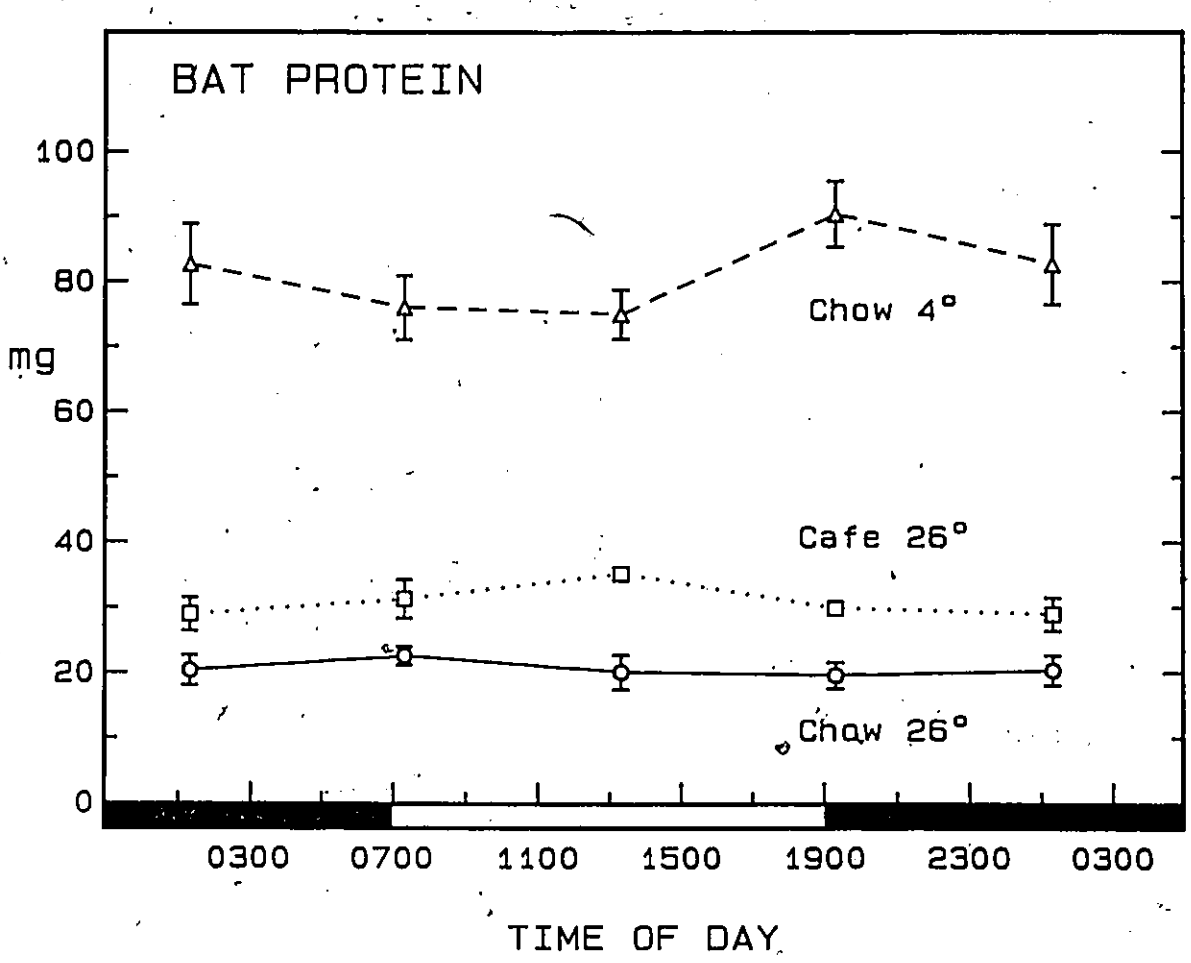

FIGURE 14. CIRCADIAN STUDY: PROTEIN.

Rats were treated and statistical analyses were performed as described in the legend to Table 17. Values shown are means for n = 6; error bars represent S.E.M. (they are too small to be shown for some data points). Lights-on is indicated by the light area on the time axis; lights-off by the dark area.

One-way ANOVA showed that the only pairwise significant differences were for cold-acclimated rats, where the value for 19h30 was greater than those for 07h30 and 13h30. Two-way ANOVA showed that there is a significant effect of treatment on total protein in BAT. Total protein was not significantly affected by the time of day.

this index of BAT function. However, no significant effect of time of day was found.

Circadian variations in GDP binding are shown in Figure 15. From this it can be seen that there is no obvious pattern of circadian change in warm-acclimated rats but there is in cafeteria-fed rats (maximum at 07h30, just after lights-on) and in cold-acclimated rats (maximum at 13h30, in the middle of the lights-on period, when muscular activity is at its minimum). The variation (in relative terms) is most marked for the cafeteria-fed rats.

Variations in total activity of thyroxine 5'-deiodinase (T5'D) are shown in Figure 16. As shown above in Results section B1, cafeteria-feeding failed to increase T5'D activity above the level seen in chow-fed, warm-acclimated rats. This was true no matter what time of day the rats were studied. In contrast, cold-acclimated rats had greatly increased T5'D activity, as expected from previous findings (Results section B1). While, from the appearance of Figure 16 one might suspect that there is a circadian variation in T5'D activity in cold-acclimated rats, in fact there are no statistically-significant differences between any of the time points for these rats. (If all treatments are considered together (two-way ANOVA), there is a significant effect of time of day on T5'D activity, with the maximum at 07h30 and the minimum at 01h30. However, such an analysis is invalid because the T5'D activities in cold-acclimated rats are so different from those found in the other two groups that the population as a whole is not normally distributed.)

Conclusions: Circadian variations in body weight, body temperature, and wet weight of BAT were found in this study. Circadian variation was

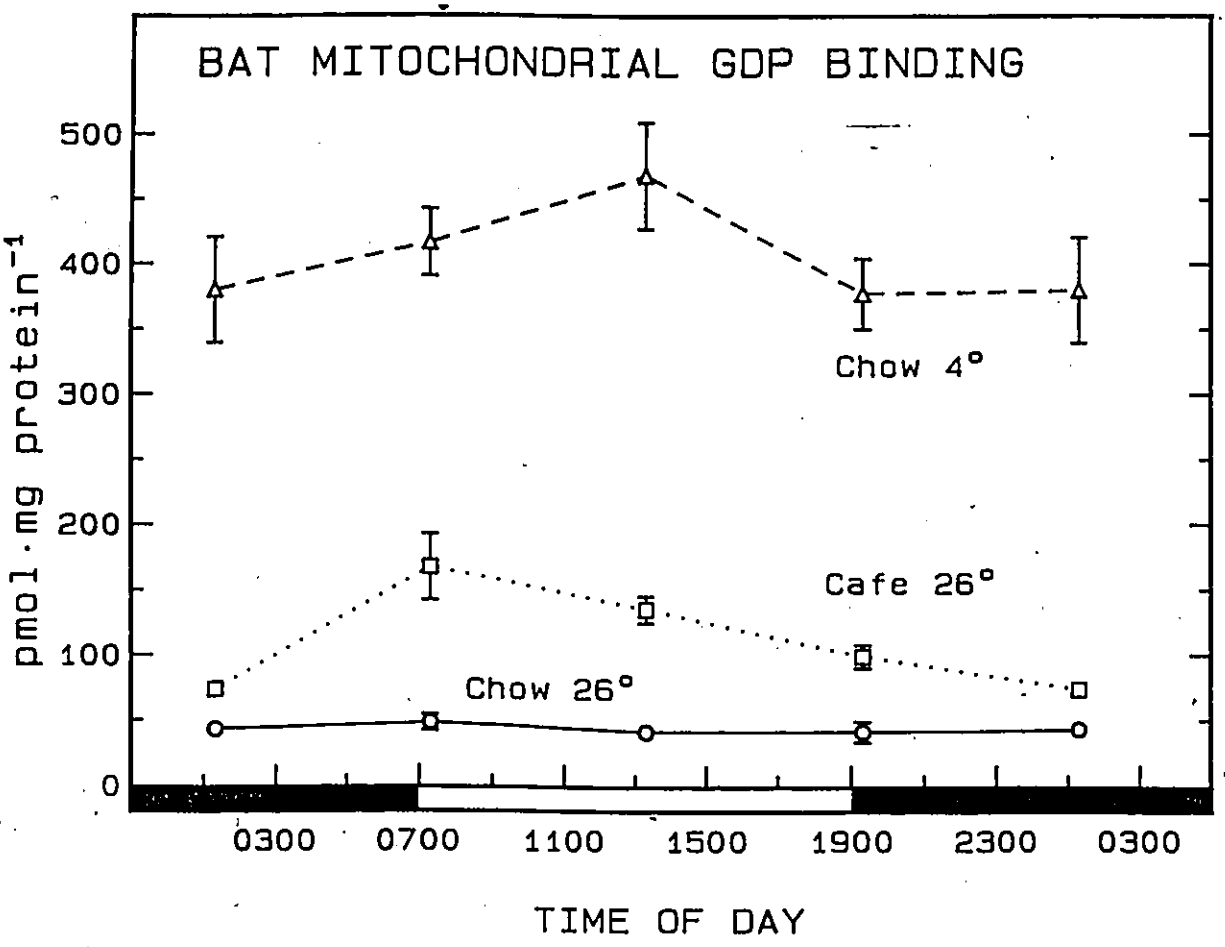

FIGURE 15. CIRCADIAN STUDY: GDP BINDING.

Rats were treated and statistical analyses were performed as described in the legend to Table 17. Results are presented as described in the legend to Figure 14.

By one-way ANOVA, there were significant pairwise differences within two treatment groups. For cafeteria-fed rats, the value at 07h30 was greater than those for 19h30 and 01h30. In the cold-acclimated rats, the mean at 13h30 was different from those at 19h30 and at 01h30.

Two-way ANOVA showed that there were significant main effect for both treatment and time of day.

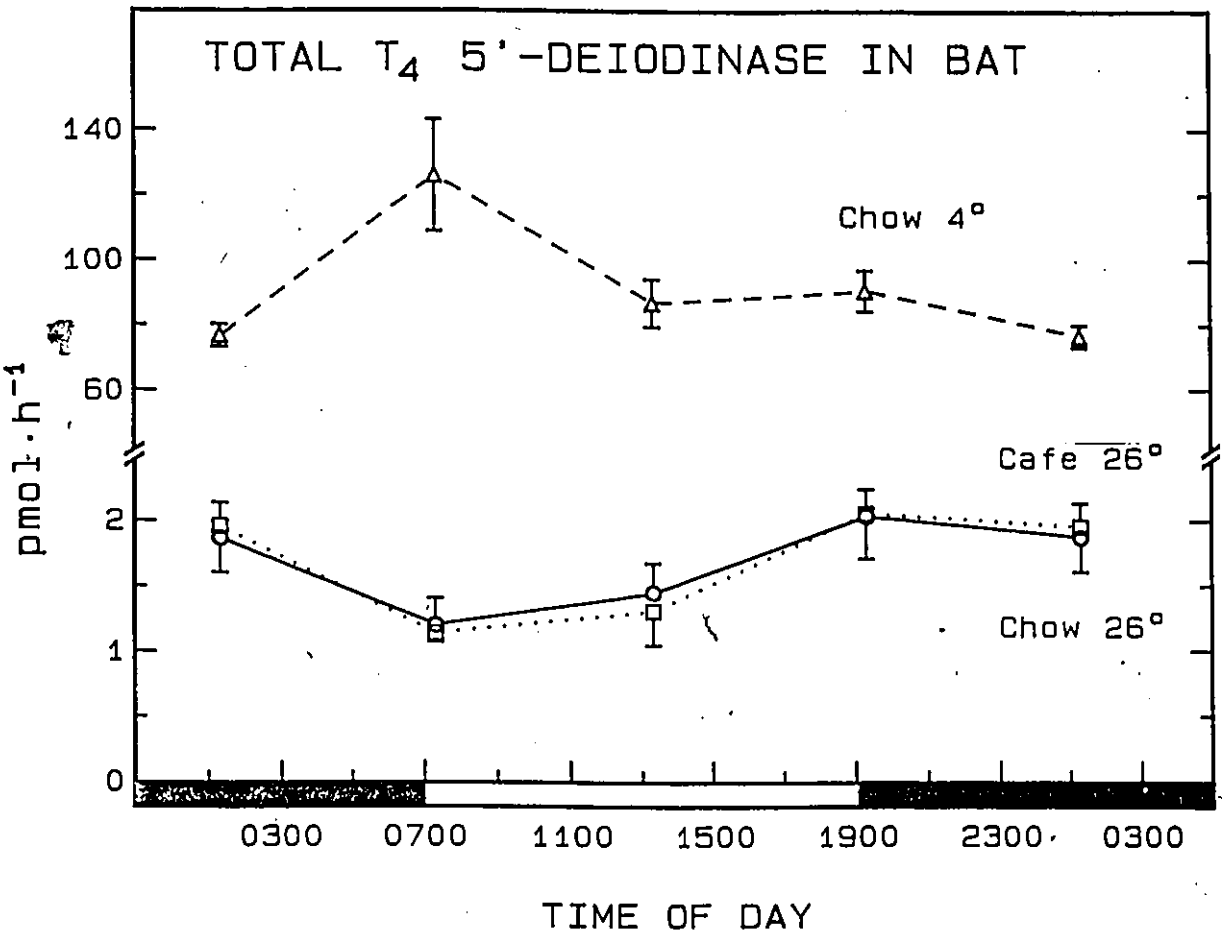

FIGURE 16. CIRCADIAN STUDY: THYROXINE 5'-DEIODINASE ACTIVITY.

Rats were treated and statistical analyses were performed as described in the legend to Table 17. Results are presented as described in the legend to Figure 14. Note the break in the Total Activity (ordinate) axis.

By one-way ANOVA, the only pairwise difference within any treatment group was in cold-acclimated rats, where the value at 07h30 was significantly different from all other points in this treatment group. Two-way ANOVA indicated that there were a significant effects for both treatment and time of day.

found in GDP binding of cold-acclimated and cafeteria-fed rats. There was no circadian variation in wet weight of white adipose tissue or in total protein content of BAT. As well, when considered separately by treatment groups (chow-fed, cold-acclimated, or cafeteria-fed), there was no significant effect of the time of day on T5'D activity. Therefore, the finding from Results section B1 is confirmed and expanded: cafeteria-feeding has no effect on T5'D activity at any time of day.

Section 3. The role of thyroxine 5'-deiodinase in the response of brown adipose tissue of thyroidectomized rats to acute and long-term exposure to cold.

Objectives: The objective of this study was to determine whether endogenous production of triiodothyronine (T_3) from thyroxine (T_4) by the thyroxine 5'-deiodinase (T5'D) in BAT is necessary for the acute thermogenic response, or the long-term growth, or both, of BAT in cold-exposed rats. A second goal of this project was to study the changes in composition of BAT mitochondrial proteins with the newly-developed radioimmunoassay for UCP.

Design: From work done previously in our laboratory by Dr. Joan Triandafillou (Triandafillou et al. 1982), it had been concluded that thyroid hormones were needed only in a permissive role for BAT response to cold. In that study, T_4 was injected into thyroidectomized rats. With the discovery of the T5'D in BAT, it was possible to re-interpret the findings of that study as representing conversion in situ of T_4 to T_3. Therefore, the role of T_3 might be more than simply permissive. To test the validity of the previous study, the present experiments were performed by adding a condition that ruled out conversion of T_4 in BAT: the injection of T_3 as the replacement thyroid hormone.

To determine whether endogenously-produced T_3 was necessary for BAT function, the source of thyroid hormones was removed. Thus, thyroidectomized (T_x) rats supplemented with either T_4 or T_3 (or vehicle) were studied after acute (15 hours) or long-term (3-4 week) exposure to cold. If the deiodinase were playing an important role, T_3-injected rats

would be similar to vehicle-injected rats; if they were similar to T_4-injected rats, the original conclusion would be supported. 15 hours was used as an acute stimulus because it was known that this was sufficient time to allow a significant response by T5'D (see Results section B1).

The acute effects of cold-exposure were studied in the first experiment. The benchwork in this experiment, except the determination of serum T_3 and T_4 levels, was done by Mr. David Mount under my co-supervision and is here reported for completeness. Forty male rats, initially weighing 200 to 225 g, were used. Twenty-seven were thyroidectomized (T_X) and thirteen were sham-operated by the supplier (Charles River). Rats were shipped two weeks after surgery.

On arrival, rats were kept under standard holding conditions except that, instead of water, T_X rats were given 0.125 % calcium solution to drink. For four weeks after arrival, rats were given one of four treatments: a) nine T_X rats were injected with T_3 (s.c., 0.5 µg per 100 g); b) ten T_X rats were injected with T_4 (s. c., 2.5 µg per 100 g): c) eight T_X rats were injected with vehicle (10% rat serum in normal saline); d) sham-operated rats were not given any special treatment. Injections were performed daily between 16h00 and 17h00.

After four to five weeks of treatment, some rats were exposed to cold (4°C) for 15 h beginning at 17h00 (for n-values, see tables below). Rats were killed by decapitation and standard preparations were made; preparations were done for seven days.

In the second experiment, the long-term effects of cold-acclimation were studied. Forty-eight rats (35 T_X, 13 sham-operated) were used. Rats were treated as in experiment 1 except that 14 T_X rats were injected with

T_3, 14 with T_4, and 7 with vehicle. These treatments were continued for 40 days. Then 7 each of the sham-operated, T_3-, and T_4-injected rats were placed for two days at 14°C, then for 18-23 days at 4°C, while all other rats (7 T_3-injected rats, 7 T_4-injected rats, 7 vehicle-injected T_X rats, and 6 sham-operated rats) remained at 26°C. The injections were continued for this period. Rats were killed by decapitation and standard preparations made.

Results: a) Acute exposure to cold. The acute effects of exposure to cold (4°C) on body weights and body temperatures in T_X and sham-operated rats are presented in Table 19. As can be seen from the change in body weight from beginning to end of the experiment, vehicle-injected T_X rats had an almost complete failure of growth (as expected) while the T_3-injected rats lagged in growth. Vehicle-injected rats were also hypothermic; other rats were normothermic.

The acute effects of cold-exposure on the size of BAT and on serum thyroid hormone levels are shown in Table 20. The wet weight of BAT was significantly reduced in T_3- and T_4-injected rats while total protein content was not affected by any treatment.

If protein is expressed per body weight, then it was significantly increased in vehicle-injected thyroidectomized rats (Table 20). The brief 15 hour exposure to cold did not affect the wet weight of BAT or its protein content in any treatment group.

Serum T_3 and T_4 levels were greatly reduced by thyroidectomy, as expected, although T_4 levels in cold-exposed, vehicle-injected rats were surprisingly high (Table 20). T_3 levels were reduced in both T_3- and T_4-

TEMPERATURE	Sham operated	Vehicle injected	T_4 injected	T_3 injected
		n VALUES		
WA	5	4	5	5
CA	8	4	4	5
		Initial body weight (g)		
WA	256 ± 5.4	237 ± 4.0	236 ± 5.0	227 ± 4.6
CA	252 ± 3.3	240 ± 6.6	224 ± 2.5	225 ± 7.9
		Final body weight (g)		
WA	438 ± 21.7	239 ± 3.8	404 ± 6.5	375 ± 10.2
CA	431 ± 9.9	270 ± 10.8	391 ± 10.9	375 ± 12.2
		Change in body weight (g)		
WA	181 ± 17.3	2 ± 7.3*	168 ± 5.6	147 ± 6.8*
CA	180 ± 8.9	30 ± 13.6*	168 ± 8.7	150 ± 4.7*
		Body temperature (°C)		
WA	37.3 ± 0.2	36.3 ± 0.3*	37.1 ± 0.2	37.1 ± 0.1
CA	37.6 ± 0.1	36.1 ± 0.6*	37.8 ± 0.2	37.3 ± 0.1

TABLE 19. EFFECTS OF ACUTE COLD-EXPOSURE IN THYROIDECTOMIZED RATS: BODY WEIGHTS AND BODY TEMPERATURES.

Rats were either **sham-operated** or thyroidectomized and injected with **vehicle**, T_4, or T_3 for 4 weeks, then either left at 26°C (**WA**, warm-acclimated) or exposed to 4°C for 15 hours (**CA**, cold-acclimated). Values shown are means ± S.E.M. for n values as shown. An asterisk indicates values having a statistically significant effect of a treatment compared to the corresponding **sham** group (Student's t-test; $p < 0.05$ or smaller for all comparisons). "Initial body weight" refers to the weight of rats at the beginning of the experimental period; "final body weight", to the weights at the end of the experiment.

Two-way ANOVA was also performed. It indicated that there was no significant effect of temperature and a significant effect of treatment for all four measures reported in this Table.

TEMPERATURE	Sham operated	Vehicle injected	T_4 injected	T_3 injected
		Brown adipose tissue:		
		Wet weight (mg)		
WA	499 ± 24	570 ± 58	334 ± 31*	334 ± 53*
CA	460 ± 39	435 ± 79	309 ± 16*	303 ± 26*
		Protein (mg)		
WA	31.6 ± 3.81	29.9 ± 5.54	27.2 ± 4.03	26.4 ± 4.87
CA	39.1 ± 3.76	37.1 ± 7.09	31.5 ± 2.36	31.0 ± 3.05
		Protein per body weight (mg/100g)		
WA	5.5 ± 0.89	10.4 ± 1.65*	6.5 ± 1.05	5.7 ± 1.16
CA	6.7 ± 0.38	10.1 ± 2.26*	7.0 ± 0.50	7.1 ± 0.99
		Serum T_3 (ng/dL)		
WA	41.4 ± 6.5	< 5*	16.9 ± 3.6*	17.4 ± 0.8*
CA	56.1 ± 5.7	< 5*	43.1 ± 11.8§	18.2 ± 2.1*
		Serum T_4 (ng/mL)		
WA	32.1 ± 1.6	0.5 ± 0.3*	39.5 ± 3.3	3.5 ± 2.1*
CA	36.7 ± 1.9	5.7 ± 3.2§	52.3 ± 4.3*	1.1 ± 0.6*

TABLE 20. EFFECTS OF ACUTE COLD-EXPOSURE IN THYROIDECTOMIZED RATS: BROWN ADIPOSE TISSUE AND SERUM THYROID HORMONES.

Rats were treated and statistical analyses were performed as described in the legend to Table 19. An asterisk indicates a significant effect of treatment (compared to corresponding sham-operated control) while a dagger indicates a significant effect of cold-exposure (compared to warm-exposed rats in the same treatment group), as determined by Student's t-test, with $p < 0.05$ or smaller for all differences reported as significant. Note that the limit of detection for the T_3 assay was 5 ng/dL.

For the measures of the size of BAT, two-way ANOVA showed that there was no significant effect of cold-exposure. For wet weight of BAT, and for protein content of BAT per 100 g body weight, there were significant effects of treatment; there was no effect of treatment on total protein content of BAT. For serum T_3 and T_4 levels, ANOVA showed significant main effects for both treatment and exposure to cold.

injected rats; T_4 levels were low in T_3-injected rats, as expected. Exposure to cold caused a significant increase in T_3 levels in T_4-injected rats.

GDP binding results for these rats are shown in Figure 17. It can be seen that both T_3- and T_4-injected rats were able to increase GDP binding in response to acute cold-exposure in a normal way; vehicle-injected rats could not.

The effects of acute cold-exposure on total thyroxine 5'-deiodinase (T5'D) activity are shown in Figure 18. Cold-exposure greatly increased the T5'D activity in all treatment groups; thyroidectomized rats (in each of the three treatment groups) showed an enhanced response to cold compared to sham-operated rats. Note also that warm-acclimated, vehicle-injected rats had a very high level of activity. Thyroidectomy is thus one of the few ways by which the activity of T5'D can be increased in animals kept at temperatures near thermoneutral.

Conclusions: Brown adipose tissue of thyroidectomized rats which are supplemented with either T_3 or T_4, then acutely exposed to cold, is able to activate non-shivering thermogenesis normally and shows a supernormal increase in thyroxine 5'-deiodinase activity. Injected or endogenously-produced T_3 were equally effective in allowing activation of thermogenesis. Lack of T_3 prevented the cold-induced increase in GDP binding.

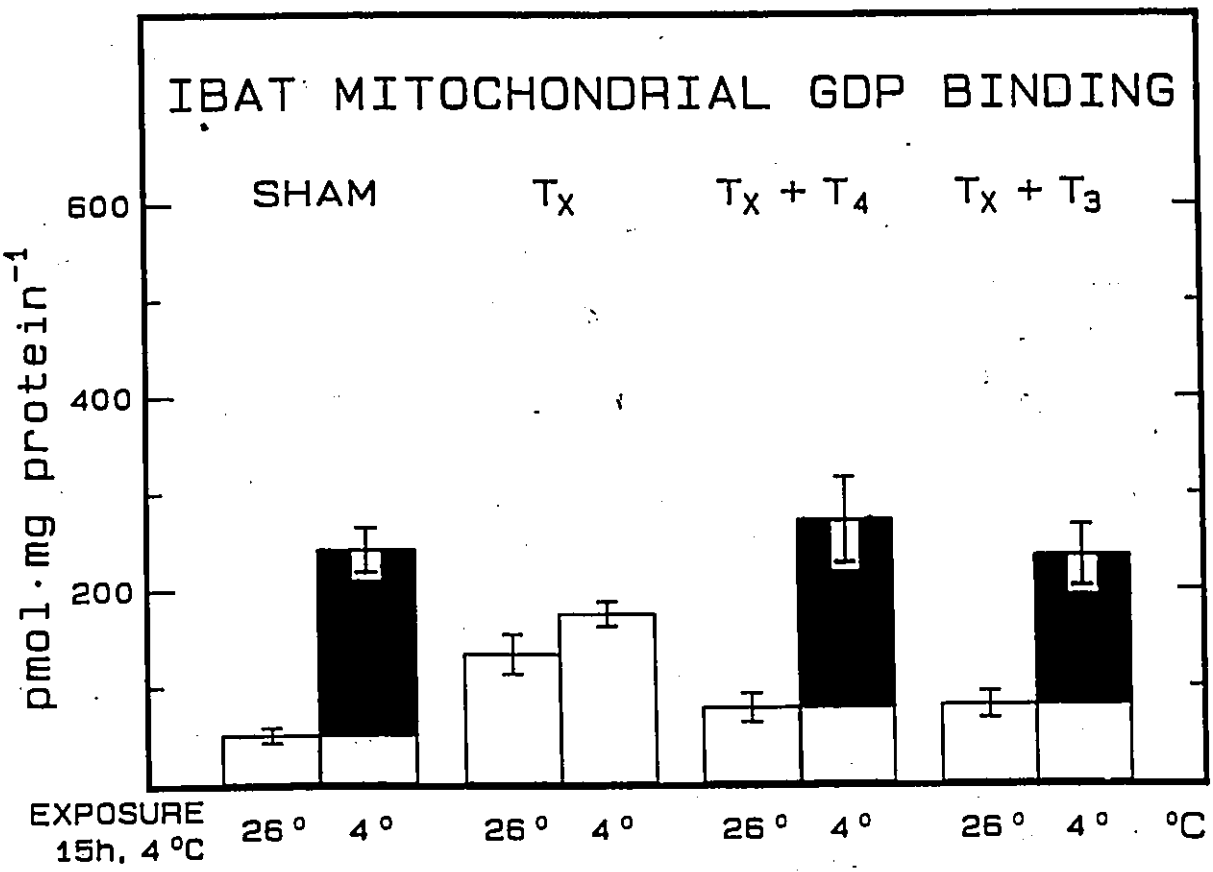

FIGURE 17. EFFECTS OF ACUTE COLD-EXPOSURE IN THYROIDECTOMIZED RATS: GDP BINDING.

Rats were treated and statistical analyses were performed as described in the legend to Table 19. Values shown are means with error bars representing the S.E.M.; black areas indicate a significant increase in GDP binding (in each treatment group) compared to warm-exposed rats of the same treatment group. Comparisons were made by Student's t-test with $p < 0.05$ or smaller for all differences reported as being statistically significant.

Two-way ANOVA showed that there was no effect of treatment on GDP binding but that there was a significant effect of cold-exposure.

Symbols: **SHAM** = sham-operated (controls)
T_X = thyroidectomized, vehicle-injected
$T_X + T_4$ = thyroidectomized, T_4-injected
$T_X + T_3$ = thyroidectomized, T_3-injected

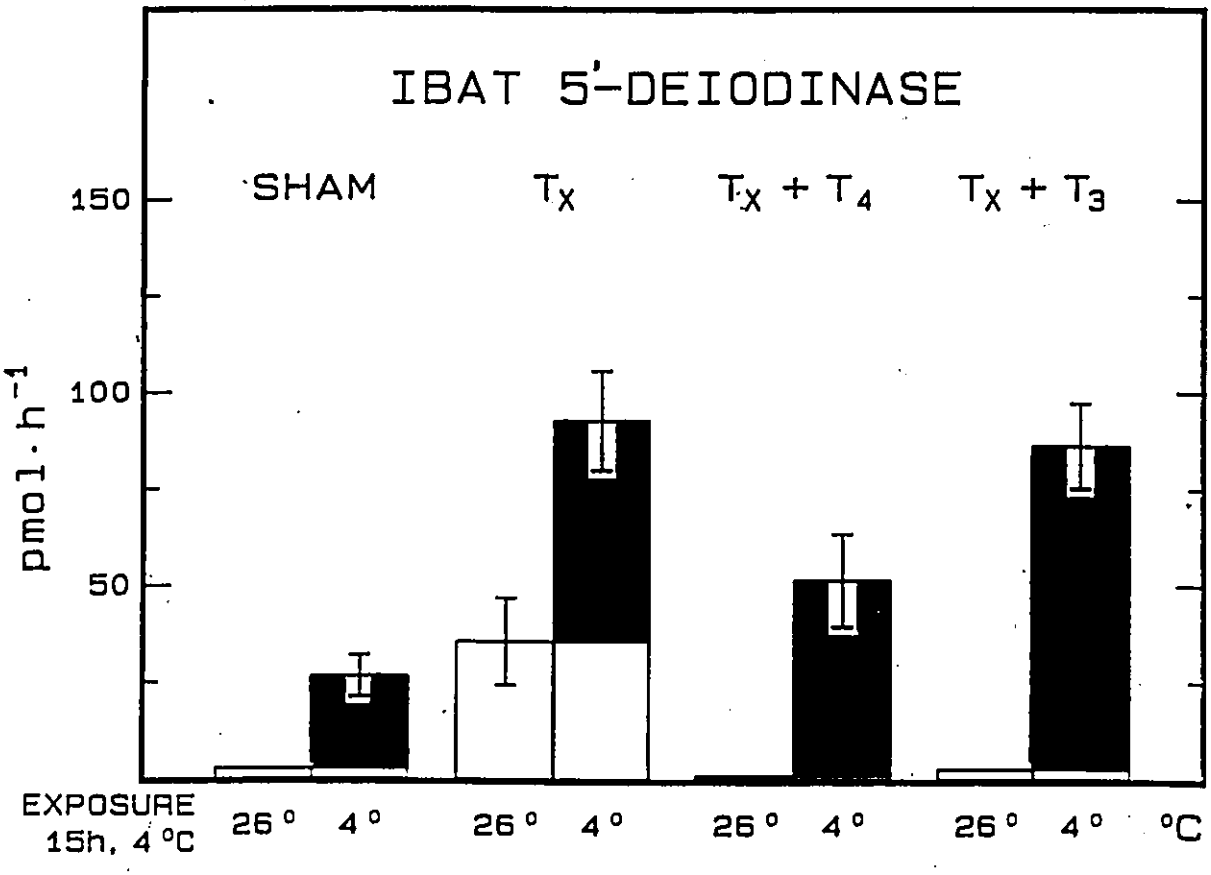

FIGURE 18. EFFECTS OF ACUTE COLD-EXPOSURE IN THYROIDECTOMIZED RATS: THYROXINE 5'-DEIODINASE ACTIVITY.

Rats were treated and statistical analyses were performed as described in the legend to Table 19; the format of this figure and the key to symbols used are as described in the legend to Figure 17.

Two-way ANOVA showed that there were significant main effects of both treatment and exposure to cold.

b) Acclimation to cold. Results for body weights and changes of body weights of the rats in this second study are given in Table 21.

Initial body weights of all thyroidectomized rats were lower than sham-operated rats but replacement by T_4 or T_3 allowed "catch-up" growth so that by the beginning of cold-acclimation only vehicle-injected rats were significantly smaller than sham-operated control rats. Cold-acclimation slowed growth in all three groups (sham-operated, T_4-injected, and T_3-injected).

Results for body temperatures, serum thyroid hormone levels, and brown adipose tissue are presented in Table 22. Vehicle-injected rats were significantly hypothermic; cold-acclimated, T_4-injected rats were hypothermic compared to warm-acclimated T_4-injected rats.

As expected, cold-acclimation significantly affected BAT wet weights and protein per 100 g body weight. This occurred in all three treatment groups subjected to cold-acclimation (sham-operated, T_4-injected, and T_3-injected), demonstrating that replacement with either T_4 or T_3 allowed normal hypertrophy in response to cold.

In sham-operated rats, cold-acclimation significantly increased T_3 levels while not affecting T_4 levels. In both T_4- and T_3-injected rats, serum T_3 levels were lower than in sham-operated rats and these levels were not increased by cold-acclimation.

Results for total BAT protein content are shown in Figure 19. It can be seen that there were no differences in protein content in warm-acclimated rats for each of the treatment groups, and that response to cold-acclimation was completely normal in both T_4- and T_3-injected rats. Similar findings were made for DNA content (Figure 20), GDP binding

TEMPERATURE	Sham operated	Vehicle injected	T_4 injected	T_3 injected
		Body weights (g): Initial		
WA	300 ± 6.2	249 ± 4.3*	229 ± 4.1*	233 ± 5.5*
CA	295 ± 6.1	--	232 ± 4.5*	240 ± 6.6*
		Start cold-acclimation		
WA	438 ± 9.5	250 ± 4.0*	376 ± 8.5	377 ± 9.9
CA	445 ± 10.5	--	370 ± 3.8	384 ± 5.1
		Final		
WA	474 ± 9.8	246 ± 3.5*	426 ± 10.4	429 ± 11.5
CA	456 ± 12.2	--	401 ± 4.0	404 ± 4.8
		Change - first period		
WA	138.5 ± 7.1	1.1 ± 5.0*	147.0 ± 6.4	144.0 ± 7.0
CA	148.9 ± 6.0	--	138.6 ± 5.1	144.6 ± 4.5
		Change - second period		
WA	35.8 ± 3.0	-3.6 ± 2.1*	50.3 ± 3.8	52.0 ± 2.5
CA	11.3 ± 2.9§	--	30.6 ± 2.6§	19.9 ± 3.3§

TABLE 21. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: BODY WEIGHTS.

Thyroidectomized rats were injected with **vehicle**, T_4, or T_3 during the first period (days 0-40). Then groups of **sham-operated**, T_4-injected, and T_3-injected rats were acclimated to cold (4°C) for 20-25 days while other groups of rats (representing each of the four treatment groups) were maintained at 26°C. Values shown are means ± S.E.M. for n = 7 (except for warm-acclimated sham-operated rats, where n = 6). Asterisks and daggers are used as described in the legend to Table 19.

"Initial body weights" refer to mean weights of rats on arrival (before injections), "start of cold-acclimation", to mean weights at the beginning of cold-acclimation, and "final" to body weights at sacrifice.

Two-way ANOVA showed that there was a significant effect of treatment on each of the measurements here reported except for change of body weight during the first period. There was no main effect of cold-acclimation on initial body weight, body weight at beginning of cold-acclimation, or change in the first period; there was a significant effect of cold-acclimation on final body weight and change in the second period.

TEMPERATURE	Sham operated	Vehicle injected	T_4 injected	T_3 injected
		Body temperature (°C)		
WA	37.7 ± 0.24	36.2 ± 0.21*	37.8 ± 0.28	37.7 ± 0.29
CA	37.5 ± 0.10	--	36.9 ± 0.24§	37.5 ± 0.12
		Brown adipose tissue		
		Wet weight (mg)		
WA	596 ± 44	669 ± 44	530 ± 32	484 ± 23
CA	926 ± 50§	--	1033 ± 30§	1163 ± 68§
		Protein per body weight (mg/100 g)		
WA	6:0 ± 0.24	16.6 ± 1.40	9.3 ± 0.85	8.8 ± 0.95
CA	23.1 ± 1.85§	--	28.7 ± 2.14*§	26.4 ± 0.95§
		Serum T_3 (ng/dL)		
WA	30.7 ± 3.2	< 5*	13.1 ± 1.4*	13.9 ± 2.6*
CA	47.6 ± 2.8§	--	17.9 ± 1.7*	13.9 ± 1.9*
		Serum T_4 (ng/mL)		
WA	32.3 ± 3.1	0.2 ± 0.1*	48.0 ± 3.6	2.6 ± 1.1*
CA	30.1 ± 1.6	--	36.7 ± 2.7	1.6 ± 1.0*

TABLE 22. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: BODY TEMPERATURES, BROWN ADIPOSE TISSUE AND SERUM THYROID HORMONES.

Rats were treated and statistical analyses were performed as described in the legend to Table 21. Note that 5 ng/dL is the limit of detection for the radioimmunoassay for T_3.

Two-way ANOVA showed that there was a significant effect of cold-acclimation for all indices reported in this Table. There was no significant main effect of treatment on body temperature or on the wet weight of brown adipose tissue. There were significant effects of treatment on protein per 100 g body weight, and on serum T_3 and serum T_4.

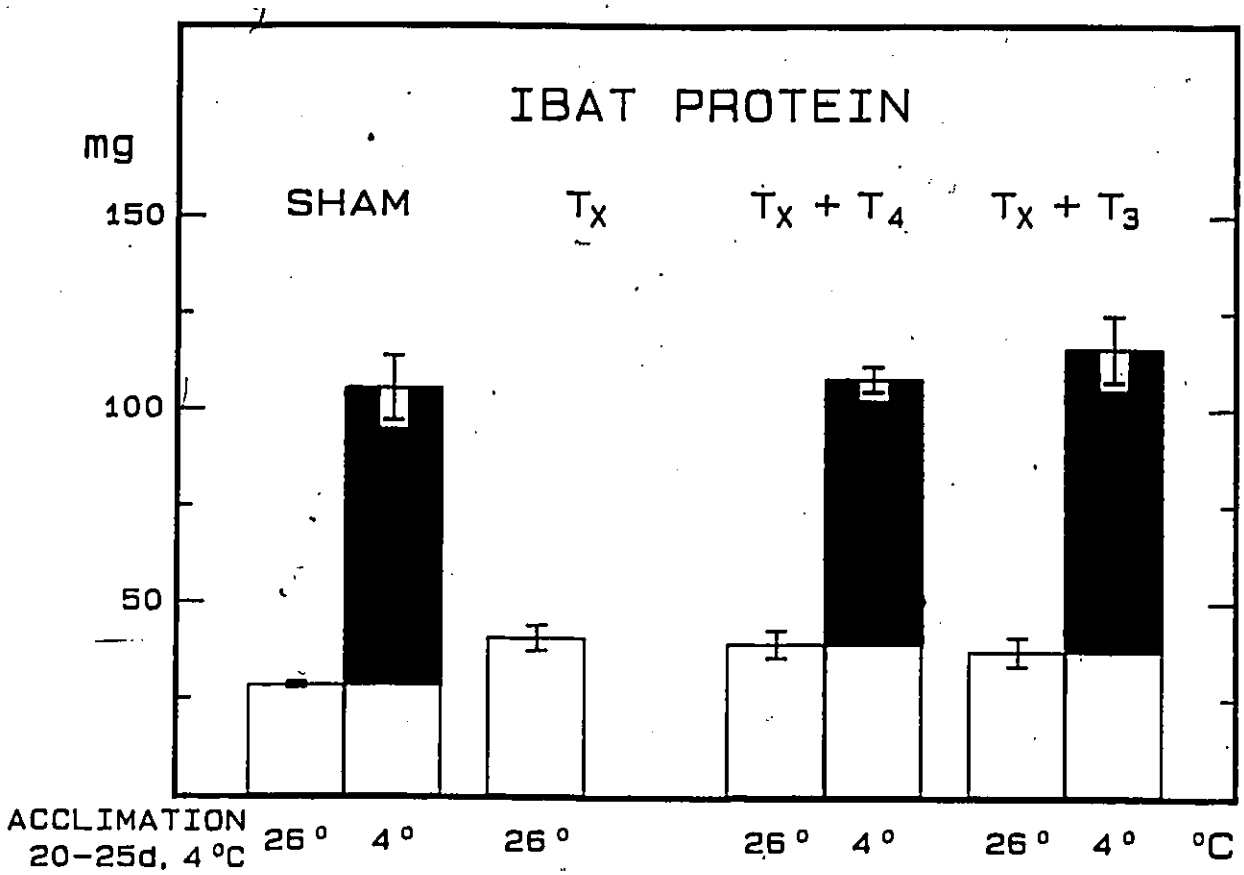

FIGURE 19. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: PROTEIN.

Rats were treated and statistical analyses were made as described in the legend to Table 21. Values shown are means; error bars represent S.E.M. for n values as listed in the legend to Table 21. Black areas indicate a significant effect of cold-acclimation for each treatment group (compared to the corresponding warm-acclimated animals); differences were determined by Student's t-test with $p < 0.05$ or smaller for significant differences. Symbols used are as described in the legend to Figure 17.

Two-way ANOVA showed that there was no significant main effect of treatment on protein content but that there was a significant effect of cold-acclimation.

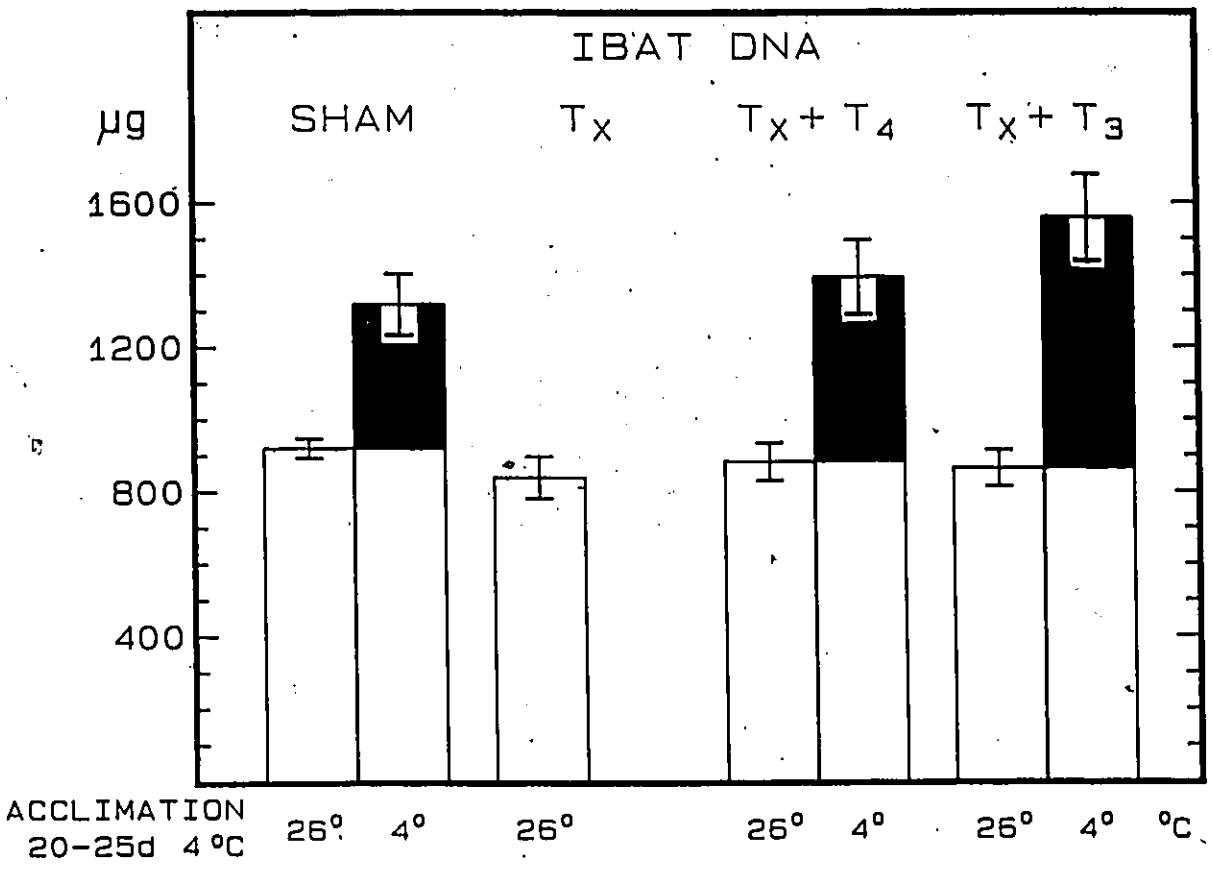

FIGURE 20. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: DNA.

Rats were treated and statistical analyses were performed as described in the legend to Table 21. Other information about the format of this figure may be obtained from the legend to Figure 17.

Two-way ANOVA showed that there was no significant main effect of treatment on DNA content; there was a significant main effect of cold-acclimation.

(Figure 21), and specific UCP levels (Figure 22). In other words, BAT of T_4- and T_3-injected, thyroidectomized rats was able to respond to cold-acclimation in a normal manner, as judged by these indices.

Results for thyroxine 5'-deiodinase (T5'D) activity are presented in Figure 23. As shown in the first part of this study (Figure 18), warm-acclimated rats had very low levels of activity. Nevertheless, while it had been found in that experiment that warm-acclimated, vehicle-injected T_X rats had an increased activity of T5'D, this result was not reproduced in the long-term experiment. Acclimation to cold caused the usual very great increase in activity for all three treatment groups; the increase was particularly marked in the T_3-injected rats.

Conclusions: Brown adipose tissue of thyroidectomized rats supplemented with either T_3 or T_4 responds normally to long-term cold-acclimation, as determined by a variety of indices (BAT total protein, DNA content, GDP binding, specific UCP levels, and T5'D activity). These results, taken with those from the first part of the study (acute exposure to the cold), suggest that circulating T_3 alone may be sufficient to permit the cold-stimulated growth of BAT and that endogenous production of T_3 by T5'D is not necessary for growth of the tissue in response to acute and long-term cold-exposure.

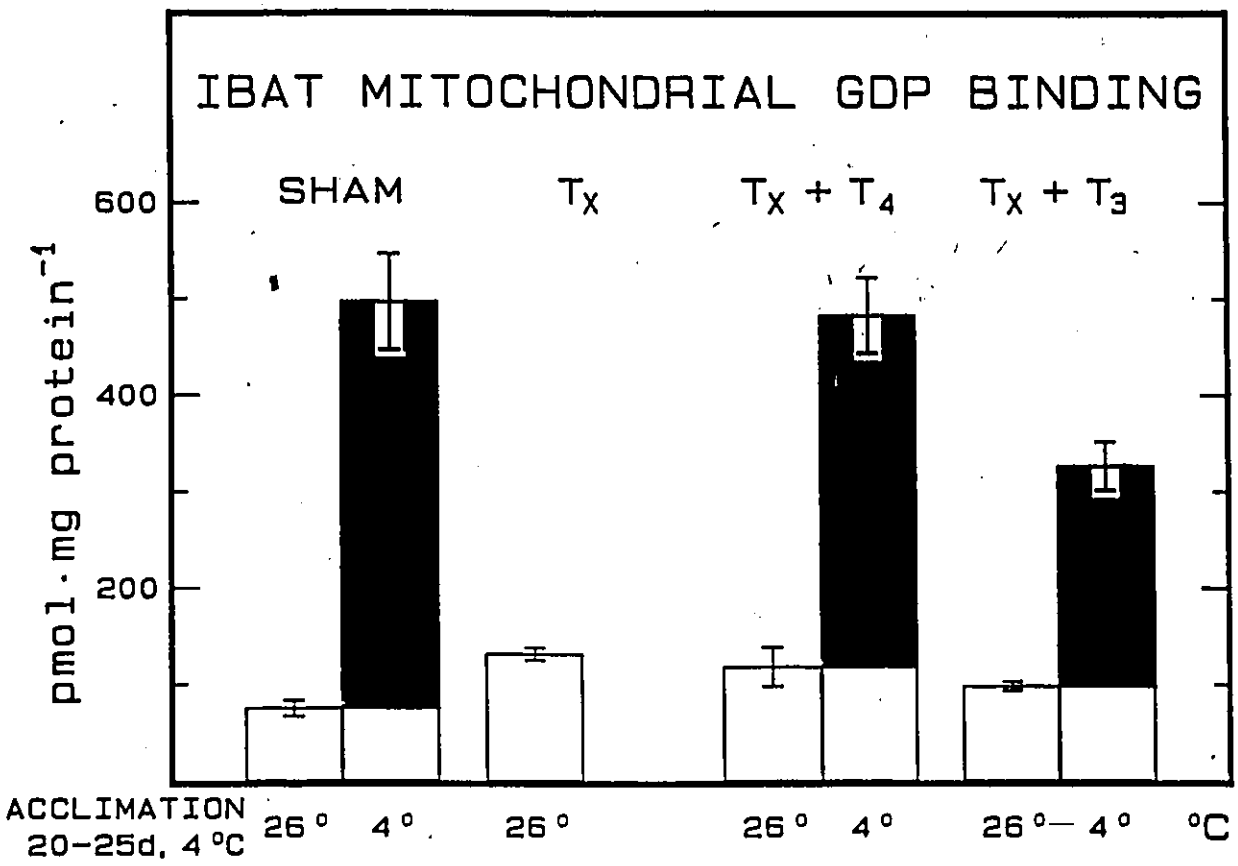

FIGURE 21. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: GDP BINDING.

Rats were treated and statistical analyses were performed as described in the legend to Table 21. Other information about the format of this figure may be obtained from the legend to Figure 17.

Two-way ANOVA demonstrated that there were significant main effects of both treatment and cold-acclimation on GDP binding.

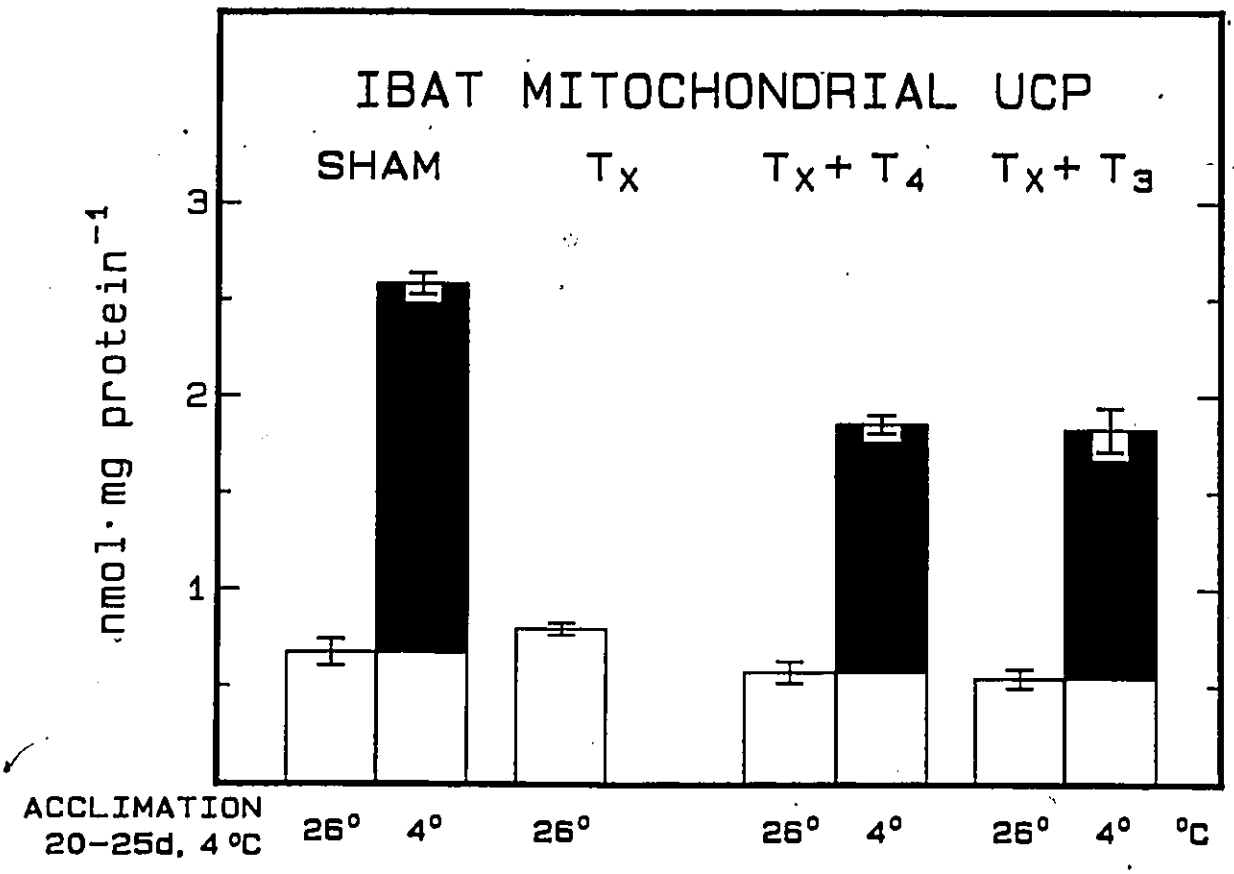

FIGURE 22. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS:SPECIFIC UNCOUPLING PROTEIN.

Rats were treated and statistical analyses were performed as described in the legend to Table 21. Other information about the format of this figure may be obtained from the legend to Figure 17.

Two-way ANOVA showed that there were significant main effects of both treatment and cold-acclimation for specific UCP content.

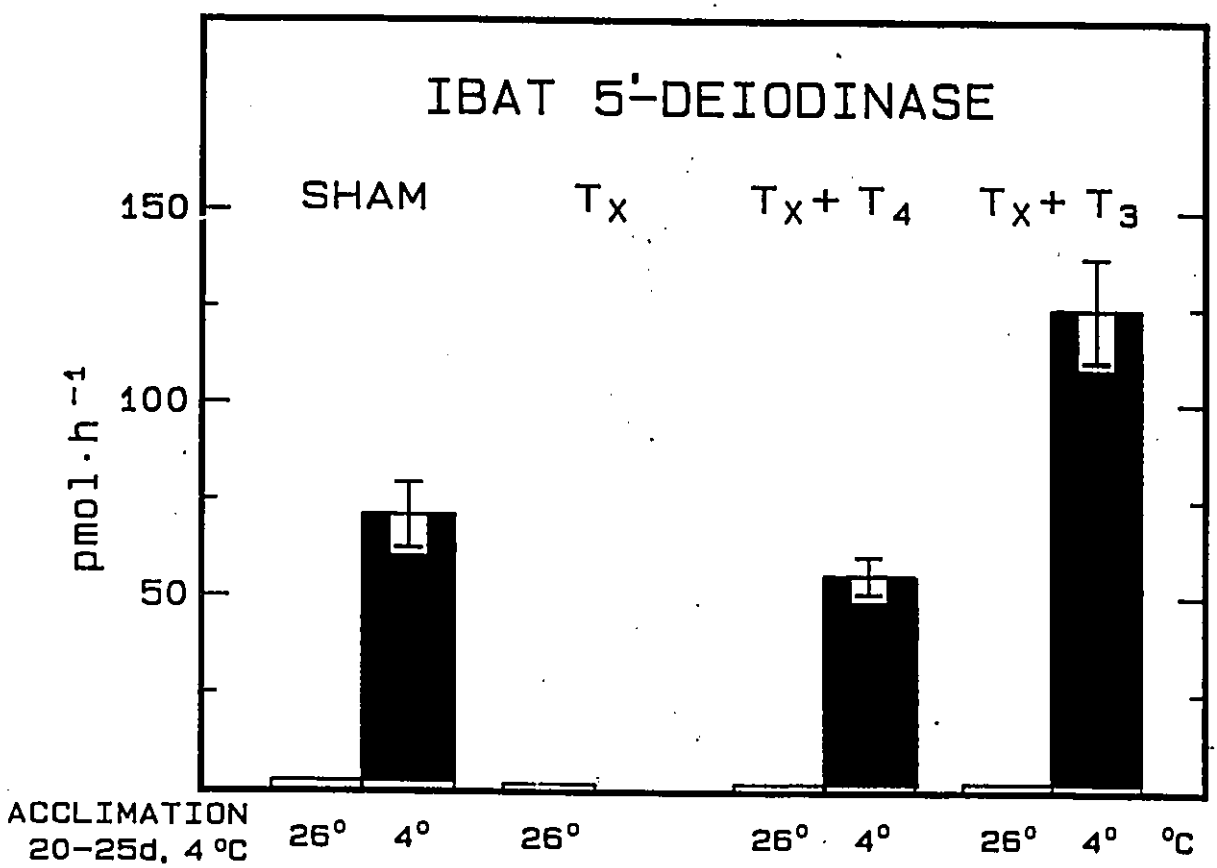

FIGURE 23. EFFECTS OF LONG-TERM COLD-ACCLIMATION IN THYROIDECTOMIZED RATS: THYROXINE 5'-DEIODINASE ACTIVITY.

Rats were treated and statistical analyses were performed as described in the legend to Table 21. Other information about the format of this figure may be obtained from the legend to Figure 17.

Two-way ANOVA showed that there were significant main effects of both treatment and cold-acclimation on total thyroxine 5'-deiodinase activity.

Section 4. The effects of sympathetic denervation on the growth and function of brown adipose tissue.

Objective: The objective of these experiments was to determine the importance of sympathetic innervation in both the acute and long-term responses of brown adipose tissue to cold-exposure, including its role in the maintenance of the cold-acclimated state.

Rationale: The study was done in three parts. In part a, the effectiveness of surgical denervation, as judged by decreased noradrenaline content of the pad, was determined. The effects of unilateral denervation were compared to those of bilateral denervation in part b. Here, several indices of BAT function and growth were studied in both warm- and cold-acclimated rats. The time-course of changes in intact versus denervated BAT was studied in part c. In the first part of that study, rats were exposed to cold, after surgery, to demonstrate the importance of innervation to growth of BAT in response to cold-exposure and cold-acclimation. In the second part, surgery was performed on rats that already had been cold-acclimated, to demonstrate the importance of the innervation in maintaining the changes caused by cold-acclimation.

In general, unilateral surgical denervation of the interscapular half-pads was performed. In this way, each animal could act as its own control. Some parts of the study were used to validate this procedure (parts a and b).

a) Effectiveness of surgery.

Objectives: The objective of these studies was to validate the technique of surgical denervation by determining the amount of residual noradrenaline remaining in denervated tissue at various times after surgery. A second objective was the demonstration of the unilaterality of the nerve supply of the half-pads of the interscapular depot.

Design: Two experiments were performed. In the first, rats were first grouped on the basis of equal body weights. One side of the interscapular pad of BAT was surgically denervated under ether anaesthesia ("unilateral denervation"; see Materials and Methods section 2b). Groups of five rats were subjected to one of three treatments for 3 or 26 days. The treatments were cold-exposure (at 4°C), cafeteria-feeding; and chow-feeding (standard holding conditions). Rats were killed by decapitation and BAT quickly removed, cleaned of adhering white fat and muscle, and frozen on dry ice. (Half-pads were treated separately.) Later, the weights of the frozen tissues were found and acid extracts (for determination of noradrenaline) were made as described in the Materials and Methods (section 12). Tissue noradrenaline content was determined by HPCL analysis as described in the same section.

In the second experiment, the effectiveness of bilateral denervation was compared to that of unilateral denervation. Rats were grouped by body weights and interscapular BAT of 16 rats was unilaterally denervated: bilateral denervations were performed in 16 other rats. Half of each group was exposed to cold (4°C) and the other half was kept warm (26°C) for

two or six weeks. Rats were killed by decapitation and tissue prepared as before. Half-pads were treated separately for both unilaterally- and bilaterally-denervated rats. Tissue noradrenaline content was determined as before. Statistics used are described in the legends to the Tables.

Results: Cold-acclimation had the usual effect of causing a slowing of growth. However, surgery did not affect the growth of either warm- or cold-acclimated rats, as shown by ANOVA of final body weights (data not shown).

Results from the first study are shown in Table 23. These results show that denervation reduced noradrenaline content to very low levels and that they remained low over the time period studied. The tissue noradrenaline content was not reduced to zero and presumably this was due to remaining sympathetic nerves innervating blood vessels.

Results from the second study are shown in Table 24. The results from two weeks post-surgery confirm the findings from the first study, that surgical denervation greatly reduced noradrenaline content of BAT in both warm- and cold-acclimated rats. These results also show that unilateral and bilateral denervation cause similarly great reduction of the noradrenaline content, and suggest that previous findings of the unilaterality of the innervation of BAT are indeed correct (Foster *et al*. 1982).

By 6 weeks post-surgery, there is some recovery of noradrenaline content, especially in cold-acclimated rats. This recovery was more rapid in unilaterally-denervated rats, possibly indicating growth of new axons from the intact side.

SURGERY	Control	Cold-acclimated	Cafeteria-fed
		3 days after surgery:	
		Tissue weights (mg)	
Sham-operated side	183 ± 15.2	205 ± 13.4	257 ± 19.2
Denervated side	233 ± 14.4	269 ± 32.0	288 ± 22.1
		Noradrenaline content (ng)	
Sham-operated side	258 ± 7.3	321 ± 19.7	178 ± 12.1
Denervated side	13 ± 1.0	10 ± 1.3	26 ± 4.1
		26 days after surgery:	
		Tissue weights (mg)	
Sham-operated side	230 ± 27.2	531 ± 44.9	404 ± 36.5
Denervated side	229 ± 30.5	219 ± 38.9	380 ± 55.1
		Noradrenaline content (ng)	
Sham-operated side	334 ± 28.8	477 ± 24.0	389 ± 40.9
Denervated side	25 ± 5.9	25 ± 5.6	26 ± 3.4

TABLE 23. DENERVATION STUDY: EFFECTIVENESS OF SURGICAL DENERVATION OF BROWN ADIPOSE TISSUE (STUDY 1).

Interscapular brown adipose tissue was unilaterally denervated and rats were then kept for 3 or 26 days under one of three treatments: standard holding conditions (CONTROL), acclimation to cold (4°C) (COLD-ACCLIMATED), or cafeteria-feeding (CAFETERIA-FED). Noradrenaline content of separated half-pads was determined by HPCL with electrochemical detection. Values shown are means ± S.E.M. for n = 5.

Three-way ANOVA was performed. It showed significant main effects of time after surgery and of treatment on wet weight of BAT; there was no significant effect of operation on this measurement. There were significant main effects of time, treatment, and surgery on the noradrenaline content of the half-pads.

TEMPERATURE	BILATERAL		UNILATERAL	
	Left	Right	Sham	Denervated
		2 weeks after surgery:		
		Tissue weights (mg)		
WA	241 ± 33.7	281 ± 26.1	195 ± 21.8	240 ± 39.0
CA	231 ± 36.2	244 ± 14.1	376 ± 8.5	205 ± 27.9
		Noradrenaline content (ng)		
WA	9 ± 1.7	8 ± 1.1	223 ± 19.8	16 ± 0.5
CA	8 ± 1.7	9 ± 1.5	335 ± 42.3	6 ± 2.2
		6 weeks after surgery:		
		Tissue weights (mg)		
WA	206 ± 25.9	196 ± 20.1	286 ± 51.5	280 ± 27.9
CA	341 ± 8.0*	374 ± 46.1*	560 ± 55.2	359 ± 29.6
		Noradrenaline content (ng)		
WA	19 ± 3.4	24 ± 2.7	332 ± 24.9	64 ± 10.2
CA	46 ± 12.6*	52 ± 15.2*	455 ± 32.6	96 ± 6.7

TABLE 24. DENERVATION STUDY: EFFECTIVENESS OF SURGICAL DENERVATION OF BROWN ADIPOSE TISSUE (STUDY 2).

Interscapular brown adipose tissue of rats was denervated, either unilaterally or bilaterally, as noted in the Table. Rats were then kept either under standard holding conditions (WA, warm-acclimated) or at 4°C (CA, cold-acclimated) for 2 or 6 weeks after surgery. Values shown are means ± S.E.M. for n = 4 (except as noted with *, where n = 3).

Three-way ANOVA was performed on the results. It showed that there were significant main effects of time after surgery, type of surgery (sham or denervated), and temperature of acclimation.

Conclusions: These results demonstrate that surgical denervation of BAT virtually abolishes its noradrenaline content and that this reduction of noradrenaline content persists for at least 26 days (longer than the time period used in the experiments reported below).

b) Comparison of unilateral and bilateral denervation on growth and function of brown adipose tissue in cold-exposed and cold-acclimated rats.

Objectives: The objective of these experiments was to further validate the equivalence of unilateral and bilateral denervation as shown in part a. A second objective was to investigate the effect of denervation on a number of indices of growth and function of BAT in animals acclimated to cold before or after surgery.

Design: Two experiments were done. In the first experiment, rats were grouped by body weights and growth rates at the time of operation. Twelve rats were bilaterally denervated, twelve were unilaterally denervated, and twelve were given sham denervations (anaesthetized only). Halothane was the anaesthetic. Half of each group was placed in the cold (4°C) for 12 days while the other half was kept under standard holding conditions for the same time. Rats were killed by decapitation and standard preparations made; half-pads of unilaterally-denervated rats were treated separately.

In the second experiment, rats were first grouped by body weights and growth rates. Two groups of twelve rats were cold-acclimated or kept in the warm. After two weeks, BAT of half of each of the two groups was bilaterally denervated while the other half of the rats received sham operations (anaesthetic only). Following surgery, rats were returned to their previous holding conditions. Fourteen days after surgery, rats were killed by decapitation and standard preparations made. (Results from unilaterally-denervated rats from the 14-day point of the second

experiment of part c were compared to the results of this part.) Statistics used are described in legends to Tables and Figures.

Results: i) Acclimation to cold after surgery. Results for body weights, body temperature, and gonadal white adipose tissue are given in Table 25. There were no significant effects of type of surgery or temperature of exposure on initial body weights, body weights at operation, or the change in body weight in this time period (data not shown). After the denervations, there was an effect of surgery on body weights (see change in body weight) for warm-exposed rats but not cold-exposed rats. Surgery affected rats having unilateral lesions and bilateral lesions to the same extent. As always, the growth rate of cold-exposed rats was lower than in warm-exposed rats.

Surgery had no effect on thermoregulation (as judged by the body temperatures) or wet weight of gonadal white fat. (Cold-exposure reduced wet weight of gonadal white adipose tissue, as expected.)

Results for brown adipose tissue are presented in Table 26. In warm-acclimated rats, denervation had little effect on any indices measured. In rats acclimated to cold after surgery, denervation reduced but did not eliminate the usual effect of cold. Thus protein levels are half those found in BAT having intact innervation, but still twice the levels found in warm-acclimated rats. GDP binding was also half the normal value but 4-5 times the warm-acclimated level. T5'D was much more dependant on sympathetic innervation yet was also 5-6 times the level found in warm-acclimated rats. Specific UCP levels, again less than half those seen in BAT having

Ambient temperature	Sham operated	Unilateral denervation	Bilateral denervation
		Body weight (g)	
		At surgery	
WA	252 ± 4.7	241 ± 4.1	241 ± 7.0
CA	245 ± 3.3	243 ± 1.9	239 ± 4.5
		Final	
WA	329 ± 3.3	309 ± 5.7	302 ± 4.1
CA	284 ± 2.8	280 ± 4.3	271 ± 9.4
		Change	
WA	87 ± 7.0	68 ± 5.4	61 ± 7.7
CA	39 ± 3.4	37 ± 4.7	33 ± 5.9
		Body temperature (°C)	
WA	37.5 ± 0.15	37.5 ± 0.08	37.0 ± 0.12
CA	37.2 ± 0.22	37.1 ± 0.26	37.0 ± 0.32
		Gonadal white adipose tissue (g)	
WA	2.7 ± 0.18	2.6 ± 0.12	2.7 ± 0.17
CA	1.7 ± 0.10	1.6 ± 0.09	1.5 ± 0.15

TABLE 25. COMPARISON OF UNILATERAL WITH BILATERAL DENERVATION: WHOLE-ANIMAL RESULTS.

Rats were subjected to one of three treatments: (surgical) bilateral denervation of interscapular brown adipose tissue (IBAT), unilateral denervation of IBAT, or sham operation. They were then kept under standard holding conditions (**WA**, warm-acclimated) or exposed to cold (4°C) for 12 days (**CA**, cold-acclimated). Results shown are means ± S.E.M. for n = 6.

Two-way ANOVA was performed on the results. There were no differences in body weight between any groups at the time of operation. There were significant main effects of surgery and of temperature of exposure on both final body weight and change of body weight. There were no differences in body temperature between any groups. There was a significant main effect of temperature of exposure, but not of surgery, on wet weight of white adipose tissue.

Ambient temperature	Sham operated	Unilateral (sham side)	Unilateral (denervated)	Bilateral denervation
		Wet weight (mg)		
WA	475 ± 26.8	225 ± 18.9	231 ± 13.6	455 ± 20.8
CA	837 ± 51.7	435 ± 47.5	230 ± 11.4	459 ± 32.8
		Protein content (mg)		
WA	21.9 ± 3.37	9.4 ± 0.82	9.7 ± 1.42	18.8 ± 2.06
CA	88.4 ± 9.87	39.1 ± 1.67	17.7 ± 1.05	39.4 ± 8.12
		GDP binding (pmol/mg)		
WA	50 ± 7.5	61 ± 6.3	44 ± 3.3	35 ± 4.7
CA	456 ± 26.0	523 ± 48.9	257 ± 56.3	268 ± 26.9
		T_4 5'-deiodinase (pmol/h)		
WA	2.2 ± 0.13	0.9 ± 0.07	1.2 ± 0.16	1.5 ± 0.13
CA	94.4 ± 4.90	44.0 ± 2.78	5.5 ± 1.05	10.4 ± 1.17
		Specific UCP (nmol/mg)		
WA	0.44 ± 0.028	0.42 ± 0.034	0.30 ± 0.022	0.37 ± 0.028
CA	2.24 ± 0.066	2.21 ± 0.099	0.98 ± 0.062	0.99 ± 0.051

TABLE 26. COMPARISON OF UNILATERAL WITH BILATERAL DENERVATION: BROWN ADIPOSE TISSUE.

Rats were treated and statistical analyses were performed as described in the legend to Table 25.

Two-way ANOVA showed that there were significant main effects of both operation and temperature of acclimation on all indices reported in this Table.

intact innervation, were nevertheless 2-3 times the levels found in warm-acclimated rats.

These results (Table 26) demonstrate the equivalence of unilateral and bilateral denervation. Values for half-pads having intact innervation are almost exactly half those for sham-operated rats (where both sides of the pad were combined and the preparation made of the whole pad); likewise, results for denervated half-pads were almost exactly half those for bilaterally-denervated rats (again, here both sides of the BAT pad were prepared together). For specific measurements (specific GDP binding and specific UCP levels) the results are directly comparable (sham-operated BAT to tissue having intact innervation in unilaterally-denervated rats, and bilaterally-denervated BAT to unilaterally-denervated rats). Two-way ANOVA showed no significant effect of type of surgery (unilateral versus bilateral denervation) for any index measured; sham-operated tissues were also not different from one another.

Conclusions: For the indices of BAT function tested here, unilateral denervation was equivalent to bilateral denervation and, therefore, unilateral denervation is a valid experimental design. Furthermore, these findings confirm the previous findings (from analysis of tissue noradrenaline content after denervation; see part a above) that there is no cross-innervation of BAT. In rats placed in the cold after surgery, denervation reduced but did not prevent the cold-induced increases in the indices of BAT function measured here.

ii) Acclimation to cold before surgery. In the second experiment, the effects of bilateral denervation were compared to those of sham-operation and of unilateral denervation in rats previously warm- or cold-acclimated. Rats were studied 14 days after denervation.

Results for body weights and changes of body weights are given in Table 27. As expected, cold-acclimation reduced the growth rate of rats (change - period 1). Continued exposure to cold after surgery did not cause a further difference in growth rate (change - period 2). Rats having unilateral denervation had a greater growth rate in period 2 than did the other two groups. This was because the results reported here for unilaterally-denervated rats are repeated from the first part of this experiment (the 14-day point) where (for logistical reasons) faster-growing rats had been used.

Results for body temperatures and tissue wet weights are presented in Table 28. Neither surgery nor cold-acclimation significantly affected thermoregulation (as indicated by body temperature). Likewise, there was no significant main effect of surgery on the wet weight of gonadal white fat; as before, cold-acclimation significantly reduced the wet weight of gonadal white fat.

Results for brown adipose tissue are shown in Table 29. The results were very similar to those presented above for rats not previously acclimated to cold (Table 26). As before, these results demonstrate the equivalence of unilateral and bilateral surgical denervation under this experimental protocol. Results of wet weight of BAT (Table 28), total protein, and total thyroxine 5'-deiodinase activity for half-pads having either intact or severed innervation were almost exactly half of those for

Ambient temperature	Sham operated	Unilateral denervation	Bilateral denervation
		Body weight (g)	
		Initial	
WA	134 ± 1.9	138 ± 3.6	131 ± 2.2
CA	130 ± 4.0	137 ± 3.3	131 ± 2.6
		At surgery	
WA	285 ± 5.9	286 ± 2.7	290 ± 4.8
CA	249 ± 5.0	257 ± 3.8	253 ± 2.5
		Final	
WA	350 ± 9.0	379 ± 2.7	360 ± 7.6
CA	305 ± 5.7	348 ± 6.0	311 ± 2.6
		Change - Period 1	
WA	151 ± 6.5	149 ± 2.4	158 ± 5.8
CA	120 ± 7.5	120 ± 5.5	122 ± 4.4
		Change - Period 2	
WA	65 ± 3.5	94 ± 4.1	71 ± 3.6
CA	56 ± 3.7	91 ± 4.7	57 ± 0.9

TABLE 27. COMPARISON OF UNILATERAL WITH BILATERAL DENERVATION IN RATS PREVIOUSLY ACCLIMATED TO COLD: BODY WEIGHTS AND CHANGES.

Rats were first kept either under standard holding conditions (WA, warm-acclimated), or placed in the cold (4°C) for two weeks (CA, cold-acclimated), then subjected to one of three treatments: bilateral denervation of interscapular brown adipose tissue, unilateral denervation of IBAT, or sham-operation. The rats were then maintained as before for 14 days. Results shown are means ± S.E.M. for n = 6. Period 1 refers to the time between the beginning of treatment (warm- or cold-acclimation) to surgery; period 2 is the time from surgery to the end of the experiment.

Results were analyzed by two-way ANOVA. There were no significant main effects of temperature of acclimation or of type of surgery on initial body weight. At operation (and for change of weight during period 1) there was no effect of type of surgery but there was an effect of temperature of acclimation. There were significant main effects of both type of surgery and temperature of acclimation on final body weight and change in body weight during period 2.

Ambient temperature	Sham operated	Unilateral denervation	Bilateral denervation
	Body temperature (°C)		
WA	37.5 ± 0.18	37.1 ± 0.20	37.2 ± 0.21
CA	37.3 ± 0.27	36.7 ± 0.19	37.6 ± 0.25
	Gonadal white adipose tissue (g)		
WA	3.2 ± 0.16	3.6 ± 0.23	3.0 ± 0.27
CA	1.5 ± 0.06	1.9 ± 0.22	2.1 ± 0.05

	Brown adipose tissue (mg)			
	Sham operated	Unilateral (sham side)	Unilateral (denervated)	Bilateral denervation
WA	547 ± 24.6	297 ± 11.6	293 ± 11.8	592 ± 38.5
CA	977 ± 50.0	622 ± 33.0	451 ± 34.2	858 ± 28.1

TABLE 28. COMPARISON OF UNILATERAL WITH BILATERAL DENERVATION IN RATS PREVIOUSLY ACCLIMATED TO COLD: BODY TEMPERATURES AND TISSUE WET WEIGHTS.

Rats were treated and the statistical analyses were performed as described in the legend to Table 27.

Results were analyzed by ANOVA. Two-way ANOVA showed that neither type of operation nor temperature of acclimation affected body temperatures. There was a significant effect of temperature of acclimation on the wet weight of gonadal white adipose tissue: there was no effect of type of operation. Both type of surgery and temperature of acclimation had significant main effects on the wet weight of BAT. When sham operations were compared alone (unilateral sham operation versus rats receiving anaesthetic only), these main effects persisted. When denervated pads were compared alone (unilateral versus bilateral denervation) there was no main effect of type of surgery: there was an effect of the temperature of acclimation.

Ambient temperature	Sham operated	Unilateral (sham side)	Unilateral (denervated)	Bilateral denervation
		Protein (mg)		
WA	17.0 ± 1.57	8.1 ± 0.99	6.2 ± 0.55	18.8 ± 1.91
CA	109 ± 13.2	53.9 ± 1.31	23.1 ± 1.09	47.4 ± 6.00
		GDP binding (pmol/mg)		
WA	53 ± 10.2	49 ± 3.2	37 ± 3.0	38 ± 3.7
CA	376 ± 26.5	376 ± 20.0	64 ± 3.3	95 ± 16.0
		T_4 5'-deiodinase (pmol/h)		
WA	1.9 ± 0.15	1.0 ± 0.05	0.9 ± 0.05	2.0 ± 0.18
CA	93.4 ± 6.66	51.1 ± 4.15	1.1 ± 0.15	6.2 ± 0.99
		Specific UCP (pmol/mg)		
WA	14.5 ± 1.28	13.5 ± 0.56	10.5 ± 0.59	11.2 ± 0.92
CA	70.0 ± 2.93	75.9 ± 4.16	20.8 ± 1.13	17.5 ± 1.86

TABLE 29. COMPARISON OF UNILATERAL WITH BILATERAL DENERVATION IN RATS PREVIOUSLY ACCLIMATED TO COLD: BROWN ADIPOSE TISSUE.

Rats were treated and the statistical analyses were performed as described in the legend to Table 27.

Results were analyzed by two-way ANOVA. Results from rats receiving sham operations were compared with those from the half-pad having intact innervation of unilaterally-denervated rats; likewise, results from rats receiving bilateral denervations were compared to those from rats having unilateral denervation of BAT. For all indices reported in this Table, there was a significant effect of temperature of acclimation. Unilateral denervation was equivalent to bilateral denervation (i.e., no significant main effect) for all indices measured except for thyroxine 5'-deiodinase activity, where there was a significant difference between the unilaterally- and bilaterally-denervated groups.

the corresponding whole-pad results (i.e., for sham-operated or bilaterally-denervated rats); for specific GDP binding and specific uncoupling protein, results for tissues with intact innervation, or severed innervation, are very similar no matter whether the surgery was unilateral or bilateral.

Conclusions: As before, for the indices of BAT function measured here, unilateral denervation was equivalent to bilateral denervation. Again, the independence of innervation to the two sides of the interscapular depot of brown adipose tissue has been confirmed.

c) Time course of changes in denervated brown adipose tissue in cold-acclimation of rats and in cold-acclimated rats.

Objective: The objective of this study was to continue the work described in part b by following the time course of the changes following unilateral denervation of BAT.

Design: Two experiments were performed. In the first, rats were exposed to cold after denervation and changes were studied as a time course. In the second, changes in rats subjected to denervation after previous cold-acclimation was studied to demonstrate the importance of the innervation in maintaining the cold-induced trophic changes in BAT.

In the first experiment, 48 rats weighing 200 to 230 g were grouped on the basis of equal growth rates, with animals grouped to minimize differences in body weights at the times of operations and sacrifice. Rats were unilaterally denervated under halothane anaesthesia as described in the Materials and Methods (section 2b). Half were then placed in the cold (4°C) and the other half were kept under standard holding conditions. Rats were killed by decapitation 1, 3, 6, or 12 days after surgery (four rats per preparation, two of each of the warm-exposed and cold-exposed groups at each time point, with preparations staggered over three days to give a total of six animals per treatment for each of the four time points) and standard preparations made. Half-pads were prepared separately.

The second experiment was similar to the first except surgery was performed after acclimation to cold. Groups of 24 rats weighing 120 to 150 g were grouped by body weight and growth rate and given one of two

treatments for two weeks: cold-exposure (4°C), or standard holding conditions. Unilateral denervations were then performed under halothane anaesthesia. Rats were killed by decapitation 1, 3, 7, or 14 days after surgery (preparations were staggered as described for the previous study in part c) and standard preparations made. Half-pad were treated separately. Statistics used are described in the legends to the Tables and Figures presented below.

Results: i) Acclimation to cold after surgery. Results for body weights and growth rates of rats for the first part of the first experiment (acclimation to cold in rats having unilaterally-denervated BAT) are given in Table 30. Body weights at surgery for the 1-day and 12-day groups were lower than those for the other two groups because surgery was performed first on the 1-day and 12-day groups for logistical reasons. More importantly, the growth rates of all groups before surgery were not statistically different from one another (data not shown). After surgery, growth rates for warm-acclimated rats continued to be similar to one another; growth rates for the cold-exposed rats were considerably less than for the warm-acclimated rats, as expected.

Results for body temperatures and tissue wet weights are given in Table 31. Cold-exposed rats were able to thermoregulate normally, as shown by the mean body temperatures. (There was a significant main effect of temperature of exposure on body temperature, by two-way ANOVA, but the means were very little different from one another (mean values 37.7°C for warm-acclimated rats versus 37.2°C for cold-acclimated

Temperature	1 Day	3 Days	6 Days	12 Days
		Body weights (g)		
		At surgery		
WA	209 ± 2.3	224 ± 2.0	224 ± 3.8	209 ± 4.1
CE	208 ± 1.2	223 ± 2.3	224 ± 2.6	209 ± 2.8
		Final		
WA	223 ± 1.8	254 ± 2.7	275 ± 4.0	315 ± 5.6
CE	213 ± 3.1	230 ± 2.4	242 ± 4.4	262 ± 10.3
		Change		
WA	14 ± 1.3	31 ± 2.1	52 ± 2.1	106 ± 2.0
CE	5 ± 2.1	7 ± 1.8	19 ± 5.3	54 ± 9.3
		Growth rate (g/d)		
WA	13.8 ± 1.28	10.2 ± 0.71	8.6 ± 0.35	8.8 ± 0.17
CE	4.5 ± 2.14	2.3 ± 0.59	3.1 ± 0.88	4.5 ± 0.77

TABLE 30. DENERVATION TIME-COURSE STUDY (1): CHANGES IN BODY WEIGHTS OF RATS EXPOSED TO COLD.

Rats were subjected to unilateral denervation (surgical) of their interscapular brown adipose tissue, then held under standard holding conditions (WA, warm-acclimated) or exposed to cold (4°C) (CE, cold-exposed) for 1, 3, 6, or 12 days. The values shown are means ± S.E.M. for n = 6.

Two-way ANOVA showed that there were differences in body weights at the time of surgery (these are explained in the text). Rats that were put into the cold did not have different body weights at the time of surgery compared to the corresponding rats kept in the warm. ANOVA also showed that there were significant main effects of time after surgery and temperature of exposure on final body weight, on change of body weight after surgery, and on growth rates post-surgery.

Temperature	1 Day	3 Days	6 Days	12 Days
		Body temperature (°C)		
WA	37.5 ± 0.22	37.3 ± 0.11	37.9 ± 0.22	37.9 ± 0.21
CE	37.6 ± 0.20	37.0 ± 0.17	37.1 ± 0.34	37.2 ± 0.19
		Gonadal white fat (g)		
WA	1.5 ± 0.06	1.8 ± 0.11	2.1 ± 0.06	2.7 ± 0.13
CE	1.3 ± 0.08	1.5 ± 0.07	1.4 ± 0.06	1.3 ± 0.10
		Brown adipose tissue (mg)		
WA				
Sham	171 ± 11.4	192 ± 10.5	219 ± 11.5	233 ± 16.4
Denervated	189 ± 7.5	173 ± 5.1	226 ± 8.0	247 ± 12.9
CE				
Sham	141 ± 9.3	186 ± 12.2	232 ± 25.2	392 ± 47.8
Denervated	180 ± 10.8	189 ± 11.1	205 ± 25.4	360 ± 57.0

TABLE 31. DENERVATION TIME-COURSE STUDY (1): BODY TEMPERATURES AND TISSUE WET WEIGHTS IN RATS EXPOSED TO COLD.

Rats were treated and statistical analyses preformed as described in the legend to Table 30.

Two-way ANOVA showed that there was no significant effect of time after surgery on body temperature; there was an effect of temperature of exposure. There were significant main effects of both time and temperature on the wet weight of gonadal white adipose tissue.

Three-way ANOVA was performed on the results for wet weight of brown adipose tissue. There were significant main effects of temperature of exposure and of time after surgery. There was no effect of surgery (denervated side versus sham-operated side) of wet weight of BAT.

rats). By one-way ANOVA (Duncan's post-hoc test), only the highest two temperatures were different from the lowest two.)

Wet weights of gonadal white adipose tissue increased with longer treatment times in warm-acclimated rats but not in cold-acclimated ones. This increase in size was likely due to the continuing growth of the rats in the warm.

In warm-acclimated rats, wet weight of BAT was relatively unaffected by denervation. The expected increase in wet weight of BAT half-pads having intact innervation in cold-exposed rats did occur; this increase was paralleled in the denervated side.

Results for other indices of BAT function are shown in Figures 24, 25, 26, and 27. In warm-acclimated rats, neither denervation nor time of treatment post-surgery affected the indices measured and these rats will not be described further.

In cold-exposed rats, the time-course of changes of total protein in BAT half-pads having intact innervation is presented in Figure 24. As expected, there was a significant increase within three days, which increased with further cold-exposure. Denervation prevented the cold-induced increase in protein for 1, 3, and 6 days post-surgery, but by 12 days there was a significant increase in protein content of BAT, long before there could have been regrowth of sympathetic nerves (see Results section B4a).

GDP binding increased within one day for BAT half-pads having intact innervation; the levels approached their maximum by 3 days (Figure 25). In denervated half-pads, there was a significant increase in GDP binding within one day (albeit smaller than the increase seen in half-pads having

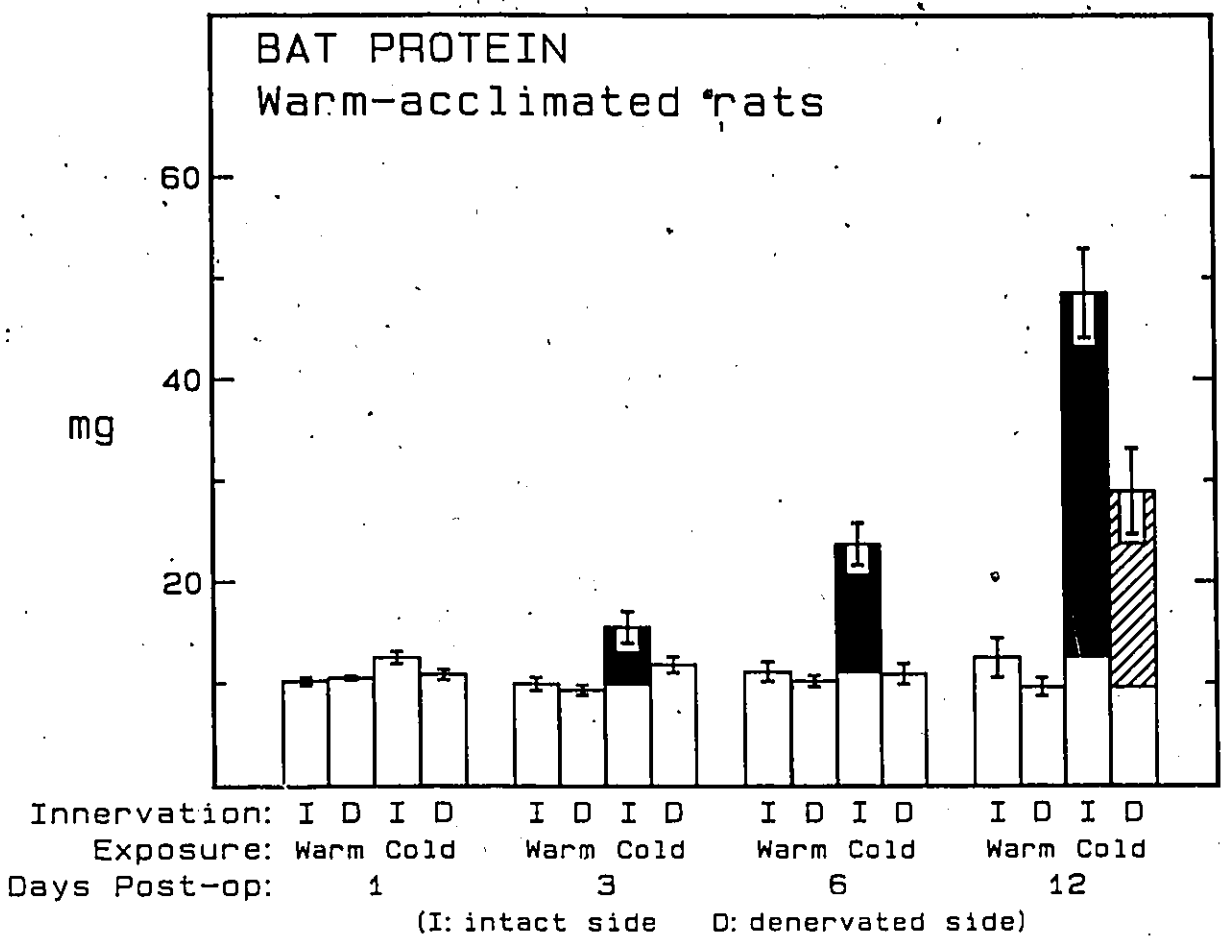

FIGURE 24. DENERVATION TIME-COURSE STUDY (1): PROTEIN OF RATS EXPOSED TO COLD.

Rats were treated and statistical analyses were performed as described in the legend to Table 30. Bars represent means for n = 6, with error bars representing S.E.M. Black or hatched areas in bars indicate a significant increase over tissue of warm-acclimated rats having the same surgical treatment (denervation or intact innervation). These comparisons were made by Student's unpaired t-test with $p < 0.05$ or smaller for all differences indicated as being significant.

Three-way ANOVA was also performed. It showed that there were significant main effects for time after surgery, temperature of exposure, and surgery (intact versus denervated).

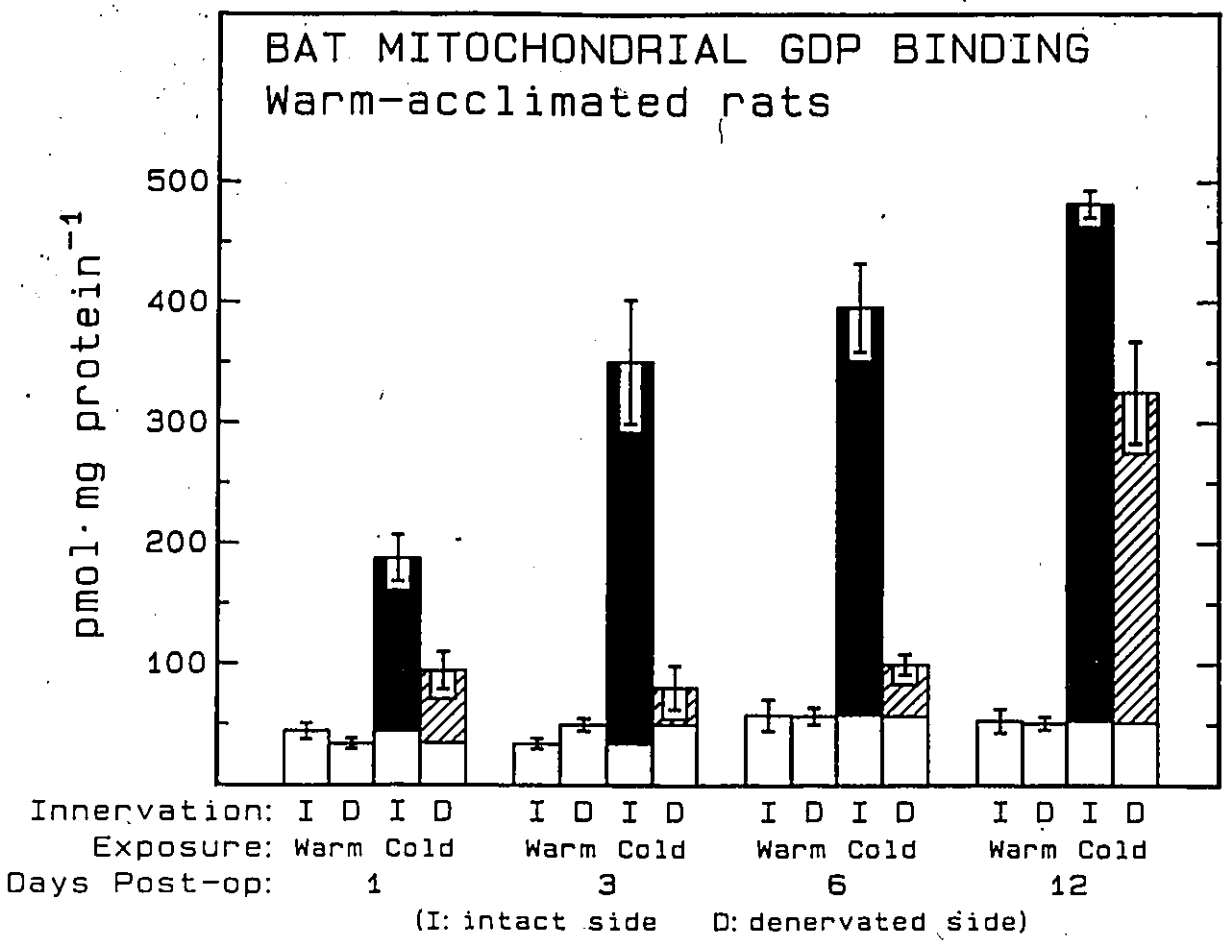

FIGURE 25. DENERVATION TIME-COURSE STUDY (1): GDP BINDING OF RATS EXPOSED TO COLD.

Rats were treated and the statistical analyses were performed as described in the legend to Table 30. The format of the Figure is as described in the legend to Figure 24.

Three-way ANOVA was also performed. It showed that there were significant main effects for time after surgery, temperature of exposure, and surgery (intact versus denervated).

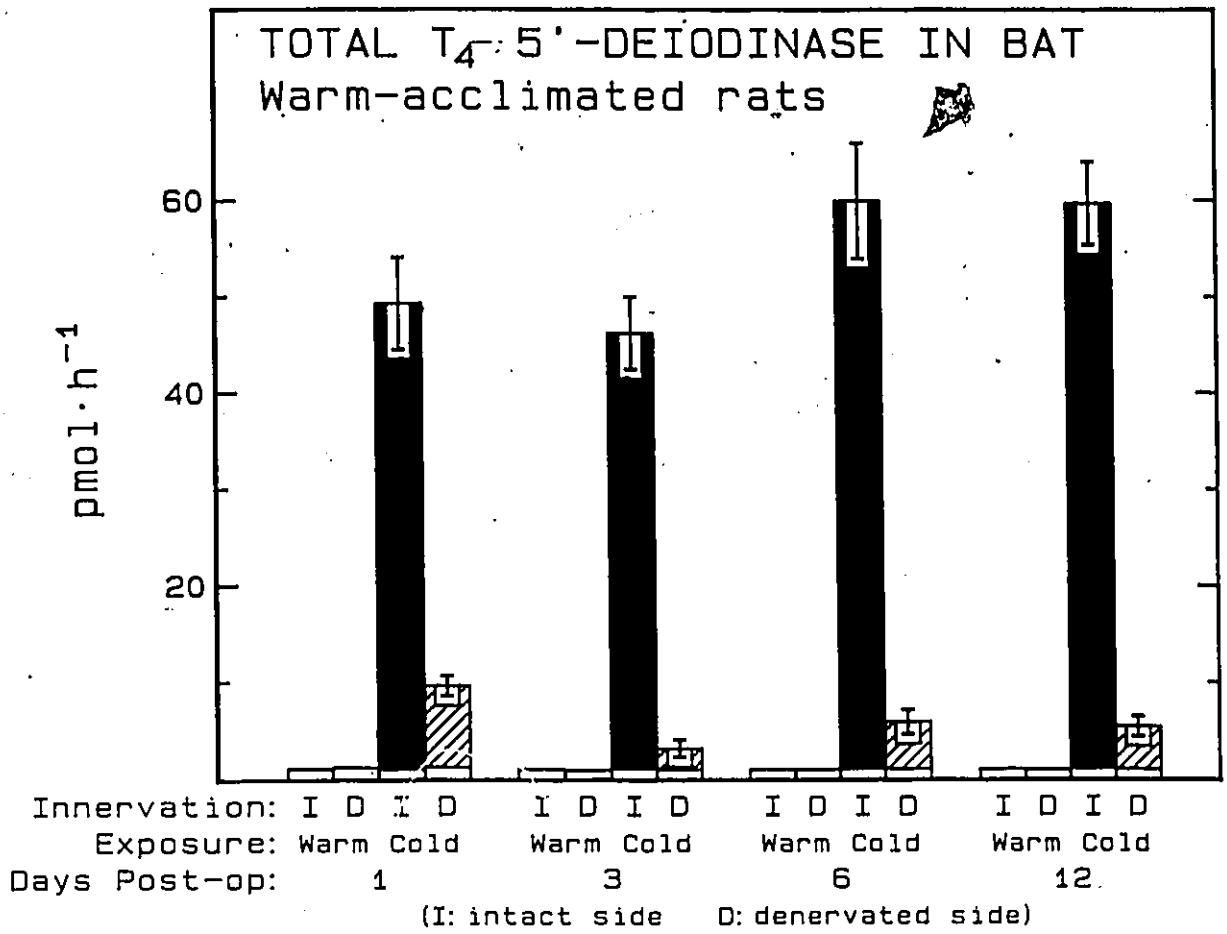

FIGURE 26. DENERVATION TIME-COURSE STUDY (1): THYROXINE 5'-DEIODINASE ACTIVITY OF RATS EXPOSED TO COLD.

Rats were treated and the statistical analyses were performed as described in the legend to Table 30. The format of the Figure is as described in the legend to Figure 24.

Three-way ANOVA was also performed. It showed that there were significant main effects for temperature of exposure and surgery (intact versus denervated); there was no significant effect of time after surgery.

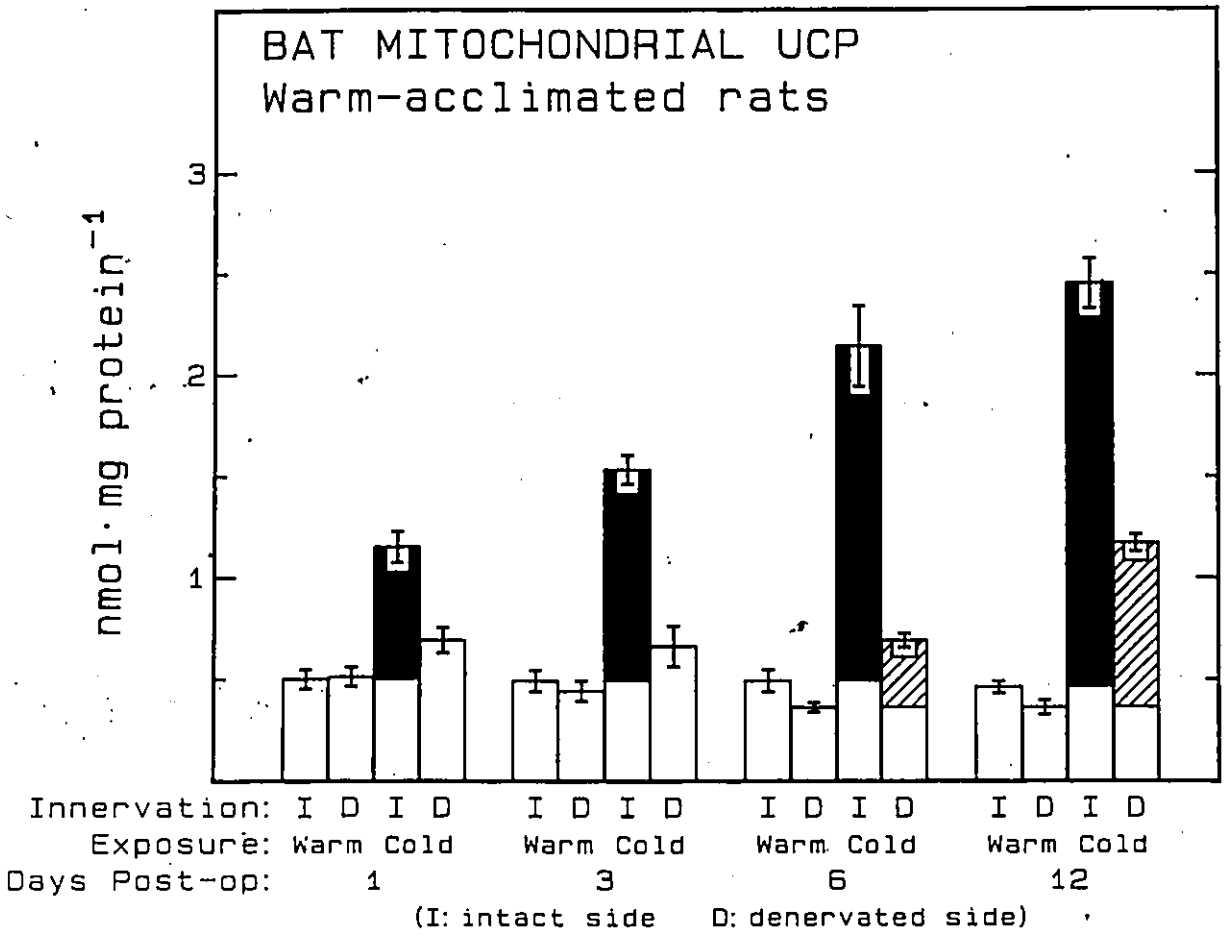

FIGURE 27. DENERVATION TIME-COURSE STUDY (1): SPECIFIC UNCOUPLING PROTEIN OF RATS EXPOSED TO COLD.

Rats were treated and the statistical analyses were performed as described in the legend to Table 30. The format of the figure is as described in the legend to Figure 24.

Three-way ANOVA was also performed. It showed that there were significant main effects for time after surgery, temperature of exposure, and surgery (intact versus denervated).

intact innervation). By the twelfth day post-surgery, there was a large increase in GDP binding, paralleling the increase in protein content.

In contrast to the results for protein content and GDP binding, the cold-induced increase in the activity of thyroxine 5'-deiodinase (T5'D) was almost totally dependant upon intact sympathetic innervation (Figure 26). Cold-exposure caused maximal increase within one day in BAT half-pads having intact innervation, as previously found (Results section B1). Cold-exposure caused a statistically significant increase in activity of T5'D in denervated half-pads, but these increased levels were very much smaller than those in half-pads with intact innervation; indeed, maximal levels in denervated half-pads were achieved in the first day.

Figure 27 shows the pattern of increase in specific levels of the uncoupling protein (UCP) (nmol UCP per mg mitochondrial protein). The pattern was similar to that seen for GDP binding and protein content. In half-pads having intact innervation, there was a steady increase in UCP content. In denervated half-pads, there was a small increase by 6 days and a larger one by 12 days.

Conclusions: The time-courses of the cold-induced changes in BAT protein, GDP binding, T5'D, and UCP (as seen in half-pads having intact innervation) were not the same, with T5'D increasing to maximum within one day while GDP binding rapidly increased to reach a maximum by three days. The greatest increase in protein was after six days, while the increase in UCP content of mitochondria was steady over the 12-day period.

The importance of sympathetic innervation in acclimation to cold was demonstrated by the effect of denervation. The cold-induced increase in T5'D activity was almost entirely dependent upon sympathetic innervation, while the increases in the other three indices were reduced but not eliminated by sympathetic denervation (as previously found; see Results section B4b). In particular, there seems to be some process which begins after 6 days and which allows increases of UCP, GDP binding, and protein to approach appreciable fractions of those seen in half-pads having intact innervation.

ii) Acclimation to cold before surgery. In this second experiment, rats were first cold-acclimated (or warm-acclimated) for two weeks, then subjected to denervation and studied 1, 3, 7, or 14 days after surgery.

Results for body weights are given in Table 32; results for changes in body weight and for growth rates in Table 33. Not surprisingly, rats that had first been cold-acclimated weighed less at the time of surgery than did warm-acclimated rats; they also had slightly smaller growth rates during this time ("period 1"). After surgery, growth rates of cold- and warm-acclimated rats were the same (period 2), so the difference in body weights at the time of surgery was maintained until the end of the experiment.

Results for body temperatures and tissue wet weights are given in Table 34. The temperature of acclimation did not affect thermogenesis (as indicated by body temperature). As usual, cold-acclimation reduced the wet weight of gonadal white adipose tissue; in both warm- and cold-acclimated

Temperature	1 Day	3 Days	7 Days	14 Days
		Body weights (g)		
		Initial		
WA	141 ± 1.9	141 ± 0.9	136 ± 1.3	138 ± 3.6
CA	145 ± 2.4	142 ± 1.3	136 ± 1.5	137 ± 3.3
		At surgery		
WA	294 ± 2.8	296 ± 4.9	287 ± 5.3	286 ± 2.7
CA	264 ± 4.5	270 ± 2.9	260 ± 3.2	257 ± 3.8
		Final		
WA	308 ± 3.7	318 ± 4.8	336 ± 6.3	379 ± 2.7
CA	281 ± 3.9	292 ± 4.7	299 ± 6.1	348 ± 6.0

TABLE 32. DENERVATION TIME-COURSE STUDY (2): BODY WEIGHTS OF RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were acclimated to cold (4°C) for two weeks (**CA**, cold-acclimated), or kept under standard holding conditions (**WA**, warm-acclimated), then subjected to unilateral denervation of interscapular brown adipose tissue. They were then studied 1, 3, 7, or 14 days after surgery (being maintained under the same treatment conditions as previously). Values shown are means ± S.E.M. for n = 6.

Results were analyzed by two-way ANOVA. There was a significant effect of time after operation on initial body weight because operations had to be staggered for logistical reasons. There was no significant effect of treatment on initial body weight. There were significant main effects of both time after surgery and treatment on the body weight at surgery and the final body weight.

Temperature	1 Day	3 Days	7 Days	14 Days
		Change in body weight (g)		
		Period 1		
WA	153 ± 4.1	156 ± 5.6	151 ± 6.2	149 ± 2.4
CA	120 ± 6.2	129 ± 4.0	125 ± 4.2	120 ± 5.5
		Period 2		
WA	14 ± 1.9	22 ± 3.0	49 ± 2.2	93 ± 4.1
CA	16 ± 2.1	22 ± 2.6	38 ± 4.7	91 ± 4.7
		Growth rates (g/d)		
		Period 1		
WA	8.0 ± 0.09	7.1 ± 0.26	6.9 ± 0.19	7.8 ± 0.19
CA	6.3 ± 0.29	5.9 ± 0.17	5.6 ± 0.13	6.3 ± 0.25
		Period 2		
WA	13.8 ± 1.85	7.3 ± 0.99	6.9 ± 0.31	6.6 ± 0.29
CA	16.3 ± 2.12	7.3 ± 0.85	5.5 ± 0.68	6.5 ± 0.34

TABLE 33. DENERVATION TIME-COURSE STUDY (2): CHANGES OF BODY WEIGHTS AND GROWTH RATES OF RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were performed as described in the legend to Table 32. Period 1 refers to the time between the beginning of treatment (warm- or cold-acclimation) and surgery; period 2 is the time between the surgery and the end of the experiment.

Analysis by two-way ANOVA showed that neither change in body weight nor growth rate was significantly affected by the treatment. During period 1, there was a significant effect of time after surgery on change of body weight but not on growth rate. During period 2, there was no effect of time after surgery on change of body weight, but there was a significant effect on growth rate.

Temperature	1 Day	3 Days	7 Days	14 Days
		Body temperature (°C)		
WA	37.5 ± 0.19	37.0 ± 0.10	36.9 ± 0.11	37.1 ± 0.20
CA	37.5 ± 0.23	37.4 ± 0.24	37.1 ± 0.24	36.7 ± 0.19
		Gonadal white fat (g)		
WA	2.2 ± 0.12	2.2 ± 0.14	2.5 ± 0.18	3.6 ± 0.23
CA	1.2 ± 0.11	1.3 ± 0.15	1.6 ± 0.14	1.9 ± 0.22
		Brown adipose tissue (mg)		
WA				
Sham	262 ± 22.6	249 ± 18.6	299 ± 13.5	297 ± 11.6
Denervated	322 ± 24.1	308 ± 18.6	309 ± 8.6	293 ± 11.8
CA				
Sham	387 ± 24.6	455 ± 12.3	541 ± 22.2	622 ± 33.0
Denervated	425 ± 24.1	390 ± 18.2	419 ± 19.3	451 ± 34.2

TABLE 34. DENERVATION TIME-COURSE STUDY (2): BODY TEMPERATURES AND TISSUE WET WEIGHTS IN RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were performed as described in the legend to Table 32.

Results were analyzed by ANOVA. Two-way ANOVA showed that body temperature was significantly affected by time after surgery but not by type of treatment. The wet weight of gonadal white fat was significantly affected by both time and treatment.

Wet weight of BAT was analyzed by three-way ANOVA. This showed that there were significant main effects of time after surgery, treatment, and operation (sham-operation or denervation).

rats, the gonadal white fat continued to grow with time after operation as the rats continued to grow.

As in the first experiment, denervation had little effect on wet weight of BAT in warm-acclimated rats. In cold-acclimated rats, BAT half-pads having intact innervation continued to show increases in wet weight for the duration of the experiment. Wet weights of denervated BAT in cold-acclimated rats did not increase, but neither did they decrease to the levels seen in the warm-acclimated rats.

Results for the other indices of BAT function are shown in Figures 28, 29, 30, and 31. As in the first half of the study, neither denervation nor time of treatment after surgery affected the indices measured in warm-acclimated rats and thus these results will not be described further.

In general, in cold-acclimated rats, indices of BAT function did not change in the half-pads having intact innervation; rather, they were maintained at fully cold-acclimated levels for the duration of the experiment. Total protein levels in BAT increased slowly (Figure 28) as the tissue continued to grow (protein content increased in concert with the increases in wet weight of BAT shown in Table 34). Specific GDP binding had declined slightly by 7 and 14 days after surgery (Figure 29) but this change was small. The total activity of thyroxine 5'-deiodinase (T5'D) (Figure 30) and the specific uncoupling protein (UCP) levels (Figure 31) were almost unchanged.

The importance of sympathetic innervation in maintaining the trophic changes in BAT caused by cold-acclimation can be seen from the results for denervated half-pads of the cold-acclimated rats. Total protein content declined within a day of surgery (Figure 28) but did not decline much

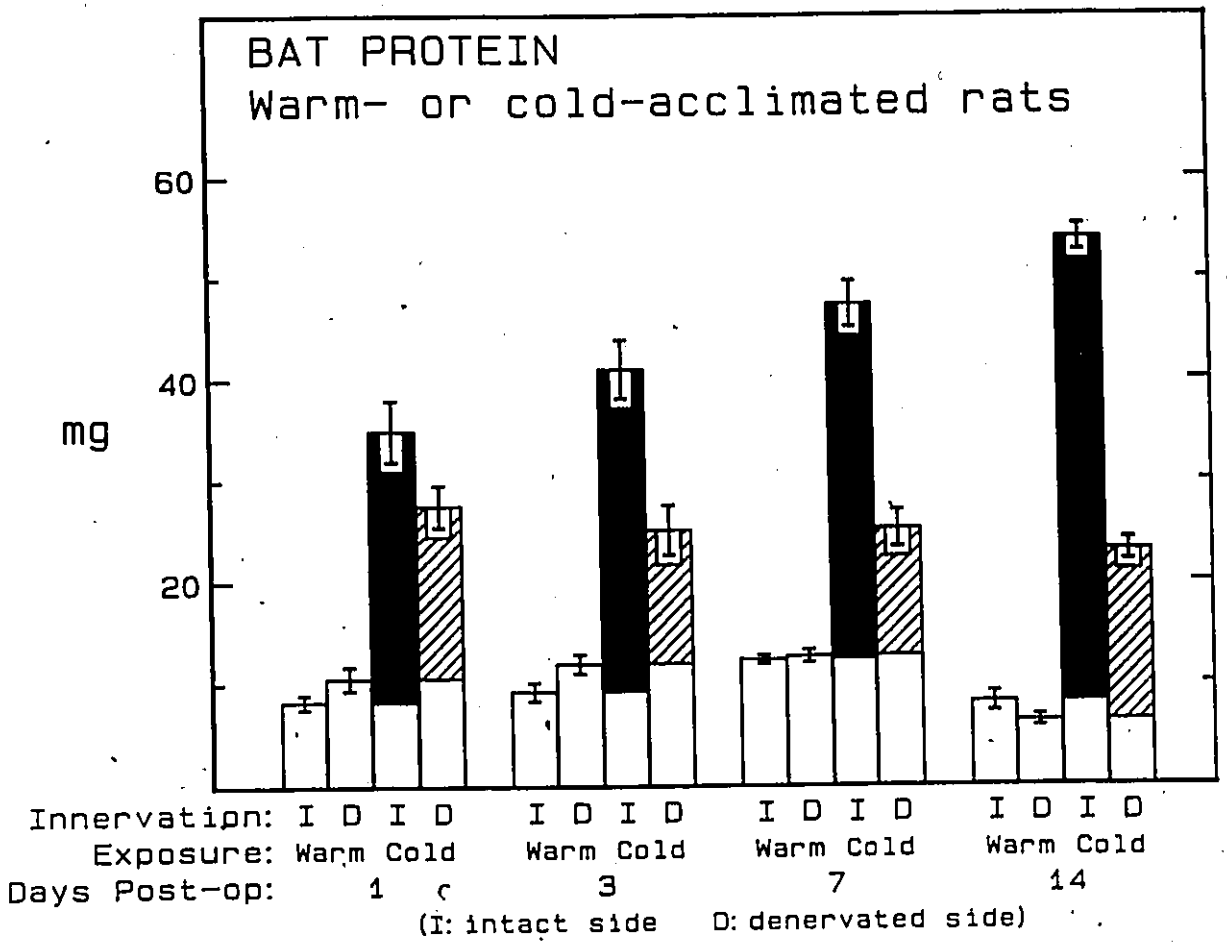

FIGURE 28. DENERVATION TIME-COURSE STUDY (2): PROTEIN IN RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were preformed as described in the legend to Table 32. The format of the Figure is as described in the legend to Figure 24.

Three-way ANOVA showed significant main effects of treatment (warm- or cold-acclimation), time after surgery, and operation (sham-operation or denervation).

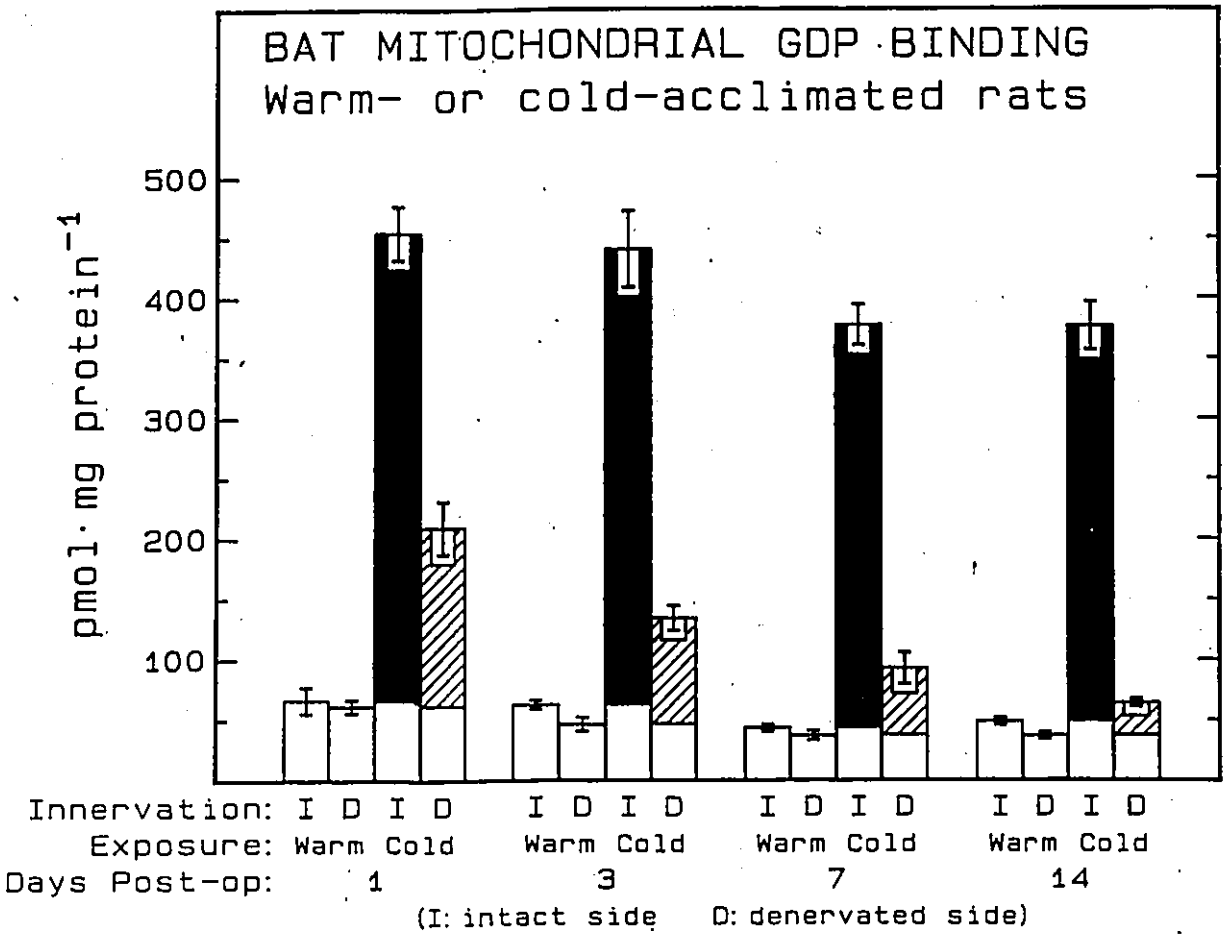

FIGURE 29. DENERVATION TIME-COURSE STUDY: GDP BINDING IN RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were preformed as described in the legend to Table 32. The format of the Figure as is described in the legend to Figure 24.

Three-way ANOVA showed significant main effects of treatment (warm- or cold-acclimation), time after surgery, and operation (sham-operation or denervation).

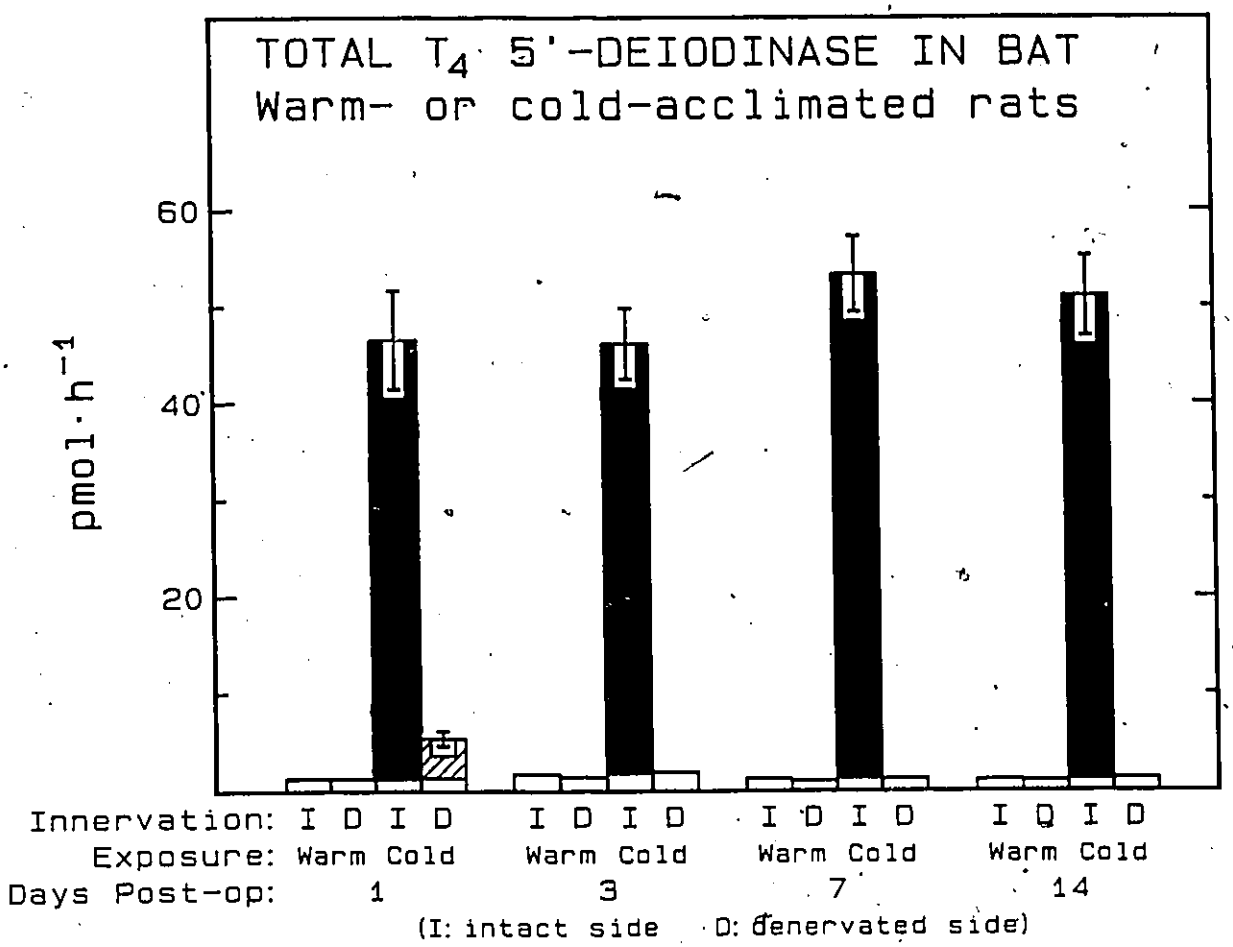

FIGURE 30. DENERVATION TIME-COURSE STUDY (2): THYROXINE 5'-DEIODINASE ACTIVITY IN RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were preformed as described in the legend to Table 32. The format of the Figure is as described in the legend to Figure 24.

Three-way ANOVA showed significant main effects of treatment (warm- or cold-acclimation) and operation (sham-operation or denervation). There was no significant effect of time after surgery.

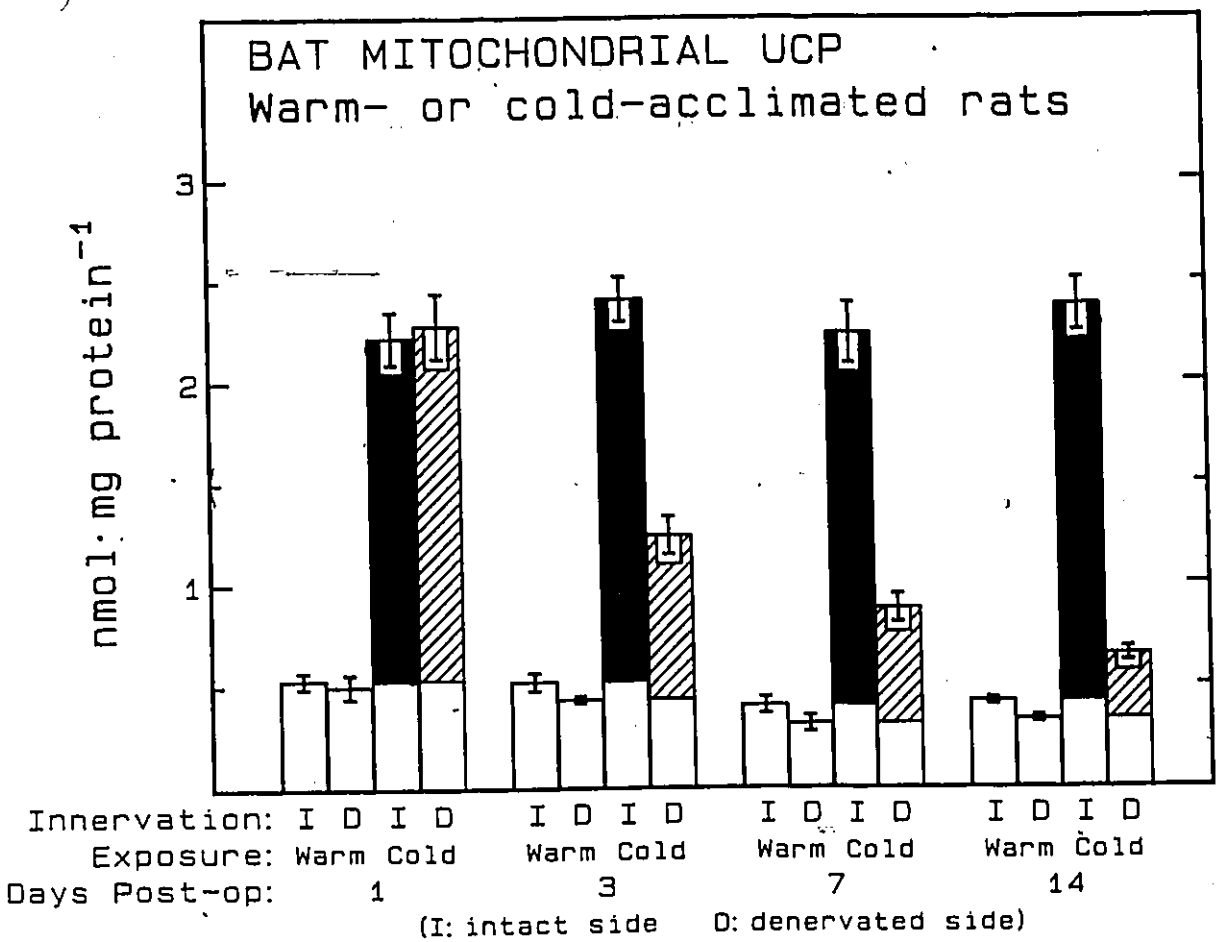

FIGURE 31. DENERVATION TIME-COURSE STUDY (2): SPECIFIC UNCOUPLING PROTEIN IN RATS PREVIOUSLY ACCLIMATED TO COLD.

Rats were treated and the statistical analyses were preformed as described in the legend to Table 32. The format of the Figure as is described in the legend to Figure 24.

Three-way ANOVA showed significant main effects of treatment (warm- or cold-acclimation), time after surgery, and operation (sham-operation or denervation).

further for the rest of the experimental period. Since the protein content in the half-pad having intact innervation continued to increase, the gap in protein levels between sides having intact and severed innervation increased with the time after surgery. Nevertheless, by the fourteenth day after surgery, protein levels in denervated BAT half-pads in cold-acclimated rats were still significantly greater than the levels found in warm-acclimated rats and still about 40% of the level in half-pads having intact innervation in cold-acclimated rats.

Denervation caused a rapid decrease (within one day of surgery) in specific GDP binding of BAT mitochondria from previously cold-acclimated rats (Figure 29) and this decline continued for the rest of the experiment: by the fourteenth day after surgery, levels had declined almost to the levels found in warm-acclimated rats.

In the first part of the study (Figure 26), it was found that the activity of T5'D was extremely dependent upon presence of an intact sympathetic innervation; this finding was confirmed in this part of the experiment (Figure 30). Denervation caused an almost immediate decrease in T5'D activity, which reached levels seen in warm-acclimated rats within 3 days of denervation.

The specific UCP levels also declined after denervation (Figure 31). Unlike the decline in GDP binding, this decrease did not occur at once; rather, it was only three days after surgery that there was any decline. This suggests that "masking" of GDP binding sites occurred in the first day following denervation, before the decline in specific UCP levels. As with GDP binding, by the fourteenth day post-surgery specific UCP levels were approaching levels seen in warm-acclimated rats.

Conclusions: As shown in the first part of this study, sympathetic innervation is the most important, but not the only, factor regulating the changes in BAT seen in cold-acclimated rats. In previously cold-acclimated rats, denervation had a more profound effect on the indices of BAT function than it had on these indices in rats placed in the cold for the first time after surgery. In other words, sympathetic innervation is more important in the maintenance of the cold-acclimated state than it is for acclimation to cold. Thyroxine 5'-deiodinase activity is the most sensitive to denervation, followed by GDP binding, followed by specific UCP levels, and finally total protein levels, which were somewhat resistant to the effects of denervation.

DISCUSSION

"All of Nature speaks to me -
If only I could figure out
What it's trying to tell me"

- L. Anderson

PART A. HYPOTHALAMIC CONTROL OF BROWN ADIPOSE TISSUE.

1. Acute effects. The principal finding of this study was that neither LH lesion nor VMH lesion caused the acute activation of thermogenesis in BAT, as judged by GDP binding (Figure 7). In fact, except for an effect of LH lesions on body temperature, the lesions had no effects on any of the measured indices (Tables 1 and 2). The fact that LH-lesioned rats were hyperthermic (Figure 8) supports the histological finding that the lesions were correctly placed. Further support for that conclusion is that other rats, operated on in the same way and at the same time, exhibited the LH syndrome over a longer time period (Results section A2). Therefore, I conclude that the LH lesions were successful.

The finding that LH lesions did not acutely activate thermogenesis is, at first, surprising. From the work of others cited in the Introduction (Yoshida et al. 1983, Arase et al. 1987), an increase in BAT thermogenesis would be predicted. In fact, it has been reported by one group that there was a significant increase in GDP binding soon after LH lesions (Lupien et al. 1986).

Balanced against this are the findings of Stevenson and Montemurro (1962), that the acute increase in metabolic rate of VMH-lesioned rats was even greater than LH-lesioned rats, despite the fact that, in the long

term, VMH rats become markedly hypometabolic. This suggests that the acute effect of the lesions is non-specific. For example, the marked edema found around the lesions (Figure 5) could contribute to a non-specific hyperthermia. (It is also of interest to note that hyperthermia is not present in LH rats studied after the acute recovery (Results section A3), supporting the idea of a non-specific hyperthermia.)

Furthermore, it is possible that BAT does contribute significantly to the hyperthermia in the time before the measurements made in these studies.

The hyperthermia observed acutely following LH lesion (i.e., occurring within the first one or two days following the lesion) is reminiscent of endotoxin-induced hyperthermia, the acute rising phase of which has been associated with increased thermogenesis in BAT (Harris et al. 1985. Jennings and Elia 1987, Jepson et al. 1988). Interestingly, once an elevated body temperature is attained, it is not sustained by BAT thermogenesis (Harris et al. 1985, Jepson et al. 1987); in fact, it is likely that thermogenesis in BAT is then suppressed, as is seen in other states where other organs cause an increase in body temperature such as exercise (Arnold et al. 1986) and hyperthyroidism (Triandafillou et al. 1982). Such "sparing" of BAT thermogenesis by other heat sources could explain the results of my studies.

Another possible explanation for the discrepancy between my results and those of Lupien and coworkers is the nature and placement of the lesions. In my studies, heat-lesions were used, while Lupien et al. used electrolytic lesions; the problems associated with the latter were described in the Introduction. More importantly, Lupien et al. placed their lesions

considerably more anterior than did we, and may have injured the anterior hypothalamus. Injury to this structure may have removed its tonic inhibitory effect on BAT thermogenesis (Imai-Matsumura and Nakayama 1987) which, combined with the cooler housing conditions used by Lupien et al. (19-22°C), could explain the increased GDP binding found by them.

The hypothesis offered here, that other thermogenic mechanisms "spared" BAT thermogenesis in the acute period following hypothalamic lesion, could be tested by placing different groups of lesioned rats into a graded set of cold stresses (e.g., 26°C, 19°C, 12°C, and 4°C). I would expect that, if other thermogenic mechanisms were compensating for BAT thermogenesis, there would be a decreased elevation of GDP binding in lesioned animals, especially at the higher temperatures.

2. **Knife-cuts.** The principal finding of this study was that KC rats were unable to normally activate BAT thermogenesis in response to overfeeding (Table 6). Taken with the work done on this model of obesity by Dr. Susan Hogan in our laboratory (Coscina et al. 1985), which showed that KC rats were able to respond normally to cold, it can be concluded that KC rats are like VMH rats (Hogan et al. 1982) in that they have normal response in BAT to cold but not to overfeeding. The general conclusion from this work is that the hypothalamic regulation of response to cold is distinct from the regulation of the response to diet.

In the study of the response to acute cold-exposure, it was shown that KC rats were able to increase protein and GDP binding in BAT in a manner similar to normal animals (Coscina et al. 1985). In chow-fed, warm-acclimated KC rats in this same study, there was a failure to activate BAT

thermogenesis despite the fact that these rats became hyperphagic. In non-lesioned rats that overeat, such as in cafeteria-fed rats, there in normally an increase in GDP binding in BAT (Himms-Hagen et al. 1981; see also Table 6). This did not happen in that first study or in my study, even when KC rats were given the additional thermogenic stimulus of cafeteria-feeding (Table 6). Indeed, the only manipulation that did induce an increase in GDP binding in KC rats was a 36 hour fast in KC rats that had been previously cafeteria-fed. I speculate that this may have been due to an increased level of free fatty acids in the BAT of the fasted rat. caused by the starvation, which could have displaced purine nucleotides from the UCP.

That the BAT of KC rats could not respond to the stimulus of overfeeding (both due to the behavioral changes (i.e., hyperphagia) caused by the knife-cut, and to the cafeteria-feeding) suggests that BAT is the site for at least part of the metabolic component (hypometabolism) of the weight gain in these animals

Interestingly, pair-feeding KC rats to the chow intake of non-lesioned rats largely prevented the abnormalities of growth and body weight (Table 4) but did not cause a suppression of GDP binding below normal levels, which might have been expected since such animals are, effectively, starving. (Starving tends to reduce BAT size and thermogenic activity (Rothwell and Stock 1982b, Hayashi and Nagasaka 1983, Rothwell et al. 1984b). It may be that the BAT of such KC rats is at the minimal possible level of thermogenesis, consistent with the fact that they were kept at 26°C. their thermoneutral temperature, and hence were under no thermal stress.

The similarity of KC rats to VMH rats has been noted. One area in which this is particularly notable is food intake (Table 5). As in a similar study of VMH rats (Hogan et al. 1985), KC rats ate more total energy, and more of each of the three macronutrients. This effect was further exaggerated by cafeteria-feeding.

In the present study, KC rats had lowered serum T_3 levels but normal T_4 levels, suggesting that the results of this study could be at least partially explained by hypothyroidism. This was particularly notable in cafeteria-fed rats, where the increase in T_3 levels found in non-lesioned rats was not found in KC rats. However, when the GDP binding results were re-analyzed by Analysis of Covariance to equate for the different T_3 levels, the statistical differences in GDP binding results remained, suggesting that hypothyroidism alone does not explain the findings of this study.

The failure to increase serum T_3 levels in KC rats, coupled to the finding such rats do not increase GDP binding when overfed, suggests that T5'D activity in BAT of KC rats might be subnormal. However, the finding that this enzyme has low activity in rats kept at thermoneutral temperatures, even if rats are overfed (Results sections B1 and B2), tends to rule this out.

The overall conclusion from this study is that the effects on BAT of KC (Coscina et al. 1985) are similar to those of VMH lesion (Hogan et al. 1982, 1985) and that the pathways for the activation of BAT thermogenesis and growth in response to diet are distinct from those pathways mediating the response to cold.

The KC rat has been extensively studied by Vander Tuig, Romsos, and coworkers. They have shown that KC rats fed chow ad lib. have normal GDP binding despite the marked increase in calories ingested (Vander Tuig et al. 1985) and that pair-feeding of KC rats suppressed GDP binding. The first result is in agreement with my findings (Table 6); the second is not. (It should be noted that Vander Tuig et al. found much higher basal levels of GDP binding than I did, possibly because the rats were mildly cold-stressed in that they were held at 23°C.) The general conclusion of this work was in agreement with mine: decreased thermogenesis in BAT of KC rats contributes to their lower energy expenditure and, hence, their obesity.

In a later work (Vander Tuig et al. 1986) it was found that KC rats kept in the cold for 16 days after surgery had lower GDP binding than did non-lesioned rats, but that KC rats were similar to normal rats by 30 days after surgery. The first finding is contradictory to the finding of Dr. Hogan in our laboratory (Coscina et al. 1985) but, again, the final conclusion is the same, that the KC rat does (at least eventually) respond normally to cold.

This research group has also shown, in KC rats fed a high-fat diet, that adrenalectomy both suppresses hyperphagia and, of more interest to us, increases energy expenditure (Romsos et al. 1987); this latter finding was correlated with increases in GDP binding. (See the Introduction for further discussion of the effects of adrenalectomy.) This study also supports my conclusion that decreased diet-induced thermogenesis in BAT is part of the overall decreased energy expenditure in KC rats.

Most recently, this group has shown a decreased turnover of noradrenaline in BAT of KC rats both 3 days and 4 weeks after surgery (Vander Tuig et al. 1987). (This occurred despite a long-term activation of other sympathetically-innervated organs, as shown by increased noradrenaline turnover rates found in the heart and pancreas at 4 weeks post-surgery.) This finding is also consistent with, and helps to explain, my findings.

It appears that parasagittal knife-cut is a relatively specific manipulation that results in preventing the BAT response to feeding while leaving intact the response to cold. (Given its greater specificity, parasagittal knife-cuts should be preferred over VMH lesions as an experimental system.) Further work could be aimed at trying to find the pathway or pathways responsible for the response to cold (although I suspect that this would be extremely difficult since thermoregulation is one of the most critical homeostatic functions of the hypothalamus and disruption of it might not be compatible with life; perhaps comparative studies of poikilotherms and heterotherms would be of use).

Although, as mentioned above, I would predict that T5'D activity in KC rats reared at thermoneutral temperatures would be normally low, it would be of interest to verify this. Of greater interest would be the response of this enzyme to cold-exposure of KC rats; given the normal response of other indices of BAT function, I would expect it to increase normally. Likewise, it would be of interest to study the effect of KC on UCP levels, in cafeteria-fed rats, in cold-exposed and cold-acclimated rats, and particularly in pair-fed KC rats, where it might help to show whether in fact BAT is at its minimal thermogenic capacity, as suggested above.

3. Lateral hypothalamic lesions. The principal findings of this study were that LH rats have more BAT per body weight than do normal rats (Table 14 and Figure 11), that such rats are capable of the normal cold-induced increase of BAT thermogenesis (Figure 12), and that they have an exaggerated diet-induced growth of BAT (Table 14 and Figure 11).

Beyond the histological findings, there is evidence that the LH lesions were successful. In the first week following the lesions, LH rats lost even more weight, and had a markedly lower food efficiency, than did pair-fed rats (Table 9). The smaller body weights and rates of weight gain typical of the LH rat were found, even in cafeteria-fed rats (Tables 10 and 11). The smaller size of gonadal white fat (Table 12) indicates that the LH rats were leaner than normal rats, also consistent with the LH syndrome. As previously noted, LH rats were not hyperthermic (compare to the hyperthermia acutely after lesions: Figure 8).

The most important results concern BAT protein and GDP binding (Table 14 and Figures 11 and 12). Increases in total protein were found in LH rats; these differences are made more distinct when expressed per gram BAT wet weight (Table 14) or per metabolic body mass (Figure 11). The key finding was that LH rats fed a cafeteria diet showed an exaggerated response (Figure 11). It is possible that this difference did not occur in chow-fed rats because, as in KC rats previously discussed, the lack of thermogenic stimulus prevented expression of this difference.

Like VMH rats and KC rats, LH rats were able to normally activate BAT thermogenesis (increase GDP binding) is response to acute cold-exposure (Figure 12), reinforcing the idea that the hypothalamic response

to cold and to diet (as seen by the effects on BAT) are mediated by different pathways.

LH rats fed cafeteria food had smaller weight gains than non-lesioned rats despite the fact that the two groups had similar food intakes (Table 11). This is consistent with the idea that there is a metabolic component to the LH syndrome (Keesey and Corbett 1984, Corbett et al. 1985). The finding that the pair-fed non-lesioned rats were essentially the same as the normally-fed non-lesioned rats reinforces the idea that the previous feeding history does not fully explain the findings in LH rats, further supporting the idea of a metabolic component. The finding of an exaggerated growth of protein in BAT of LH rats suggests that BAT is likely a site involved in this increased energy expenditure.

As mentioned in the previous section of the Discussion, restricted food intake or starvation usually causes a suppression of BAT thermogenesis. This did not happen in the present study (although, at the times measured, food intake of the LH rats was not lower than normally seen), suggesting that either the LH rat fails to suppress BAT thermogenesis, or that the level seen represents the minimal possible activity of the tissue. One experiment that could test these hypotheses would be the measurement of GDP binding in food-restricted LH rats; if the former idea is correct, a suppression of GDP binding would be found; if the latter idea is correct, no lowered GDP binding would be found.

Because of the minimal requirement for BAT thermogenesis at thermoneutral holding temperatures, it took the cafeteria-feeding regimen to demonstrate the exaggerated BAT response in LH rats. Since it is essentially impossible to produce a series of cafeteria menus with stepwise

variation of energy content (the rats, having a free choice of food items, tend to eat differently from one another), one could not quantitate the relationship between degree of overeating and degree of BAT hypertrophy. However, such a study could be done by force-feeding sucrose solutions. Such a regimen would have the additional advantage that non-lesioned rats could be pair-fed for the entire duration of the experiment, something that was impossible in the current study.

On the other hand, cold-exposure is such a powerful stimulus to thermogenesis that differences between LH and non-lesioned rats may have been obscured. Several additional experiments could be performed to further test the idea that BAT is hyperreactive in LH rats. First, a range of milder cold stresses could be used (as described in the previous section of the Discussion); if the BAT of LH rats is hyperreactive, an increase in GDP binding would be found at a higher ambient temperature (although a quantitative interpretation of the results of GDP binding is difficult to make). Second, a study of the time-course of changes in cold-exposed LH and non-lesioned rats would be of use; here, a faster response would be predicted for the LH rats. Third, a study of UCP levels in LH rats would be useful. Both specific and total UCP could be determined in such an experiment. That would also allow a determination of total GDP binding, since percent recovery of BAT mitochondria could be found. Measurements of total GDP binding and of total UCP would be the single best ways of showing that LH rats have more active BAT than do non-lesioned rats.

Holt and York (1988) studied lean (Fa/?) and obese (fa/fa) Zucker rats and found an increase in GDP binding in lean, LH-lesioned rats and a decrease in GDP binding in non-lesioned rats pair-fed to the levels eaten

by lesioned rats. These results are somewhat contradictory to mine in that I found no effects of these two treatments on GDP binding (Figure 12).

There are several possible reasons for the different results. Holt and York reared their rats at 23°C, a mild cold stress for rats, and they did have higher basal GDP binding levels than I usually find for such rats. Second, they studied rats 10 days after lesion while my study was done much later (4 weeks or more after lesion). Third, the kinds of lesions used differed both in kind (I used non-irritating radio-frequency lesions while Holt and York used electrolytic lesions) and location (in my study, the lesions were more anterior). Finally, it may be difficult to compare Holtzman rats, which I used, to lean Zucker rats, about two-thirds of which were, presumably, heterozygous for the fa allele.

PART B. THYROXINE 5'-DEIODINASE IN BROWN ADIPOSE TISSUE: CONTROL AND FUNCTION.

1. Effects of cold-exposure and cafeteria-feeding. The principal findings of these studies were that exposure or acclimation to cold greatly increased the activity of thyroxine 5'-deiodinase (T5'D), while cafeteria-feeding had no effect on this enzyme's activity (Results section B1), and that these findings were independent of the time of day (Results section B2).

Exposure of warm-acclimated rats to cold (4°C) caused a statistically significant increase in T5'D activity within 2 hours and a very large (greater than 50-fold) increase after 10 hours. This finding is in good agreement with the work of others (Silva and Larsen 1983). The increased T5'D activity fell slightly thereafter but remained massively elevated above the levels seen in the warm-acclimated controls. The rate of increase of T5'D activity was much greater than the increase in total protein content of BAT (Table 15), indicating that a specific process is responsible for the activation of T5'D.

The ten-hour lag before maximal response suggests that a rapid activation process, such as protein phosphorylation or dephosphorylation, is not acting here. Rather, the time-course is more suggestive of a process involving de novo synthesis of the enzyme. In fact, the work of Jones et al. (1986) shows that gene transcription and protein synthesis are required for the increase in T5'D activity in animals subjected to cold or to injection of noradrenaline. This conclusion is supported by the finding that

cycloheximide and actinomycin D block noradrenaline-induced increase in T5'D activity (Silva and Larsen 1986).

Given the importance of sympathetic stimulation for the increase in T5'D activity (indeed, as shown in Results section B4, sympathetic stimulation is an almost absolute requirement for the increase in activity of T5'D in cold-exposed rats), and the known increase in sympathetic activity in cafeteria-fed rats, it was somewhat surprising that cafeteria-feeding failed to increase T5'D activity in the present study (Table 16). The cafeteria feeding did increase GDP binding (Table 16), so presumably sympathetic activity was indeed increased. One possibility is that sympathetic stimulation is "necessary but not sufficient"; that is, that some other factor necessary for the increase in enzyme activity is elaborated in cold-exposed rats but not in cafeteria-fed ones. Alternately, cafeteria-feeding may cause the release of some factor inhibiting the sympathetically-mediated stimulation of enzyme activity. (A parabiosis experiment could be used to test these ideas.) A third possibility, tested in the second study discussed here (Results section B2), is that an effect of cafeteria-feeding would be seen if T5'D activity were determined at different times of the day.

In short, despite the known circadian cycle in sympathetic activity, no circadian variation in T5'D activity was found for any experimental treatment used (chow-feeding, cafeteria-feeding, or cold-acclimation) (Figure 16). (The finding that there is no circadian variation in T5'D activity in chow-fed rats is in agreement with a recent report (Murakami et al. 1988).) There is evidence that the cafeteria-feeding was successful (increases in total protein (Figure 14) and GDP binding (Figure 15) were

found) and circadian variations in GDP binding were found in both cafeteria-fed and cold-acclimated rats. The finding of a circadian cycle in GDP binding in cafeteria-fed rats, with a maximum at the end of the dark period, is in general agreement with the previous findings of others (Rothwell et al. 1983). One might expect this to be the time for the maximal activation of diet-induced thermogenesis since it corresponds to the end of the feeding period for rats, when the level of free fatty acids in BAT, which activate thermogenesis, would be highest. The maximum of GDP binding in cold-acclimated rats, which occurred in the middle of the light phase, can be explained by the fact that the rats are least active at this time and thus have a greater requirement to non-shivering thermogenesis in BAT at that time. The relative constancy of GDP binding in warm-acclimated, chow-fed rats can be explained by the fact that these rats were at their thermoneutral temperature, so BAT thermogenesis was at a minimum at all times.

As with the studies of the effects of lateral hypothalamic lesions and parasagittal knife-cuts, the present studies offer evidence of the differences in the regulation of response to diet versus cold. The present studies could be explained by a difference in regulation in the central nervous system, or a difference in peripheral (hormonal) regulation, or both. Further studies would be focused on testing these ideas. One peculiar finding already made is that the two factors most strongly increasing T5'D activity are noradrenaline and insulin (Silva and Larsen 1986), whose actions are usually considered to be opposite. The understanding of the complex regulation of T5'D activity would be greatly advanced by an explanation of this phenomenon.

2. Thyroidectomy. The principle finding of these studies is that T5'D activity is not absolutely required for either the acute or the long-term responses of BAT to cold. Some thyroid hormone is required for these responses, but circulating T_3 was as effective as T_3 produced in situ by T5'D.

In the first study, of the effects of acute cold-exposure, vehicle-injected thyroidectomized rats were hypothermic and failed to grow, as expected (Table 19); for the other groups, the dosages of injected T_3 and T_4 were sufficient to completely normalize body temperatures and growth. The finding that vehicle-injected rats had higher BAT protein per body mass (Table 20) was probably due to the increased need for BAT thermogenesis in the face of reduced thyroid hormone-mediated thermogenesis elsewhere. This idea is supported by the increased GDP binding found in warm-exposed, vehicle-injected rats (Figure 17).

The conclusion that T5'D activity is not required for acute response to cold is supported by the results for GDP binding (Figure 17) and T5'D activity (Figure 18). The cold-induced increase in GDP binding was normal in both T_4-injected rats and T_3-injected rats. The increase in T5'D activity was present and even somewhat exaggerated, especially in the T_3-injected rats, but again the pattern is similar to that seen in the sham-operated rats. Thus even low levels of circulating T_3 were sufficient to sustain a normal cold-induced response.

Lack of thyroid hormone (vehicle-injected rats) prevented the cold-induced increase in GDP binding, demonstrating that there is an absolute requirement for some thyroid hormone for normal response. Interestingly, the activity of T5'D was very high. One could argue, teleologically, that

the elevation of this activity is an attempt to utilize whatever small amount of T_4 might be available. The same thing was seen in the T_3-injected rats. This suggests a possible role of T_4 as a negative feedback inhibitor of T5'D activity.

In the second study, of the effects of long-term cold-acclimation, injection of T_3 or of T_4 permitted regain of weight lost due to thyroidectomy (Table 21). As in the first study, vehicle-injected rats were hypothermic (Table 22); cold-acclimated, T_4-injected rats were also hypothermic compared to similar warm-acclimated rats, for reasons that are unclear (T_3-injected rats had similarly-low T_3 levels yet were not hypothermic).

The major conclusion, that circulating T_3 is as effective in mediating the adaptation to cold of BAT, is supported by Figures 19-23. Both T_3- and T_4-injected rats had protein, DNA, GDP binding, specific UCP, and T5'D responses that were very similar to sham-operated rats, sometimes almost exactly the same. There was a somewhat greater increase in T5'D activity in T_3-injected rats, as seen in the first study. GDP binding in T_3-injected rats was lower than in sham-operated or T_4-injected rats, perhaps indicating a submaximal response and therefore a preference for endogenously-produced T_3; still, the increase over the levels found in warm-acclimated T_3-injected rats was substantial. Finally, the increase in specific UCP levels was submaximal in both T_3- and T_4-injected rats, but the response in the supplemented rats was equal in each. Thus, except for the GDP binding results, injection of T_3 was as effective as T_4 in normalizing the responses. Therefore, the original conclusion of

Triandafillou et al. (1982), that thyroid hormones play only a permissive role, has been supported in these studies.

There are two problems with the work presented here. The first is the discrepancy between the findings for T5'D activity in warm-acclimated, vehicle-injected rats in the two studies; in the first study, the activity was very high, while in the second, it was at the level normally seen in animals held at their thermoneutral temperature. The reason for this difference is unclear, other than the fact that the assays were done by different people. It might be worthwhile to repeat this part of the study.

The second, and more serious, problem is the disagreement between my results and those of Bianco and Silva (1987a, b). They studied the effect of acute exposure to cold (48 hours) and concluded that serum T_3, even at supernormal levels, could not mediate the cold-induced increase in UCP levels that were produced by T_4. They calculate that even the highest dosages of injected T_3 could produce only 90-95% occupancy of nuclear T3 receptors, while it is thought that 100% occupancy is required for complete expression of UCP synthesis (Bianco and Silva 1987c, 1988). (In contrast, animals reared at room temperature have an occupancy of about 70%, a condition associated with minimal levels and synthesis of UCP (Bianco and Silva 1987c, 1988).)

To resolve the difference, several suggestions may be made. First, it may be that in thyroidectomized rats maintained on low levels of T_3 or T_4, as ours were, may somehow adapt to permit full activation despite incomplete occupancy of receptors. If, for example, there is an increase in the number of receptors, a critical number of hormone-receptor complexes,

sufficient to fully activate protein synthesis, may be formed without producing complete occupancy.

Second, it is important to distinguish GDP binding from UCP levels. As described in the Introduction, the phenomenon of "unmasking" of GDP-binding sites has been well-characterized. Coupled with the finding that UCP levels change slowly even in cold-exposed animals (Trayhurn et al. 1987), the discrepancy can be explained by the statement that endogenous production of T_3 is not required for the increase of GDP binding caused by unmasking (which has been postulated as being due to mitochondrial swelling (Nedergaard and Cannon 1987)) but is required for (maximal) stimulus of the synthesis of UCP. (This idea is also compatible with my finding of normal cold-induced synthesis of UCP in thyroidectomized animals kept in the cold for a long time since, even at submaximal rates of synthesis, there would have been enough time to synthesize the UCP to a maximum final level.

This model is also compatible with the findings from Results section B4 (discussed in more detail in the next section). Here, there was an almost total dependence on sympathetic stimulation for the maintenance of T5'D activity (Figure 26). Yet there was significant synthesis of UCP, 6 and 12 days after denervation, in the absence of significant T5'D activity. Likewise, in Results sections B1 and B2, it was shown that there is a dissociation between increases in GDP binding and T5'D activity in cafeteria-fed rats. Therefore, I conclude that T5'D activity (endogenous production of T_3 in BAT) is not required for the cold-induced increase in GDP binding. It is required for maximal synthesis of UCP in the acute period following cold-exposure (Bianco and Silva 1987a, b) but its absence

does not completely prevent a slower synthesis of UCP, mediated both by the primary noradrenaline signal and by circulating T_3 causing a partial increase in occupancy of nuclear T_3 receptors (Silva 1988).

It is clearly desirable to do more experiments. Ideally, GDP binding, UCP levels, and nuclear T_3 receptor occupancy would all be done on the same sample. A time-course, such as 0, 2, 10, 24, 48 hours and 5 and 10 days after cold-exposure, would be the best design. Another attractive model would be the study of denervated BAT in thyroidectomized rats. Since denervation essentially eliminates the activity of T5'D, even in animals kept in the cold (Figure 26), there would be almost no production of T_3 in BAT. Such a model could be used to further test the conclusions reached in the present study.

3. Denervation. The effect of denervation on the adaptation of BAT to cold was studied in the last part of the thesis (Results section B4). The principal findings of these studies were that surgical denervation effectively removes sympathetic stimulation from BAT, that unilateral and bilateral denervations have equivalent effects on a variety of indices of BAT function, that the key indices of BAT function measured (protein, specific UCP levels, GDP binding, and T5'D activity) were dependent to different degrees on sympathetic stimulation (protein least, then UCP levels, then GDP binding, then T5'D activity), and that sympathetic stimulation is more important in maintaining the cold-acclimated state than it is in mediating the acclimation. In general, the sympathetic innervation is the single most important factor regulating the indices measured, but

other factors must also operate because denervation alone does not completely abolish the effects of cold for any of the indices measured.

In part a, the effectiveness of surgery as a means of removing the sympathetic innervation of interscapular BAT was demonstrated. In the first study, the noradrenaline content of the denervated tissue was almost abolished by surgery (Table 23) and this reduction was maintained for at least 26 days. Therefore, in the later experiments, where the longest time studied after denervation was 14 days, there could not have been measurable regrowth of sympathetic nerves.

In the second experiment, there was evidence of some regrowth of nerves in that, six weeks after surgery, noradrenaline levels were significantly greater than levels two weeks after surgery (Table 24), in one case reaching 21% of the level found in the sham-operated half-pad. It is of interest to note that the recovery on noradrenaline content was faster in unilaterally-denervated BAT, suggesting that local trophic factors may be involved.

At two weeks after surgery, a time period more germane to the rest of the studies here, unilateral denervation was as effective in reducing noradrenaline content as bilateral denervation, supporting the use of unilateral denervations in later studies. I found no evidence to support the claim of Girardier and Seydoux (1986) that there is cross-innervation. My results are in excellent agreement with those of Foster et al. (1982). Like them, I found residual noradrenaline content after denervation, presumably from the innervation of the rich vasculature of BAT.

The studies in part b were done to continue the work begun in part a and to characterize the long-term effects of denervation on BAT of

animals raised in the cold before or after the surgery (Tables 25 to 29). These studies showed that denervation greatly reduced but did not abolish the trophic effect of cold-acclimation on the indices of BAT function, and they showed that unilateral and bilateral denervation had equivalent effects on the indices of BAT function studied here, since, for measures of total quantities (such as protein) unilateral denervations were almost exactly half those of bilateral denervations; for specific quantities (such as GDP binding) unilateral and bilateral denervations gave almost exactly the same results.

These studies also showed that the importance of intact sympathetic innervation was different for the different indices, with T5'D activity being almost totally dependent on sympathetic innervation, UCP level less so, GDP binding still less, and protein levels most resistant to the loss of sympathetic stimulation. It is difficult to interpret these findings; it may be that protein levels are regulated by a number of factors while T5'D is regulated by few factors, or even exclusively by the sympathetic nervous system.

The most information was gained from part c. (See Figures 24-31.) This study was a continuation based on the results of part b. By use of a time-course it was possible to more clearly determine the relative importance of the sympathetic innervation for the indices of function studied. The other thing learned from this study is that the sympathetic innervation is more important in maintaining the cold-acclimated state than in mediating the transition to the cold-adapted state, since in the latter case all the indices atrophy from the maxima previously achieved by cold-

acclimation while, in the former, there is actually growth from the warm-acclimated state. (This is further discussed below.)

The extremely rapid decline of T5'D activity in denervated BAT of previously cold-acclimated rats (Figure 30), and its failure to increase in BAT of previously warm-acclimated rats (Figure 26) are in good agreement with previous work showing the dependence of the enzyme on sympathetic stimulation and its short half-life (Jones et al. 1986, Silva and Larsen 1986). The rapid increase of T5'D activity in BAT having intact innervation in cold-exposed rats is in agreement with previous findings (Results section B1). The increase in protein in the present experiment was somewhat slower than in Results section B1, possibly due to the different ages of the rats.

The time course of increase of UCP levels in the BAT having intact innervation in cold-exposed rats was similar to that found by others (Bianco and Silva 1987a, b, Peachey et al. 1988) but not by Trayhurn et al. (1987) who found a slow increase for 2 days followed by a continual, more rapid increase for the next 18 days. These latter results may have been due to the rearing of the rats: before being exposed to the cold, the rats had been kept at 29°C, which could have been warm enough to cause atrophy of BAT and a slowed response to the cold.

Similar denervation studies done by two other groups deserve special attention because of the similarity of design to my work. In the first, by Rothwell and Stock (1984b), it was concluded that surgical denervation completely prevented cold-induced changes in BAT protein and GDP binding when measured 7 days after surgery. (UCP and T5'D were not measured.) They found GDP binding results (for denervated half-pads) of 116 ± 11

pmol/mg protein for cold-exposed rats and 72 ± 9 pmol/mg protein for rats kept in the warm (means ± S.E.M.). The difference is stated to be not statistically significant but, since they do not state their sample size, this cannot be verified. My findings for GDP binding at the 6-day point were similar; with n = 6, the difference was statistically significant. More significantly, since Rothwell and Stock did not go beyond 7 days, they did not find the more striking increase that had occurred 12 days after surgery. (Indeed, given the results of the present study, it would be worthwhile testing rats left in the cold for longer than 12 days, provided that it could be shown that the nerves had not regrown; assays of tissue homogenates for dopamine β-hydroxylase could be used.)

The second important study was that of Desautels et al. (1986). They used a design similar to mine in that they studied changes in unilaterally-denervated BAT caused by cold-exposure, as a 14-day time-course. They studied mice in this work. Like me, they found intact innervation is largely but not entirely responsible for the response of BAT to cold. In particular, they found that protein and UCP levels in denervated half-pads of cold-exposed mice were greater that the corresponding levels in warm-exposed mice, although the levels in denervated tissue tended to decline with time, unlike my results. They also found, as I did, atrophy of protein and UCP levels in BAT of animals that had been cold-acclimated before denervation (although this atrophy was exaggerated in their study because they placed the mice at 33°C after surgery). Unfortunately, they did not measure T5'D activity, so we cannot make this comparison to my results.

The results of part c of my studies also offer an excellent example of the phenomena of masking and unmasking, the change in the stoichiometry

of the interaction between GDP and UCP. For example, by comparing Figures 25 and 27, it can be seen that one day and three days after cold-exposure, there was a significant increase in GDP binding with a smaller increase in the levels of UCP (unmasking). In rats previously cold-acclimated, denervation caused a rapid decrease in GDP binding without a change in UCP levels (masking; compare Figures 29 and 31).

As mentioned in the preceding section of the Discussion, part c of this study demonstrated another example of the dissociation between T5'D activity and UCP synthesis. Since denervation almost abolished the activity of T5'D, the nuclear T_3 receptors were probably not saturated, preventing optimal synthesis of UCP. Yet in this study, UCP synthesis did occur, albeit likely not at its maximal possible rate. Therefore, full occupancy of T_3 nuclear receptors is not an absolute requirement for UCP synthesis.

The overall conclusion from this work is that the sympathetic nervous system is the most important factor mediating BAT response to cold, but not the only one. One focus of future studies would be an attempt to identify this factor or factors. One clue might be the pattern of atrophy found in denervated BAT of rats that had been previously cold-acclimated.

The pattern of atrophy of the various indices depends on the half-life of the component, the effect of any remaining sympathetic stimulation; and any circulating or local factors affecting BAT. It is interesting to note that previous cold-acclimation seems to prevent the partial acclimation seen in rats that had been previously in the warm. There are two possible explanations. First, it may be that the sympathetic-independent changes were occurring but were obscured by the atrophic declines from the cold-acclimated levels. Second (and more likely), there may be a critical period

after which the sympathetic-independent factors cannot operate, as other thermogenic adaptations come into play. I speculate that there is some "cold factor" (or factors) mediating the sympathetic-independent changes seen in warm-acclimated, cold-exposed rats with denervated BAT. This "factor" might mediate the proliferation and differentiation of preadipocytes; after the "critical period", it might not be elaborated, or the sensitivity to it might be changed.

The finding that the most dramatic changes in protein, GDP binding, and UCP levels in the previously warm-acclimated rats occurred after 6 days supports this idea, since it would take that long for the differentiation of brown preadipocytes. Two experiments could be done to test this. First, the changes in the cell types present in BAT following denervation could be determined by the method of Bukowiecki et al. (1986). Second, by use of a variety of times of cold-exposure, the "critical period" could be better defined.

Almost any circulating hormone, or indeed locally-released paracrine factor, could be considered as a candidate for the "cold factor". Circulating catecholamines are attractive candidates, especially given that one would predict receptor supersensitivity following the denervation. Against this is the finding that, in denervated BAT, acute cold-exposure does not cause an increase in blood flow (Foster et al. 1982).

Two other factors that may be good candidates are nerve growth factor (NGF) and glucagon. It would be expected that, following section of the nerves innervating it, BAT would produce considerable quantities of NGF. This would explain the return of noradrenaline in denervated tissue (part a). As well, in a unilateral denervation of BAT, NGF produced by a

denervated half-pad might attract growth from the intact nerve in the other half-pad, explaining reports of cross-innervation (Seydoux et al. 1977). This would also explain the more rapid recovery of noradrenaline in unilaterally-denervated BAT compared to bilaterally-denervated tissue (part a).

Glucagon is an interesting candidate. The work of Billington et al. (1987) has shown that injections of glucagon produced increases in BAT protein, DNA, cytochrome oxidase, lipoprotein lipase, and GDP binding. Most importantly, it was found that the responses of lipoprotein lipase and cytochrome oxidase to injected glucagon were preserved after surgical denervation.

Two experiments could be done to test the possibility that glucagon is the "cold factor". First, it would be useful to characterize the effect of glucagon on denervated BAT for the other usual indices of function (BAT protein, GDP binding, T5'D, and UCP levels). Second, it could determined whether the injection of anti-glucagon antiserum into cold-exposed rats having denervated BAT blocks the changes found in the present work.

THESIS AND SYNTHESIS

The purpose of the work presented here was to study the growth and regulation of brown adipose tissue. In retrospect, it has turned out that the regulation of thermogenesis and growth of BAT is more complex and more subtle than it seemed when I began. Although non-shivering thermogenesis and diet-induced thermogenesis share a "final common pathway" - brown adipose tissue - ample evidence has accumulated that there is a great difference in how these pathways are regulated. The differences in both central regulation (Part A of the thesis) and peripheral regulation (Part B) of the responses in BAT to cold and to diet are a common thread through this work.

I believe that there are evolutionary reasons explaining the different strategies of modulation of energy expenditure in these two situations. From a teleological perspective, it makes sense to have different responses to environmental coldness, which comes at regular, predictable times and persists for a long time, and overeating, which would occur unpredictably and adventitiously. From these different evolutionary pressures have come different physiological and regulatory strategies.

And while it is often frustrating to deal with the complications that these two pathways pose, ultimately it is rewarding because they offer us perspective. By comparing the two, we can learn about both.

APPENDIX 1

CAFETERIA MENU

This menu was used in the experiment on rats having lesions of the lateral hypothalamus (Section 3 of the Results). Menus used in other studies (Results Sections 2, 4, 5, 6, and 8) were similar to this menu.

DAY 1: Peanut butter on a "vegetable thin" (cracker)
1/2 soft fudge cookie
1/8 "Mr. Big" (chocolate bar)
3 "Bugles" (corn snack)

DAY 2: Pate de foi on "Ritz" cracker
Vanilla wafer cookie
1/8 "Milk Nut" (chocolate bar)
Onion ring

DAY 3: "Cheeze-whiz" (cheese spread) on corn cracker
1/2 soft mint cookie
1/8 "Malted Milk" (chocolate bar)
2 "Cheese Balls" (snack food)

DAY 4: Ham spread on soda cracker
Oreo cookie
1/8 "Sweet Marie" (chocolate bar)
4 "Honeycomb" (sugared breakfast cereal)

APPENDIX 2

INSTRUMENTATION

INSTRUMENT	MODEL
beta counter	Beckman LS6800
gamma counter	Beckman Gamma 5500
HPLC	Varian 5000 with BAS LC-4B amperometric detector
ultracentrifuges	Beckman L8-55M Beckman L2-65B
centrifuges	Sorvall RC2-B Sorvall RC-5B
benchtop centrifuges	Eppendorf 5412 Eppendorf 3200
spectrophotometers	Spectronic 20 Gilford 2400-2
analytical balance	Mettler AE 163
balances	Mettler PE 1600 Sartorius 1216MP
thermometer	Bailey Model BAT-8
polytron	Brinkman Model PCU-2-110
oxygen monitor	Yellow Springs Instruments YSI Model 52
vertical electrophoresis	Bio-Rad Model 220 cell ISCO Model 490 power supply
Western blotting	Bio-Rad Trans-Blot cell Bio-Rad Model 200/2.0 constant voltage power supply
computer	Amdahl 470V8
microcomputers	IBM XT IBM PC Radio Shack TRS-80 Model 3

APPENDIX 3

REAGENTS

Biochemicals used were of the highest purity available. Commonly-available reagents (sucrose, toluene, etc.) were obtained from the company which had the institutional contract at the time (CanLab or Fisher). Veterinary supplies (halothane, Innovar-Vet, pentobarbital, saline) were obtained from the Animal Care Services. Food for rats, also of the highest purity available, were obtained from local grocers. Other reagents were obtained as listed below.

REAGENT	SUPPLIER
A) BIOCHEMICALS AND REAGENTS	
acetic acid, glacial	Baker
acrylamide/bis-acrylamide (29:1)	Bio-Rad
ADP, sodium salt	Sigma
alumina	Baker
ammonium acetate	Sigma
ammonium persulfate	Bio-Rad
ascorbic acid	Sigma
atractyloside, sodium salt	Sigma
Bio-Beads	Bio-Rad
bovine serum albumin	
-for protein assay (fraction V)	Sigma
-for RIA (RIA grade)	Sigma
cation exchange resin (AG 50 W-X2 100-200 mesh, hydrogen form)	Bio-Rad
charcoal (activated) (NORIT)	Sigma
chloroacetic acid	Sigma
choline chloride	Sigma
chromatography paper (Whatman #1 Chr)	Fisher
Coomassie blue reagent (dye concentrate)	Bio-Rad
Coomassie blue R-250	Sigma
cytochrome c (from horse heart)	Sigma
DNA (from calf thymus)	Sigma
dextran, clinical grade	Sigma
DHBA	Sigma
diphenylamine	BDH
dithiothreitol	Sigma
EDTA	
-free acid	Sigma
-sodium salt	Sigma
-potassium salt	Sigma
filter paper (Whatman #3)	Fisher
Freund's complete adjuvant	Sigma

REAGENT	SUPPLIER
Freund's incomplete adjuvant	Sigma
glycerol	Baker
glycine	Sigma
GDP, sodium salt	Sigma
HEPES	Sigma
human serum	AMF Biologicals
Lubrol WX	Sigma
MOPS	Sigma
2-mercaptoethanol, type 1	Sigma
methanol	BDH
NCS (tissue solublizer)	Amersham
nitrocellulose	Bio-Rad
nitrogen (gas)	Air Products
noradrenaline	Sigma
perchloric acid 70%	Fisher
phenol reagent (Folin-Ciocalteu)	Fisher
polyvinylchloride microtiter plates	Becton Dickenson
potassium chloride	Baker
potassium phosphate	
-monobasic	Fisher
-dibasic	BDH
PPO	Sigma
PTU	Sigma
rotenone, grade II	Sigma
SDS, electrophoresis purity	Bio-Rad
Sephadex G-50	Pharmacia
sodium acetate	BDH
sodium azide	BDH
sodium chloride	BDH
sodium metabisulfite	Baker
sodium octyl sulfate	Eastman Kodak
sodium salicylate	Fisher
TEMED	Bio-Rad
TES	Sigma
thyroxine, sodium salt	Sigma
trichloroacetic acid	Baker
triiodothyronine, sodium salt	Sigma
Triton X-100	Sigma
Trizma (free base)	Sigma
Tween-20	Bio-Rad
X-ray film	Picker International

REAGENT	SUPPLIER
B) RADIOCHEMICALS	
[8-^{3}H]-guanosine 5'-diphosphate, ammonium salt	Amersham
[U-^{14}C]-sucrose	Amersham
[3'-^{125}I]-triiodothyronine, high specific activity	Amersham
[3',5'-^{125}I]-thyroxine, high specific activity	Amersham
^{125}I-protein A	ICN Biochemicals
C) KITS	
Amerlex T_3 kit	Amersham
Amerlex T_4 kit	Amersham
Protein assay kit	Bio-Rad

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CURRICULUM VITAE

NAME: IAN ROBERT ARCHIE PARK

DATE OF BIRTH: 10 June 1958

PLACE OF BIRTH: Calgary, Alberta

CITIZENSHIP: Canadian

MARITAL STATUS: Single

EDUCATION:

Queen Elizabeth High School
Halifax, Nova Scotia (Grade 10)

Hillcrest High School
Ottawa. Ontario (Grades 12 and 13)

Carleton University
Ottawa, Ontario
B.Sc. (First Class Honours) Biochemistry 1981

University of Ottawa
Ottawa, Ontario
Ph.D. Biochemistry (expected 1989)

University of Toronto
Toronto, Ontario
M.D. (expected 1990)

SCHOLARSHIPS AND AWARDS:

High School:
Ontario Scholar 1976
Hillcrest High School Chemistry Award 1976
Ottawa Board of Education Silver Medal 1976

Carleton:
Dr. C.J. MacKenzie Scholarship 1978
Dr. E.W.R. Steacie Scholarship 1979
Dr. F.W.C. Mohr Scholarship 1980
University Medal in Science 1981

Ottawa:
NSERC 1967 Science Scholarship 1981-1985
University of Ottawa Entrance Scholarship 1981
University of Ottawa In-course Scholarship 1982
University of Ottawa In-course Scholarship 1983

Toronto:
Dr. Isaac Ben Ezra Scholarship 1988
The Borden Award 1988

EXPERIENCE:

Teaching:
- High School - Tutoring mathematics and science
- Undergraduate - Lab. demonstrator (1979-81):
  - -Chem. 100 (2 courses)
  - -Chem. 111 (1 course)
- Graduate - Lab. demonstrator(1981-84):
  - -Physical Biochemistry (3 courses)
  - -Metabolism (2 courses)
- Graduate - Tutoring medical students in first year biochemistry (1983-86)

Summer research assistantships:
- Guelph-Waterloo Center for Graduate Work in Chemistry 1979
- NSERC Summer Research Program 1980

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

Canadian Biochemical Society
North American Association for the Study of Obesity
American Association for the Advancement of Science
Canadian Medical Association

ADMINISTRATIVE EXPERIENCE:

Graduate school:
- Student representative to Biochemistry Departmental Council
- Student representative to Graduate Studies Committee, Department of Biochemistry
- Treasurer, Health Sciences Graduate Students' Association

Medical School:
- Undergraduate Medical Curriculum Committee
- Course and Program Evaluation Committee
- Faculty Council
- Medical Society
- Course committee, Medical Ethics
- Course committee, Human Sexuality Elective

PUBLICATIONS:

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1. PARK, I.R.A., D.B. MOUNT and J. HIMMS-HAGEN. Role of T_3 in thermogenic and trophic responses of brown adipose tissue to cold. Submitted to American Journal of Physiology (under revision).

2. PARK, I.R.A. and J. HIMMS-HAGEN. Diurnal variation in thermogenic state and thyroxine 5'-deiodinase activity in cold-acclimated and cafeteria-fed rats.

3. PARK, I.R.A. and J. HIMMS-HAGEN. Circannual variation in brown adipose tissue of cafeteria-fed rats.

PROCEEDINGS OF MEETINGS (unrefereed):

1. HIMMS-HAGEN, J., and I.R.A. PARK. Role of sympathetic innervation in the growth and maintenance of brown adipose tissue in cold- adapted and cafeteria-fed rats. In: "Thermal Physiology", edited by J.R.S. Hales. New York: Raven Press, 1984, pp. 193-196.

2. HOGAN, S., J. HIMMS-HAGEN, I. PARK and D.V. COSCINA. Brown adipose tissue of rats with unilateral lateral lesions of the hypothalamus. In: "Regulation of Energy Expenditure", edited by E.S. Horton - proceedings of a satellite symposium of the IV International Congress on Obesity, 1984.

3. HIMMS-HAGEN, J., K. TOKUYAMA, J. ELEY, I.R.A. PARK, J. CUI, G. ZAROR-BEHRENS, and D.V. COSCINA. Hypothalamic regulation of brown adipose tissue in lean and obese rodents. In: "Hormones, Thermogenesis, and Obesity", edited by H.A. Lardy et al. New York: Elsevier, 1989.

ABSTRACTS (WORK PRESENTED AT MEETINGS):

1. PARK, I.R.A. and J. HIMMS-HAGEN. The effect of sympathetic denervation on brown adipose tissue [BAT] growth and thermogenesis in cold-acclimated rats. Canadian Federation of Biological Societies, Ottawa, 1983.

2. PARK, I. and J. HIMMS-HAGEN. Cafeteria-feeding does not promote growth of unilaterally-denervated brown adipose tissue. "Regulation of Energy Expenditure", a satellite symposium of the IV International Congress of Obesity, Vermont, 1984.

3. HOGAN, S., J. HIMMS-HAGEN, I. PARK and D.V. COSCINA. Brown adipose tissue of rats with unilateral lateral lesions of the hypothalamus. "Regulation of Energy Expenditure", a satellite symposium of the IV International Congress of Obesity, Vermont, 1984.

4. COSCINA, D.V., J.W. CHAMBERS, I.R.A. PARK, and J. HIMMS-HAGEN. Impaired responses of brown adipose tissue (BAT) to palatable food in rats made obese with parasagittal knife cuts (KCs). Neurosciences Society, Davis, CA., 1984.

5. PARK, I.R.A., J. HIMMS-HAGEN, and D.V. COSCINA. Brown adipose tissue and thermogenesis in rats having lesions of the lateral hypothalamus. Canadian Federation of Biological Sciences, Toronto, 1985.

6. KOPECKY, J., I.R.A. PARK, L. SIGURDSON, and J. HIMMS-HAGEN. Thyroxine 5'-deiodinase in brown adipose tissue (BAT) of hamsters and rats: the effect of diet and of cold. International Congress of Biochemistry, Amsterdam, 1985.

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12. PARK, I.R.A., D.B. MOUNT, and J. HIMMS-HAGEN. Thyroxine 5'-deiodinase in brown adipose tissue: role in cold-induced thermogenesis and growth. XXX International Congress of Physiology, Vancouver, 1986.

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