

THE CONTRIBUTION OF CORTICOSTEROIDS TO  
THE OBESE (ob/ob) SYNDROME

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## ABBREVIATIONS

BAT	Brown Adipose Tissue
DIT	Diet Induced Thermogenesis
ADX	Adrenalectomy
GC	Glucocorticoid
SNS	Sympathetic Nervous System
NE	Norepinephrine
ACTH	Adrenal corticotrophic hormone
CRF	Corticotrophin Releasing Factor
CBG	Corticosterone Binding Globulin
$O_2^-$	Superoxide radical
$H_2O_2$	Hydrogen Peroxide
$\cdot OH$	Hydrogen radical
Mn	Manganese
MnSOD	Manganese superoxide dismutase
SDH	Succinate dehydrogenase
PMS	Phenazine methosulphate
DCPIP	2,6-Dichlorophenolindophenol
MDA	Malondialdehyde
TEP	Tetraethoxypropane

## ABSTRACT

Administration of corticosteroids to human patients as well as some experimental animals is frequently accompanied by a rapid gain in body weight. However the mechanisms responsible for this weight gain are not clear. The genetically obese (ob/ob) mouse has corticosterone levels which are 4-5 times that of its lean littermate. There is some evidence that these elevated corticosterone levels may play an important role in the development of the obese syndrome. For example, adrenalectomy of the obese mouse wholly or partially ameliorates most of its abnormalities. In addition the brown adipose tissue (BAT), an energy buffering tissue, has been reported to have lower than normal levels of manganese (Mn). Mn deficiency rapidly reduces the activity of mitochondrial Mn dependent superoxide dismutase (MnSOD). SOD enzymes catalyze the conversion of superoxide, an oxygen radical, to hydrogen peroxide. Oxygen radicals are capable of peroxidizing lipid and thus of causing membrane and tissue damage. It was thus postulated that the relative Mn deficiency of BAT in the ob/ob mouse may compromise the mechanisms responsible for protecting BAT lipid from the potential peroxidative effects of superoxide. We have therefore conducted experiments to investigate a possible alteration

of Mn distribution via elevated glucocorticoid (GC) levels using two different approaches.

First, obese mice were adrenalectomized and the resulting effects of lower serum corticosterone on food intake, body weight gain, selective tissue weights, insulin and glucose levels, BAT Mn content, mitochondrial succinate dehydrogenase and MnSOD enzyme activities, and lipid peroxidation, were monitored. Secondly, changes in these same parameters were investigated following GC supplementation in lean mice which affected an elevation of GC levels in the serum of a range comparable to those observed in the obese mouse.

It was found that serum corticosterone does not appear to affect Mn distribution or metabolism in the lean nor the obese mouse. While many characteristics of the obese syndrome were improved by adrenalectomy, administration of GC to lean C57BL/6-+/+ mice did not result in obesity, hyperphagia or any of the other characteristics of the obese syndrome. Several theories are discussed in exploring this differential response to GCs observed in lean and obese mice.

## INTRODUCTION

### 1. The Genetically Obese (ob/ob) Mouse

Ingalls et al (1) first described the obese mutant of the laboratory mouse (ob/ob). A single autosomal recessive mutation on chromosome six produces the obese syndrome characterized by hyperphagia (2-5), inactivity (4,5), increased weight gain (1,4,6,8), increased fat depots (4,7), hyperinsulinemia (5,9,10), hyperglycemia (3,5,8), insulin resistance (3,5,8), an enlarged, hypersecreting adrenal cortex (11-15), hypogonadism (2,16), decreased tibial growth (17,18), and impaired thermogenesis (18-20). The mutation occurred in a non-inbred stock of mice in the V strain at the Jackson Laboratory. It has been transferred into the C57BL/6 strain for studies involving the effect of inbred background. These are the mice used for the experiments described in this thesis. The severity of the pathological traits of the obese syndrome vary depending on the species and/or strain into which the obese gene is transferred (genetic background). The reasons for this are very poorly understood. Some feel (8) that this variation in the manifestations of the obese syndromes represents more than one pathophysiological condition; others (21,22) believe it may result, instead, from a differential sensitivity to the same causative factor(s).

Sequential development of the metabolic abnormalities

accompanying the obese syndrome can be divided into several stages. First, during the pre-weaning period, the earliest (5-12 days) apparent defect is decreased thermogenesis as demonstrated by a lower resting metabolic rate (23,24), a lower rectal temperature during exposure to mild environmental temperatures, 15<sup>0</sup>C, and a reduction in oxygen consumption (25). Joosten and van der Kroon (4) produced evidence that there is an increased rate of epididymal fat cell growth as early as 12 days of age. An elevated fat accumulation, hyperinsulinemia and skeletal growth deficits were found as early as 15 days (5). After weaning there is progressive increase in hyperphagia and hyperinsulinemia (3,5), as well as a gradual transition from hypoglycemia to hyperglycemia with the progressive appearance of glucose intolerance and insulin resistance (3,5,9). Hyperphagia and hyperinsulinemia are often concomitant in animal obesities and it is sometimes considered that hypersecretion of insulin is simply a consequence of hyperphagia.

By about 12 weeks of age hyperglycemia reaches a peak (26,9); hyperinsulinemia reaches a maximum by six months (27). This period of progressive metabolic deterioration and accelerated weight gain (40-50g) is referred to as the dynamic phase. It is followed by a spontaneous slow return to normal glycemia and insulinemia (28,9) called the static phase. The body weight of the obese mouse remains elevated until the age of 13-17 months

then it starts to decrease. The mice used in this thesis were in the post-weaning (4-7 weeks) stage, that is the initial dynamic phase.

The evidence that the mature ob/ob mouse maintains elevated levels of circulating glucocorticoids (13,12), displays adrenal cortical hypertrophy (11), and normalizes many of its growth and metabolic disturbances following adrenalectomy (17,29,30,31) suggests that high levels of circulating corticosterone may be involved in the development and/or maintenance of the obese-hyperglycemic syndrome.

## 2. Brown Adipose Tissue

Brown Adipose Tissue (BAT) is a diffuse organ composed of small lobes which are found at many sites in the body. The relative amounts at these sites varies from species to species. The largest lobes and those most often studied are located between the scapulae. These two interscapular lobes were those taken for the experiments reported in this thesis. BAT is most prominent in cold-adapted rodents, hibernating mammals and the newborn of many mammalian species. Its microscopic appearance varies according to the developmental stage as well as the physiological and nutritional state of the animal (32). Brown adipocytes are morphologically distinct from cells of white adipose tissue. Whereas white adipocytes are usually unilocular with few mitochondria of broken internal cristae, brown

adipocytes have a high number of mitochondria surrounded by numerous small (multilocular) lipid droplets. These mitochondria feature numerous tightly packed regularly arranged cristae, a characteristic of mitochondria with a high respiratory capacity. Brown adipocytes have a rich capillary bed. Cells of white adipose tissue mingle with and/or surround lobes of brown adipocytes. BAT which is inactive for long periods of time is yellow and the cells may increasingly resemble those of white adipose tissue.

In the past, BAT has been erroneously regarded as part of the thymus, an organ active in blood formation as well as an endocrine gland (33). However, for the past decade the role of BAT has been recognized as that of heat production or thermogenesis (34): BAT can be a major component of overall energy expenditure and thus an important contributor to maintenance of energy balance (35).

In the mitochondria of all tissues other than BAT, oxidative phosphorylation is coupled to electron transport. Mitchell's (36) chemiosmotic hypothesis describes the mechanism by which a proton gradient is generated across the inner mitochondrial membrane as hydrogen ions are ejected from the mitochondria during electron transport. This gradient drives the synthesis of ATP as  $H^+$  passes through ATP synthetase into the matrix. In most tissues respiration is controlled by the cellular demand for ATP, and this respiratory control minimizes the dissipation of

chemical energy as heat. In BAT, however, the inner membrane of mitochondria possesses a tissue-specific 32 kDa integral protein (37-38), thermogenin, which functions as a proton short circuit capable of uncoupling respiration from ATP synthesis. Thermogenin acts as an anion translocator over the mitochondrial membrane (39). When thermogenin is in its active state the proton gradient created by the respiratory chain is annihilated by the hydroxyl ions leaking out (39,40). Substrate combustion proceeds unhampered by ATP demands and no energy is conserved - the energy is instead released as heat. The binding of purine nucleotides to thermogenin inhibits thermogenesis (41).

BAT is of major importance in the regulation of body temperature and energy balance. It is responsible for the increase in heat production seen both in rodents exposed to cold (42,43), non-shivering thermogenesis (NST), or exhibiting hyperphagia (35,44), diet-induced thermogenesis (DIT). Exposure to cold and overeating both stimulate the sympathetic nervous system (SNS). Thermogenesis in BAT is initiated by the action of noradrenaline (NA) released from sympathetic nerve endings on the plasma membrane.

It is well-documented that ob/ob mice have poor thermoregulatory control (18). More recently it has been suggested that this is due to a defect in the thermogenic capacity of their BAT (19). The ob/ob mouse shows impaired acute response to cold (19,45), reduced DIT during

overfeeding (46), a depressed thermogenic response to NA as demonstrated by decreased oxygen consumption by BAT and marked depressed activity of the proton conductance pathway (47,48). The lack of activation of BAT thermogenesis in the obese mouse is not due to a failure to promote SNS activity within the tissue (49), but rather to a refractoriness of BAT to NE (50,51).

### 3. Glucocorticoids

#### a) History

The essential role of the adrenal gland in maintaining life was first demonstrated in 1856 by Brown-Sequard in adrenalectomized (ADX) dogs (cf. 52). Elucidation of the relationship between the adrenal gland and carbohydrate metabolism began when Cori and Cori (cf. 53) in 1927 noted a decrease in blood glucose and hepatic glycogen following ADX. Corticosterone was purified in 1937 (3,4) and ensuing research in the pharmaceutical industry yielded synthesis of the steroid analogues cortisone, prednisolone and dexamethasone, shortly thereafter. By the 1950's it became apparent that glucocorticoids (GCs) could regulate the activity of a number of specific enzymes due to effects on enzyme induction. A major advance occurred in the mid 1960's when it was found that blockade of protein or RNA synthesis resulted in an inhibition of enzyme induction by GCs (54). Subsequently, Feigelson and co-workers (55) directly demonstrated an increase in a specific mRNA by GCs, solidifying the idea that GCs can regulate the

transcription of DNA into RNA. Convincing demonstration of GC receptors in responsive tissues involved detection of specific binding by cells and correlation with steroid potency (56). A concept emerged in which GC binding to nuclear receptors affected the conformation of that receptor in such a way as to promote binding of the complex to the chromatin. The mechanism by which such binding alters transcription of DNA into RNA is much less clear.

#### b) Steroid Metabolism

GCs are steroid hormones ie. derivatives of a phenanthrene core to which a cyclopentane has been attached. GCs are synthesized from cholesterol in the zona fasciculata of the adrenal cortex. The rate limiting step is the conversion of cholesterol to 5-pregnenolone (57). This substance is then converted to progesterone and through two successive hydroxylations, corticosterone, the major GC in the mouse is formed. The appropriate structural determinants of the GC must be present before binding to GC receptors and subsequent GC effects can ensue.

#### c) Regulation of Glucocorticoid Production

The adrenal is stimulated to synthesize and release GCs by adrenal corticotrophic hormone (ACTH). ACTH, a 39 amino acid polypeptide, is thought to act by binding to a receptor in the cell membrane thus activating adenylate cyclase (cf. 58). Cyclic AMP is increased, resulting in

activation of cellular kinases, which in turn stimulate steroidogenic enzymes. ACTH synthesis and release by the pituitary is regulated by corticotrophic releasing factor (CRF). The hypothalamus is the focal point at which arriving impulses can signal release or inhibition of CRF, although extrahypothalamic sites have been suggested (59). Thus there is a hypothalamic-pituitary-adrenal cortical neuroendocrine axis formed which is under a complex array of controlling mechanisms. Stress stimuli can result in the release of CRF; trauma, pain, apprehension, nausea, fever, and hypoglycemia are all stimuli for GC production (cf. 52). In addition to stress stimuli, there is a normal circadian pattern of ACTH production with increasing levels during the night, which peak in the early morning and decline throughout the day, reaching a nadir in the evening (60). It has been suggested that 5-hydroxytyramine, catecholamines, and/or melatonin levels may account for the episodic nature of CRF (ACTH) release (61). GC control is also mediated by negative feedback by corticosteroids both at the level of the anterior pituitary and at the level of the hypothalamus. In addition, ACTH can feedback directly on the hypothalamus influencing CRF release in a so-called short-circuit loop.

#### d) Biological Functions

The GCs, as the name implies, have carbohydrate regulating properties. The control on metabolic processes is both anabolic and catabolic in nature, depending on what

cell type has been activated; effects are catabolic in muscle and white adipose tissue and anabolic in liver. Corticosterone increases glucose production in the liver due to induction of gluconeogenic enzymes, permissive effects on glucagon and NA actions, and increased availability of substrate. The major source of amino acids for gluconeogenesis is muscle in which GCs induce breakdown of protein and decrease protein synthesis. In adipose tissue the GCs stimulate lipolysis, promote the lipolytic effect of NA, and induce gluconeogenesis. The free fatty acids liberated provide an alternate energy source, thereby sparing glucose.

#### e) Glucocorticoid Excess

As soon as cortisone became available, Hensch (cf. 52) employed it in the treatment of rheumatoid arthritis - he had previously noted that arthritic patients often underwent remission when pregnant or jaundiced. Upon the dramatic improvement observed in these patients, the Nobel Prize was awarded to Hensch, Kendall and Reichstein. The ensuing therapeutic use of GC's in a large number of conditions was followed by a realization of some of the serious side effects of GC excess. High circulating levels of GCs are characteristic of some obesities in humans and experimental animals. Elevated corticosterone levels are found in the ob/ob mouse (12,13), the obese Zucker rat (fa/fa) (62), the NH obese mouse (63), the A<sup>y</sup> obese mouse

(63), and mice made obese with lesions in the hypothalamus (64). The symptoms of Cushing's disease in man are caused by elevated plasma levels of free (unbound) cortisol (the major GC in man). The elevated levels are due to (cf. 58) 1) ACTH producing tumors in the anterior pituitary 2) CRF increase due to disorders in the hypothalamus 3) cortisol producing tumors of the adrenal cortex and 4) ectopic ACTH syndrome. Manifestations of Cushing's syndrome and/or therapeutic GC excess depend on steroid dosage and duration of exposure. Symptoms (65) include a tendency toward hyperglycemia, negative nitrogen balance, and a redistribution and gain of fat, accompanying moderate hypertension and a general muscular weakness. Also, impaired wound healing and rarification of the bone matrix resulting in osteoporosis are also found. Appetite is increased and sometimes psychic disorders occur. Hirsutism is common in women, menses are irregular and fertility rare. Fatigue, weakness and inactivity are common. In children there is an inhibition of growth. Many of these manifestations are common to those observed in the obese mouse. For example, hyperglycemia (3,5,8), infertility (2,16), obesity (1-8), shortened skeletal growth (17,18) and impaired immunocompetence (66).

When ADX is performed on the genetically obese mouse, food intake (16,17,67), weight gain (69,70) and obesity (16,17,68-69) are normalized. Hyperinsulinemia (17,29) and hyperglycemia (17,29) are reduced and there is an increase

in tibial growth (16,17). ADX of obese Zucker rats (fa/fa) normalized weight gain, food intake, fat deposition, fat depot weights, adipose cell size and plasma insulin levels (70-72). Moreover, GC replacement in both of these ADX obese models reproduces hyperphagia, obesity, hyperinsulinemia, and stunting (20,72).

GCs profoundly influence the course of infections by inhibiting inflammatory and immunologic processes (73) and producing lymphocytopenia. GCs may have other ill-defined actions on the central nervous system, in addition to their inhibitory influence on ACTH. Patients with Cushing's syndrome may be euphoric, depressed or psychotic and have an increased appetite (74,cf. 52). The obese mouse is hyperphagic. Evidence is mounting which demonstrate GC effects on specific biochemical reactions in the brain (75).

#### f) Corticosteroid Binding Proteins

Corticosteroids exist in plasma in both unbound (free) and protein-bound forms. The two major corticosteroid binding proteins in plasma include corticosteroid binding globulin (CBG), also called transcortin, a high affinity, low capacity, specific binding protein, and albumin, a low affinity high capacity binder of corticosteroids. The distribution of steroid among CBG-bound, albumin-bound and unbound fractions depends on the type of corticosteroid, the steroid concentration and the level of the two plasma

proteins. Although such distributions are well documented in the human, it is not known whether they apply to the mouse. Seal and Doe (76) reported that total corticosterone capacity of CSB in the mouse is 33 ug/100 ml, although this could vary amongst different strains of mice.

#### g) Variations in Cellular Sensitivity

Each tissue and cell type responds in a unique way to GCs. Thus, from one genome, different degrees and even direction of response can be generated in different cells. Since the same GC receptor is generally thought to be common to all target cells, this response must be regulated at steps distal to receptor-steroid complex formation (77). An individual cell type usually changes its GC sensitivity according to its stage of development and metabolic rate. For example, the lymphocytes of the thymus are more severely depleted by steroid treatment than are the more mature lymphocytes of the blood, spleen, and lymph nodes (cf. 52). Munck et al (cf. 52) demonstrated that certain GC effects on thymus lymphocytes require adequate intracellular ATP generation. Inter-species differences are best illustrated by the differential effects of GC administration in so called corticosteroid-sensitive animals such as the mouse, rat, and rabbit where corticoids are potent immunosuppressors causing widespread destruction of lymphocytes, versus the response in corticosteroid-resistant species such as man, guinea pig, and monkey which

are relatively resistant to the immunosuppressant effect of GCs (78).

Some of the manifestations of high GC levels may be caused by other hormones being secreted in response to corticosteroid actions. The metabolic effects of GCs are also augmented or counterbalanced by those of other hormones. Insulin is the dominant regulator of blood glucose in the fed and fasted state. Many of the above mentioned actions of GCs are antagonized by insulin. Interactions, usually permissive effects, between GCs and thyroid hormone, glucagon, growth hormone, NA, and other hormones also exist.

#### 4. Free Radicals and Lipid Peroxidation

A normal chemical bond consists of a pair of electrons opposite in spin. A free radical is simply a molecule containing an odd number of electrons rendering it chemically reactive. Oxygen radical, is a term used to indicate reactive oxygen species which are generated as intermediates in redox processes leading from oxygen to water. These reactions generally produce the superoxide anion ( $O_2^-$ ) and/or hydrogen peroxide ( $H_2O_2$ ). Although  $O_2^-$  and  $H_2O_2$  are insufficiently reactive in aqueous media to initiate deleterious reactions (79), their potential danger lies in their ability to cooperate in the formation of the more strongly oxidizing species, the hydroxyl radical,  $OH^\bullet$  (80). The hydroxy radical is powerful enough to abstract a

hydrogen atom from a non-radical, producing another free radical. Thus, a long series of chain reactions may be generated. Lipid peroxidation is the oxidative decomposition of polyunsaturated lipids, primarily involving free radical mechanisms. It is a process characterized by three phases; an induction period (hydrogen abstraction from a methylene carbon), a rapid propagation phase (chain reactions), and a termination phase (reaction of two radicals or reaction of a radical with an antioxidant). The  $\cdot\text{OH}$  radical is capable of abstracting a hydrogen from unsaturated fatty acids to initiate lipid peroxidation. As polyunsaturated fatty acid containing lipids are the backbone of cell membranes, oxygen radicals, if not adequately controlled, can disrupt the integrity and normal functioning of membranes (81). Some end products of lipid peroxidation are also toxic (82).

##### 5. Manganese

Manganese, Mn, is a transition metal of atomic number 25. It is ubiquitous in the biosphere. Although Mn occurs only in trace amounts in animal tissues, without exception, all mammalian tissues contain Mn (83). Tissues rich in mitochondria tend to possess the highest levels of Mn (84). Mn was first reported to be an essential nutrient in 1931, when poor growth in mice, and abnormal reproduction in rats (cf. 85) fed diets devoid of the element was demonstrated. Since then the essentiality of Mn in several biological

functions has been elucidated although the biochemical mechanisms remain unclear.

a) Manganese Metabolism

The homeostatic regulation of Mn levels in animal tissues is brought about primarily through excretion of Mn rather than through regulation of absorption (86,87). However, some control exists, at the level of the gastrointestinal tract as there is increased absorption of Mn during pregnancy (88) and lower Mn absorption from iron supplemented foods (89). Under ordinary conditions the bile flow is the main route of excretion (87,90). Excretion also occurs via the pancreatic juice and, in Mn overloading, the duodenum, jejunum, and terminal ileum can provide auxiliary routes (86). Manganese absorbed into the general circulation is transported chiefly by transmanganin, an  $\alpha_2$ -macroglobulin (91). A small fraction is bound to transferrin, the iron transport protein. Mn which is located within erythrocytes is incorporated during erythropoiesis (84) either as a manganoporphyrin or chelated to heme.

Mn functions in carbohydrate and lipid metabolism although the biochemical mechanisms of these involvements have not been elucidated. A relationship between Mn and pancreatic function was first suggested by Rubenstein and co-workers in 1962 (92). These authors reported the case of a diabetic patient who responded to oral Mn with a

lowering of blood glucose levels. Both excess and deficiency of Mn can affect carbohydrate metabolism (93-95). Injection of high levels of Mn produces hyperglycemia and hypoinsulinemia in rats (93). In Mn-deficient rats, glucose tolerance is abnormal and both first and second phase pancreatic insulin output by perfused pancreata is depressed (95). Recently Subasinghe et al proposed an insulin-mimetic action of  $Mn^{2+}$  involving cGMP (96). Mn also has a lipotropic action and appears to interact with choline (97). Mn-deficient mice have enlarged deposits of abdominal fat and fatty livers (98).

The first abnormality to be recognized as resulting from an inadequate intake of Mn was perosis in chickens (85). In rats Mn-deficiency produces severe shortening of the radius, ulna, tibia, and fibula in proportion to body length as well as ataxia (99). A mutant gene affecting coat color in mice, pallid, produces a congenital ataxia quite similar to that seen with Mn-deficient non-mutant mice (100,101). Supplementing the mutant diet with high (1500-2000 ppm) levels of Mn prevents the occurrence of congenital ataxia, suggesting an interaction between genotype and the metabolism of this element (101). Liver mitochondria exhibit ultrastructural abnormalities including elongation and reorientation of the cristae, as well as alterations in the integrity of their membranes (98). It has been proposed that the morphological changes observed in the mitochondria might explain the observed

lowered oxidation rate in liver mitochondria isolated from Mn-deficient mice (102).

#### b) Manganese-dependent Enzymes

Two classifications of Mn enzymes exist 1) metal-enzyme complexes and 2) metalloenzymes. Enzymes that can be activated by Mn (as co-factor) are numerous and include hydrolases, kinases, decarboxylases and transferases (103). This type of enzymatic activation is generally non-specific and enzyme activities are not affected by Mn-deficiency. One notable exception is the glycosyl transferases which catalyze the transfer of sugar from sugar nucleotides to a variety of acceptors. The enzymes are important in the synthesis of mucopolysaccharides and in glycoprotein metabolism (102). The reduced activity of these enzymes in Mn-deficiency is believed to be responsible for the observed skeletal abnormalities and congenital ataxia (101).

In contrast to other essential transition elements, relatively few Mn metalloenzymes have been identified in higher organisms. These include arginase, pyruvate carboxylase and superoxide dismutase (SOD). No pathology has been reported relating to lower arginase activity in Mn-deficiency (84). In animals fed a Mn deficient diet, magnesium substituted for Mn in pyruvate carboxylase with no apparent loss of activity (104). The third metalloenzyme containing Mn, is Mn superoxide dismutase

(MnSOD). SOD's catalyze the reaction of  $O_2^-$  to  $H_2O_2$  and oxygen. There are two isoenzymes of SOD: one containing copper/zinc is located in the cytosol; the MnSOD is located in the mitochondria. The activity of MnSOD is affected by dietary status. In Mn-deficient mice, the activity of this enzyme was significantly lower in liver, brain, heart and lung of deficient animals than in controls (105). In chickens, there was a depressed activity of MnSOD in liver after only 7 days of feeding a Mn-deficient diet to hatchlings (84). Offspring of rats fed a Mn-deficient diet had lower than normal liver MnSOD activity, and they showed higher than normal lipid peroxidation in isolated liver mitochondria (106). An increased rate of lipid peroxidation in Mn-deficient animals could be one of the underlying biochemical lesions that lead to the membrane and cristae damage seen with Mn deficiency.

#### 6. Statement of the Research Problem

The mitochondrial respiratory chain produces oxygen radicals at 2 sites: at the flavoprotein-NADH dehydrogenase and the ubiquinone-cytochrome b region, likely by autoxidation of ubisemiquinone (107). Superoxide generation by mitochondria is greatest when respiratory chain carriers located on the inner mitochondrial membrane are highly reduced (108) ie. when levels of NAD-linked substrates are high. As active BAT is very concentrated in rapidly respiring mitochondria, one can predict that high levels of  $O_2^-$  are generated although its production has

never been measured directly in this tissue. Under normal conditions, MnSOD maintains intramitochondrial  $O_2^-$  at very low steady state concentration ( $8 \times 10^{-12} M$ ), (109). Inhibition of MnSOD effects an increase in cellular  $O_2^-$  concentrations (110,111).

Dietary Mn deficiency results in abnormal accumulation of body and tissue fat, impaired lipid metabolism and defective glucose tolerance, signs which mimic the characteristics of genetically obese mice (112). Moreover, traits involving altered mitochondrial metabolism which are common to both the Mn-deficient and obese mouse include reduced oxidative capacity, decreased succinate dehydrogenase as well as MnSOD activities, and structurally abnormal cristae (112,113).

Adrenal hormones have been implicated in the regulatory control of Mn distribution (114,115), but the precise role of GCs in this control remains uncertain. Administration of exogenous GCs in mice as well as stimulation of the animal's own adrenal cortices with injected ACTH, markedly affected the tissue distribution of the radioisotope  $^{54}Mn$ . Removal of radiomanganese from the liver was accompanied by a percentile increase in the carcass as a consequence of these treatments. The tissue concentrations of  $^{55}Mn$  were changed by cortisol in the same direction as those of the radioisotope  $^{54}Mn$  (115). This proves that the GCs affected the metabolism of the

essential metal itself and not merely that of its artificial tracer. ADX did not evoke the opposite effect on the radioisotope distribution.

While investigating the mechanism of GC-induced cataract formation in the developing chick embryo, Nishigori et al (116) found that GCs caused the elevation of hepatic lipid peroxides. They concluded that cataract formation is due to oxidative damage induced by GC supplementation as this same metabolic pattern is likely to exist in other tissues also. This is supported by the finding that administration of GC is accompanied by a dose-dependent increase in  $O_2^-$  production in rat liver microsomes (117).

Subsequent to these observations, the following hypothesis was formulated and tested in this thesis. The obese ob/ob mouse has lower BAT Mn content as well as reduced BAT MnSOD activity, as opposed to that of the lean (112,113). This, coupled with information demonstrating an effect of GCs on Mn distribution (114,115), leads one to postulate that the very high levels of circulating GC observed in the obese mouse, stimulated a redistribution of Mn such that the BAT is rendered relatively deficient in Mn. Consequently,  $O_2^-$ , produced in high quantities in the rapidly respiring BAT of suckling mice, could overcome the compromised MnSOD activity. Lipid peroxidation, membrane damage to the mitochondria and loss of tissue function, might ensue. Without optimal functioning of BAT, adequate

energy balance cannot be maintained (35). Thus high serum GCs, via their effect on Mn distribution, may be a contributing factor in the obese syndrome.

In order to examine the effects of corticosterone on the metabolism and Mn status of BAT, and its relation to the development of obesity in the genetically obese mouse, two in vivo experiments were designed which combined differing levels of serum corticosterone with varying degrees of fat accumulation. The first experiment establishes the traits of BAT metabolism pertaining to Mn status of the obese mouse and monitors the changes in these traits upon ADX which lowers serum corticosterone and body weight gain. The opposite approach of supplementing lean mice with corticosterone to achieve stable circulating corticosterone concentrations of a range comparable to that of an obese mouse was accomplished by subcutaneous implantation of pellets formed from molten corticosterone and cholesterol. Mn metabolism in the BAT of the lean corticosterone supplemented mouse could then be compared to that of the obese mouse.

## MATERIALS AND METHODS

### 1. Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.): activated charcoal (untreated powder), adenine dinucleotide phosphate (ADP), bovine serum albumin (RIA grade, fraction V), cholesterol, merthiolate, b-nicotinamide adenine dinucleotide phosphate (reduced form), p-nitrobluetetrazolium, phenazine methosulphate, succinic acid (disodium salt), tetraethoxypropane, 2-thiobarbituric acid, Trizma-Base, Trizma-HCl, and xanthine.

Fisher Chemical Co. (Ottawa, Ont.) supplied the following: boric acid, 2,6-dichloro-indophenol (disodium salt), EDTA, ethanol, ferric chloride, methanol, manganese nitrate atomic absorption standard solution (1 mg/ml), phosphoric acid (85%), sodium bicarbonate, sodium carbonate, sodium citrate, sodium cyanide, sodium hydroxide, sodium phosphate (dibasic), sodium phosphate (monobasic), sucrose, and trichloroacetic acid.

Xanthine oxidase (from cow's milk) was purchased from Boehringer Mannheim Canada Ltd. (Dorval, Que.); anti-corticosterone-21-thyroglobulin from Miles Scientific Laboratories Ltd. (Rexdale, Ont.); dextran T70 from Pharmacia Fine Chemicals (Dorval, Que.); corticosterone from ICN Pharmaceuticals Inc. (Montreal, Que.), and Hoechst 33258 Dye from Calbiochem (LaJolla, CA.).

[1,2,6,7-<sup>3</sup>H]-Corticosterone was obtained from Amersham Canada Ltd. (sp. act.=84Ci/mmol),(Oakville, Ont.); Aquasol, Triton X-100 and <sup>125</sup>I-labelled pork insulin (sp. act.=100uCi/ug) were from New England Nuclear Corp. (Lachine, Que.).

## 2. Animals

Four week old male C57BL/6 +/+ and ob/ob mice were obtained from Charles River Breeding Labs (Montreal, Que.), and Jackson Laboratories (Bar Harbour, ME.) respectively. They were maintained at 24±1°C in plastic cages, four mice per cage, with free access to food (Purina rat chow 5012) and water, and a 12:12 lighting schedule.

## 3. Treatments

After 5 days acclimatization the mice were individually caged and underwent surgery (ADX or implantation) under halothane anesthesia. Mice were exposed to halothane approximately 8 minutes and recovered within the following 5 minutes.

### Experiment A: Adrenalectomy

Thirty obese mice, each weighing approximately 20g, were separated into two groups and paired by weight. One mouse of each pair was bilaterally adrenalectomized, the other sham-operated, under halothane anesthesia. The adrenal glands were gently lifted to the opening of dorsal incisions and forceps were used to carefully remove the

glands along with a small amount of surrounding adipose tissue. Care was taken not to touch the adrenal with the forceps. Sham operation consisted of locating and exposing, but not excising the adrenal gland. Incisions were closed with stainless steel wound clips.

#### Experiment B: Implantation

The lean +/+ mice were separated into 2 groups of approximately equal weight. Fifteen mice were implanted with fused pellets of 30% corticosterone, 70% cholesterol; and 15 with 100% cholesterol as controls. A small incision was made in the abdominal region and the skin gently pulled away from the underlying membrane to form a small pocket. Pellets weighing about 40 mg were inserted subcutaneously 2 cm caudal to the incision, which was then closed with steel wound clips. Pellet concentration, not size, was manipulated to achieve a variable range of doses. To obtain a 'diluted' pellet, corticosterone and cholesterol in the desired ratio were placed in a 10ml beaker, melted in a block heater at 180-210°C and thoroughly mixed. Aliquots were transferred to a plastic mold, cooled, solidified and removed from the mold. Pure cholesterol pellets were produced for use in control animals.

Drinking water for adrenalectomized mice was replaced with physiological saline (0.9%) while the sham-operated mice were offered both saline and tap water; all implanted mice were given only tap water. All mice had food (Purina rat chow 5012), and water (or saline)

available ad libitum throughout the treatment period. The reason for using rat rather than mouse chow was the Mn content (54.4 and 154 ppm respectively) as it had been shown previously (112) that Mn supplementation could affect some of the parameters of interest. Food intake was monitored three times weekly; body weights were recorded once per week. All mice were sacrificed eighteen days after surgery.

#### 4. Preparative Procedures

##### a) Blood Collection

Mice were killed by decapitation within 15 seconds of touching the cage in order to avoid stress-induced corticosterone release. Also care was taken to avoid all undue noise and movement which might excite the mice. Trunk blood was collected into non-heparinized Eppendorf tubes (1.5 ml) and centrifuged in a microfuge for two minutes. The serum was removed and stored at  $-70^{\circ}\text{C}$  for subsequent corticosterone, insulin, and glucose determinations.

##### b) Dissection

The interscapular BAT, spleen, and epididymal white fat pad were removed and placed in ice-cold isolation buffer containing 0.25M sucrose and 5mM Tris at pH 7.2. All three tissues were cleaned of adhering muscle and, where applicable, white adipose tissue, blotted dry and weighed. BAT from ten mice of each group were used to

isolate mitochondria; the remaining five were used for determination of Mn content.

c) Isolation of BAT Mitochondria

BAT of two mice were pooled to obtain a sample of sufficient size for subsequent assays. Tissues were minced with scissors and then homogenized (five strokes of a Teflon plunger operating at 400 rpm) in a glass homogenizer, and volume was adjusted to 10 ml. One ml of homogenate was removed for lipid peroxidation and protein determinations. The remainder was used to prepare mitochondria in isolation buffer (0.25M sucrose and 5 mM Tris at pH 7.2) according to the method of Cannon and Lindberg (118), omitting the initial high speed centrifugation in order to improve mitochondrial yield. Final resuspensions of the washed mitochondria was in 100mM KCl, 20mM Tris, pH7.2, at a final protein concentration of 1.5-4 ug/ul. Mitochondria preparations were stored at  $-70^{\circ}\text{C}$  until used for analysis. Storage times were not longer than 2 months. Preliminary studies proved that none of the parameters subsequently assayed was affected by storage under these conditions.

5. Analytical Procedures

a) Estimation of Mn Content in BAT By Flameless Atomic Absorbtion Spectrometry

Cleaned BAT from 5 mice of each group was stored at  $-20^{\circ}\text{C}$  for 3 months prior to analysis. Tissues were thawed and homogenized (2% w/v) with a 0.25% Triton X-100 solution

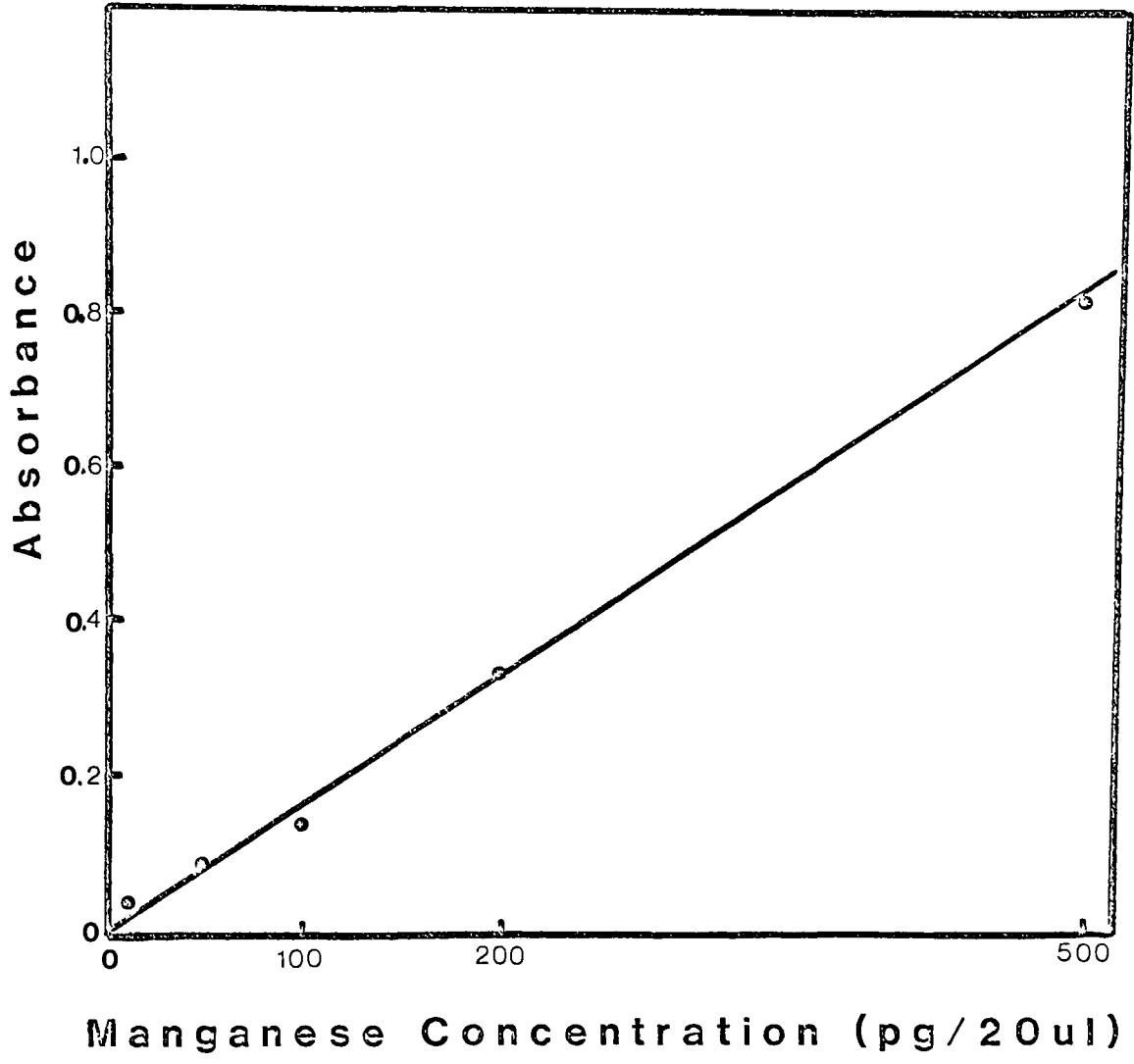
in 2N HCl using a Polytron P10 homogenizer (Brinkman Instruments, Toronto, Ont.). Acid frees the sample of matrix interferences by a pH rather than a hydrolysis type action (119), and Triton X-100 prevents creepage and spluttering of samples during the drying stage. Homogenates (20ul) were injected directly into a 551 double beam atomic absorption spectrometer (Instrumentation Laboratories (Montreal, Que.) equipped with a 655 Controlled Temperature Furnace atomizer and simultaneous deuterium arc background facilities. A Mn hollow cathode lamp was operated at 7mA with the wavelength set at 279.8 nm. Graphite rods were purged with nitrogen at a flow rate of 30/5 SCFH (Standard Cubic Feet per Hour). Temperature and ramp rates were as follows: 120°C, 10°C/s for injection; 1100°C, 20°C/s for pyrolysis and 2400°C, 0°C/s for atomization. Dilutions of a 5000 pg/20ul Mn standard solution in 0.25% Triton X-100 solution provided a standard curve (see Figure 1). Average percent recovery of Mn with this technique was 88±2.3% for 3 samples.

b) Determination of Protein

Total protein was estimated by the method of Bradford (120) with BSA as standard. The assay employs a protein-binding dye, Coomassie Brilliant Blue G250, which exists in two color forms, red and blue. The red form is converted to the blue form upon binding of the anionic dye to the protein in acid-denaturing solvent. The blue colour formed is measured at 595nm.

Figure 1 - Flameless Atomic Absorption of Mn  
Standard Curve

Standard solutions of 10, 50, 100, 200, and 500 pg/20 ul were prepared in 2N HCl containing 0.25% Triton X-100. 20 ul aliquots were injected into the furnace for atomization as described in Methods. Each point represents the mean of triplicates. Absorbance was measured at 279.5 nm.



### C. DNA Assay

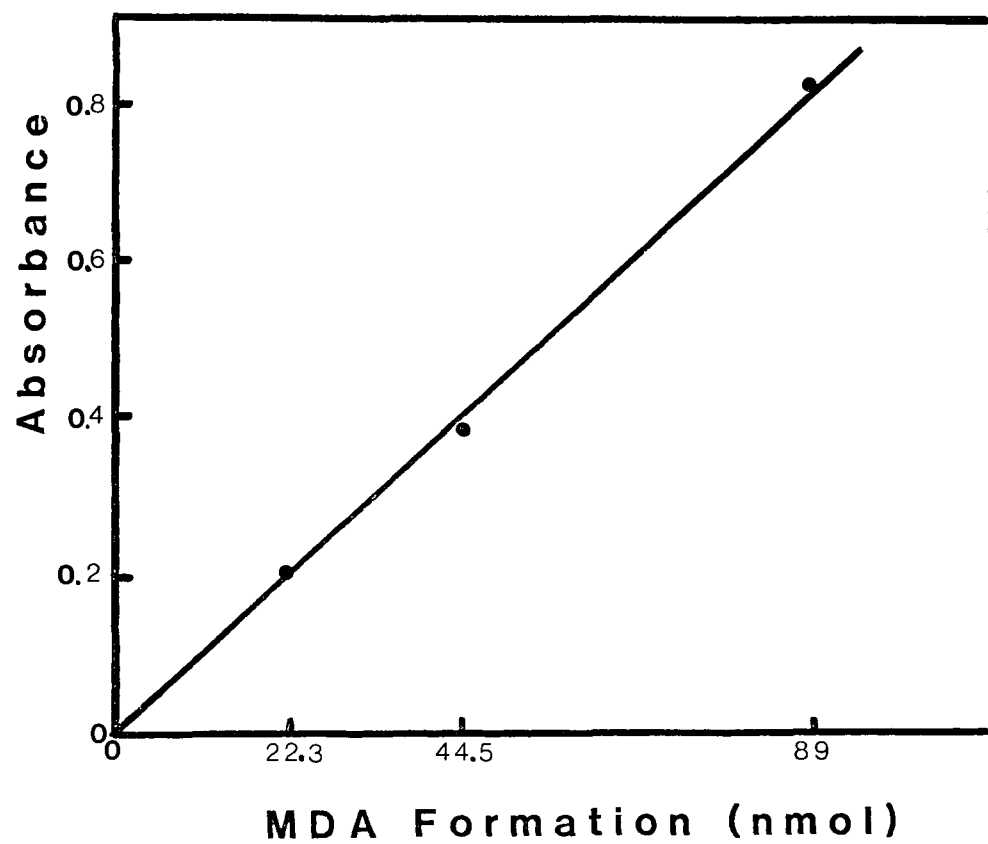
Quantitative determination of DNA in 10ul of sonicated crude BAT homogenates was accomplished by the procedures of LaBarca and Paigen (121). The method utilizes the enhancement of fluorescence seen when the dye, Hoechst 33258, binds to DNA. Fluorescence measurements were made using a Model 430 Turner Spectrofluorometer with an excitation maximum at 356 nm and an emission maximum at 458 nm.

### d) Lipid Peroxidation

The susceptibility of the BAT to undergo lipid peroxidation under peroxidizing conditions was examined using the thiobarbituric acid (TBA) reaction technique measuring malondialdehyde (MDA) formation essentially as described by Weinstein and Scott (122). Triplicate samples of homogenates (100-200ug protein) or mitochondria (~100ug protein) were pipetted into 5 ml glass tubes. A tissue blank for each sample was prepared by adding 2 mls of 20% TCA to one tube of each triplicate set, thus precipitating the protein. Standards were prepared using appropriate dilutions of tetraethoxypropane, a stable dimer of MDA. MDA itself has a very short shelf-life. After addition of 1 ml of a reaction mixture composed of oxidation initiators (2mM ADP, 2mM NADPH, and 0.2mM  $\text{FeCl}_3$ ) in 10mM Tris-HCl at pH 7.4, the tubes were incubated with shaking for 60

Figure 2 - Lipid Peroxidation  
Standard Curve

Lipid peroxidation was measured as TBA reacting products (absorbance at 532 nm) as described in Methods. Each point represents the mean of duplicates with a zero blank (MDA formation with no TEP added) subtracted.



minutes in a water bath at 37°C. Reactions were terminated by the addition of 2 ml 20% TCA (except to tissue blanks) followed immediately by centrifugation in a clinical centrifuge for 10 minutes. Two ml of a 0.5% TBA solution in water (heated to dissolve, filtered and cooled) were added to 2 ml portions of the supernatants, the tubes were capped lightly and the mixture heated in a boiling, covered water bath for exactly 15 minutes. After cooling on ice, a pink color develops which was measured at 532 nm. Absorbance values were translated into MDA formation by construction of a standard curve as shown in Figure 2.

e) Assay of MnSOD

An indirect method for quantitating the enzyme MnSOD was employed in which MnSOD competes with nitrobluetetrazolium (NBT) for available superoxide. Xanthine plus xanthine oxidase cause sufficient production of  $O_2^-$  to reduce colourless nitroblue tetrazolium to blue formazan. The method of McCord et al (123) was modified by changing the concentrations of substrate (xanthine) and dye, until appropriate inhibition was achieved. When  $1.3 \times 10^{-3} M$  xanthine oxidase,  $1.7 \times 10^{-5} M$  nitroblue tetrazolium,  $8 \times 10^{-4} M$  sodium cyanide, 0.025M sodium carbonate, and  $1 \times 10^{-4} M$  EDTA were mixed at pH 10 and at 22°C in air, a linear increase in absorbance due to the production of formazan can be followed at 560 nm in a Beckman DU7 Spectrophotometer with kinetics accessory.

Addition of 5-10 ug sonicated mitochondrial protein inhibited this colour formation by between 20-35% by competing with NBT for the available  $O_2^-$ . Rates of inhibition must not exceed 40% in this assay. Reactions were carried out for 2 minutes, in 1 ml plastic, disposable cuvettes. One unit of enzyme is defined as the amount of protein needed to cause 50% inhibition of colour formation. Cyanide is added to inhibit copper/zinc SOD and/or peroxidase activity. Copper/zinc SOD is a cytosolic enzyme, but may still be present in the mitochondrial preparation. Peroxidase could interfere by peroxidizing the formazan.

f) Assay of Succinate Dehydrogenase

Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate. Inclusion of artificial electron acceptors (reducible dyes) allows one to monitor this enzyme activity spectrophotometrically. In this instance, phenazine methosulphate (PMS) served as the intermediate electron acceptor and 2,6-dichloroindophenol (DCPIP) as the terminal acceptor (124). Each reaction mixture contained: 50 mM phosphate buffer (pH 7.4), 1mM potassium cyanide, 1.3 mM PMS, 0.15mM DCPIP and 0.02M succinic acid, in a total volume of 1 ml. The reaction is started by addition of ~20 ug mitochondrial protein. The linear reduction of DCPIP was followed at 600nm. The appropriate amount of protein is one which reduces the dye but not so fast that the

reaction becomes non-linear before 2 minutes have elapsed.

g) Corticosterone Radioimmunoassay

A direct radioimmunoassay (RIA) of serum corticosterone was developed which requires only 5-10  $\mu$ l serum. The novel features of this assay are that it does not require a time-consuming preparative extraction or chromatography step, requires a small sample volume and has much greater sensitivity than protein-binding methods (125).

The method used was based on that of Dujaili et al (126) and modified to use the commercially available antiserum and tritiated tracer. Antibody dilution (1:2) and tracer concentration (12,000 dpm) were chosen to allow sufficient radioactivity to be counted taking into account tritium counting efficiency (~35%) and optimal conditions for binding of antigen-antibody (30-40% maximum). The pH of the reaction was 3.0, a value reported to maximize the antigen-antibody binding while minimizing the binding of corticosterone to CBG (126). One major difficulty encountered was the separation of free from antibody-bound corticosterone after equilibrium had been reached. Double antibody methods are usually more specific and efficient, but in this case the pellet produced was too fragile to isolate quantitatively. Instead, use of a charcoal suspension was found to be efficient, reproducible and inexpensive.

A 1:2 dilution of antiserum generated against

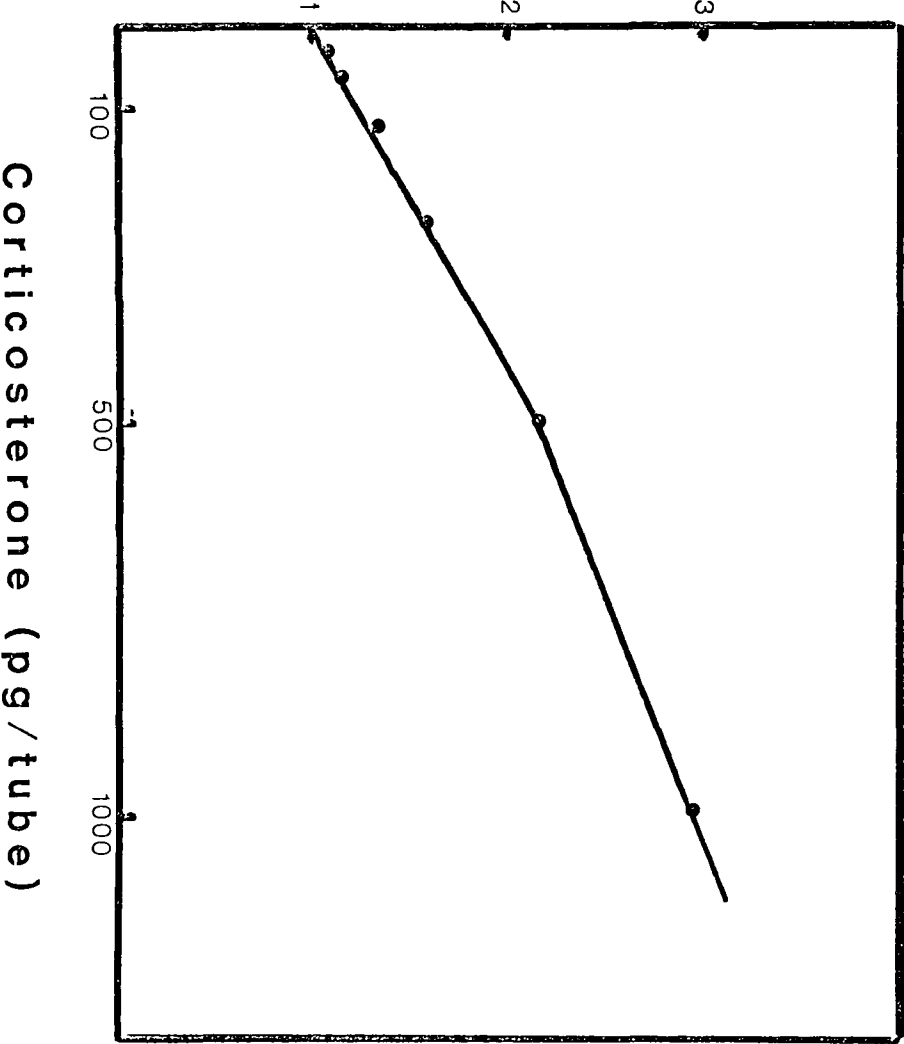
corticosterone-21-thyroglobulin (Miles Scientific Laboratories Ltd., Rexdale, Ont.), and [1,2,6,7<sup>3</sup>H]-corticosterone ( $\sim$ 12,000 dpm) were diluted to 0.55 ml with 0.5 M phosphate/0.025 M citrate buffer, pH 3, in the presence of 5-10  $\mu$ l the untreated serum for samples, or 0-1000 pg/tube cold corticosterone for standards, and incubated for 1 hour at 37<sup>o</sup>C. Separation of antibody bound from free corticosterone was accomplished by addition of dextran coated charcoal suspension (0.5% activated charcoal, 0.05% dextran T70) which adsorbs the free hormone, followed by centrifugation. A portion of the supernatant which contains the antibody bound corticosterone was used for scintillation counting in Aquasol. The sensitivity of the assay is  $\sim$ 30 pg/tube (0.15  $\mu$ g/dl serum). The average percent recovery with this technique was 101.5% (n=3). The interassay coefficient of variation was 33.1% for a low corticosterone serum sample and 7.57% for a high corticosterone serum sample. According to the data provided, the hormone with the highest ability to compete with corticosterone is 11-deoxycorticosterone with a 27% cross-reactivity at 50% displacement. A sample standard curve is provided in Figure 3.

The assay measures total corticosterone per dl serum. The major limitation with this and all other corticosterone assays developed to date, is the inability to distinguish easily between protein-bound (inactive) from free (active)

Figure 3 - Corticosterone Radioimmunoassay  
Standard Curve

The assay was conducted as described in the Methods section.  $C_0$  represents radioactivity (cpm) in a 0.4 ml aliquot of supernatant after charcoal precipitation, when no standard was added.  $C_x$  represents the amount of radioactivity in the 0.4 ml aliquot when known amounts of standard corticosterone were added.

Fraction of Trace Binding Co/Cx



corticosterone. One can assay for total CBG protein, but this still does not yield information on the fraction of corticosterone which is free in serum.

h) Insulin Radioimmunoassay

Levels of serum insulin were determined by radioimmunoassay as described by Dalpe-Scott et al (127). The procedure involves a nonequilibrium, double-antibody technique of high precision and good sensitivity, allowing small sample sizes. 50-100 ul samples of undiluted serum were assayed.

i) Glucose Determinations

Serum glucose was measured by a glucose oxidase method, using a Beckman Glucose Analyzer (Beckman Instruments, Fullerton, California). A 15 ul aliquot of serum was diluted to 60 ul with distilled water prior to analysis.

j) Statistical Procedures

Data comparing obese-ADX to obese-sham were analyzed by the Student's T-test (two-tailed) for effect of lowering serum corticosterone in the obese mouse. Likewise in experiment B, Student's T-test (two-tailed) was used to assess the effect of corticosterone supplementation in lean mice. Results were considered significant with a  $P < 0.05$ .

## RESULTS

Results of experiments A and B are tabulated together. However, data from experiment A are distinguished from those of experiment B as the table is divided ie. Table 1A, Table 1B. This setup was employed to enable the reader to compare data from lean and obese mice while recognizing that the experiments are separate. This is essential to note as manipulations were completely different in the two experiments: serum corticosterone was lowered in obese mice by ADX and raised in lean mice by supplementation.

### 1. Serum Corticosterone

It was necessary to validate corticosterone supplementation experiments by measuring the serum corticosterone levels established in the mice as a result of the two treatments. Preliminary experiments correlating the proportion of corticosterone in the implanted pellets with the resulting serum corticosterone values revealed that pellets with 30% corticosterone raised the serum corticosterone in lean mice to a level comparable to that found in obese mice of the same age provided both groups were sacrificed and blood collected at 9:00 A.M. Still such correlations were found to be difficult - the amount of corticosterone absorbed from the pellet varied with the source of corticosterone employed to form the pellet, and the melting temperature. The same difficulties were reported by Akana et al. (128) For this reason only one

TABLE 1 - SERUM CORTICOSTERONE CONCENTRATIONS

		Serum Corticosterone (ug/dl)	
1A	OBESE		
	Sham	(13)	15.9 <u>+0.78</u>
	ADX	(14)	0.23 <u>+0.02</u> <sup>*</sup>
1B	LEAN		
	Control	(16)	4.84 <u>+0.26</u>
	Suppl	(16)	15.1 <u>+0.54</u> <sup>#</sup>

Immunoreactive serum corticosterone levels were measured as described in detail in the method section in (1A) sham operated (Sham) and adrenalectomized obese mice (ADX), and in (1B) control and corticosterone supplemented (Suppl) lean mice.

Values are means+SEM for the number of animals given in parentheses.

\* Statistically significant effect of ADX.

# Statistically significant effect of corticosterone supplementation.

uniform batch of pellets (ie. prepared from one molten mixture) was used in the final study. Pellets, removed and reweighed at the end of the study, showed that no more than 50% of the hormone had been dissolved from the fused pellets. Release of cholesterol from the pellets is extremely slow (129).

As described by others (12,13,21), serum corticosterone was elevated in the obese mouse compared to the lean (compare control values in Table 1A and 1B). ADX reduced these levels significantly (Table 1A) to values even lower than those found in the intact lean mice. Conversely, our supplementation protocol greatly increased the levels of corticosterone in the lean mouse (Table 1B) so that the levels attained in the supplemented group were similar to those typical of the obese mouse.

## 2. Body weight gain, food intake and feed efficiency

The efficiency of ADX in reducing weight gain and food intake of the ob/ob mouse is well documented (16,17,30,31,68,69). These parameters then provide another check on the effectiveness of ADX and allow a comparison with the trends observed in these parameters upon corticosterone supplementation of intact mice.

As previously reported (2,4,8,10,17) gain in body weight, food intake and feed efficiency were considerably higher in the obese sham group than in lean mice (compare

TABLE 2 - WEIGHT GAIN, FOOD INTAKE AND  
FEED EFFICIENCY

		Weight gain g/14 days	Food Intake g/14 days	Feed Efficiency g gained/g eaten x100
2A				
OBESE				
Sham	(5)	9.6±0.67	85.0±4.07	11.4 ±0.968
ADX	(4)	3.5±1.3*	38.6±3.54*	8.92±1.99
2B				
LEAN				
Control	(5)	4.3±0.30	66.8±3.38	6.53±0.588
Suppl	(5)	3.2±0.58	58.2±3.94	5.56±1.07

The experiments were described in the methods section for (2A) sham operated (Sham) and adrenalectomized obese mice (ADX) and (2B) control and corticosterone supplemented (Suppl) lean mice. Values are means±SEM for the number of animals given in parentheses.

Only data from the latter two weeks of the experiment were used for calculating food intakes as previous intakes were affected by recovery from surgery.

\* Statistically significant effect of ADX.

controls in Table 2A and 2B). Body weight gain and food intake were significantly lowered by ADX but feed efficiency was not significantly decreased by the treatment, although there was a trend to a lower value. It is interesting that ADX reduced food intake to levels well below those found in the intact lean mouse. Some authors (16,17) have reported a normalization of the efficiency of energy retention upon ADX. Failure to observe such a reduction in the present experiments possibly results from use of rat chow rather than the more commonly employed mouse chow in experiments involving mice. The fat content of mouse chow (11%) is more than twice that found in rat chow (4.5%). It is possible that the high fat diet may be better capable of stimulating DIT, and thus of reducing feed efficiency. Failure to note a change in feed efficiency is also in agreement with recent observations by Bégin-Heick and Reid (130) that although in the first three weeks post-surgery the usual normalization in food intake and body weight gain were observed, between 21-42 days post-ADX the rate of weight gain was not significantly different between the ADX and sham operated mice in spite of still decreased intake. Further effects of ADX (eg. increased tibial growth, body density) cannot be duplicated by pair-feeding the obese sham mouse to intake of the ADX mouse (16,17). This shows that the obese syndrome is much

more complex than a simple overeating disorder.

Although corticosterone supplementation of the lean mouse raised serum corticosterone levels, it did not produce a significant effect on weight gain, food consumption or feed efficiency (Table 2B). This shows clearly that in lean mice hypercorticism alone cannot induce hyperphagia.

### 3. Tissue Weights

The weight of the spleen and epididymal fat pad were used as further measures of the success of the treatments. GC administration produces a depletion of lymphocytes from lymph nodes, spleen and thymus, resulting in significant lymphocytopenia. In general it seems that GC excess affects distribution and traffic of cells involved in immune responses rather than their function (78). Due to effects of GCs on the immune system high circulating corticosterone should result in lower spleen weights. Corticosterone supplementation in lean mice as well as ADX in obese mice did have the predicted effects on lymphocyte distribution (Table 3A and 3B). The observed spleen weights thus complement the serum corticosterone results. On the other hand while ADX, as expected, reduced epididymal fat pad weight in the obese mouse, unexpectedly, corticosterone supplementation in lean mice also reduced the weight of this fat pad. In this respect high serum corticosterone levels had the opposite effect in lean and obese mice. A possible explanation for this result is related to the

TABLE 3 - TISSUE WEIGHTS

	Spleen mg (10)	Epididymal Fat Pad mg (10)	BAT mg (15)
<b>3A OBESE</b>			
Sham	44.7±2.6	2070±120	266.7±13.6
ADX	84.6±5.6*	1070±132	134.6± 7.8*
<b>3B LEAN</b>			
Control	75.7±5.7	196±10.5	74.6± 3.1
Suppl	42.2±3.8#	137±12.0#	118.0± 9.9#

Tissue weights were measured as described in detail in the methods section in (3A) sham operated (Sham) and adrenalectomized (ADX) obese mice and in (3B) control and corticosterone supplemented (Suppl) lean mice. Values are means±SEM for the number of animals given in parentheses.

\* Statistically significant effect of ADX.

# Statistically significant effect of corticosterone supplementation.

differential elevations in counter-effective hormones (eg. insulin, see below) in the lean versus obese mouse.

ADX reduced, but failed to normalize, the very high weight of BAT in obese mice. The composition of the tissue was clearly different in the two groups of mice. The total mitochondrial protein yield was higher in obese ADX mice than in the obese shams (Table 7). BAT of supplemented mice also appeared paler in colour than those of the control group, thus suggestive of the fatty infiltration seen in the obese mouse. However, the supplemented mouse was not an obese one, as shown by the reduced weight of the epididymal fat pad. Other researchers (67,72) have found no difference in the wet weight of BAT of lean mice and rats following ADX and steroid replacement. However these authors did not validate their method of corticosterone supplementation by measuring the resulting serum corticosterone levels. It is then impossible to compare data. Also, the lean adrenalectomized mouse is a very different model than the lean mouse with intact adrenals. In the former negative feedback and diurnal cycling by adrenal hormones is abolished.

#### 4. Carbohydrate Metabolism

One of the most often focused upon characteristics of the obese mouse is its hyperinsulinemia and hyperglycemia. Serum insulin and glucose were therefore determined in fed

TABLE 4 - CARBOHYDRATE METABOLISM

		Serum Insulin ng/ml	Serum Glucose mg/dl
4A OBESE			
Sham	(15)	>32	232.0 $\pm$ 14.1
ADX	(15)	4.95 $\pm$ 0.51*	200.0 $\pm$ 8.2
4B LEAN			
Control	(15)	2.61 $\pm$ 0.22	167.5 $\pm$ 6.1
Suppl	(15)	6.32 $\pm$ 0.45#	148.6 $\pm$ 5.4#

The experiments were described in detail in the methods section in (8A) sham operated (Sham) and adrenalectomized (ADX) obese mice and in (8B) control and corticosterone supplemented lean mice. Values are means $\pm$ SEM for the number of animals given in parentheses.

\* Statistically significant effect of ADX.

# Statistically significant effect of corticosterone supplementation.

ADX obese and supplemented lean and their respective controls. Insulin levels were much reduced, but not normalized by ADX, while serum glucose concentrations remained unchanged. It appears as though, while ADX lowered serum insulin levels, it did not improve insulin resistance and suggests that the hyperinsulinemia of the ob/ob mouse is not secondary to hyperglycemia. However, it is very difficult and presumptive to interpret blood glucose data from non-fasted mice as one cannot control the time elapsing between feeding and sacrifice, which should be identical in all mice of all groups. Solomon and Mayer (68) report a reduction in hyperglycemia of non-fasted obese mice following ADX. However, neither Tokuyama et al (20) nor Freedman et al (72) report a significant difference in serum glucose in non-fasted obese sham versus ADX obese animals.

Corticosterone supplementation significantly raised the insulin concentrations in the serum of lean mice (3x), however this fell short of the extreme hyperinsulinemia prevalent in the obese mouse. These higher insulin concentrations in the corticosterone supplemented lean group significantly ( $P < 0.05$ ) reduced serum glucose concentrations suggesting that the lean mouse is more sensitive to insulin than the obese.

TABLE 5- BROWN ADIPOSE TISSUE

		BAT Weight mg	Total DNA ug	Total lip ug	Lip Concentration ug/g BAT      ug/mg DNA	
5A OBF SE						
Sham	(4)	202.2±16.9	190.4±21.0	0.484±.03	2.62±0.33	2.27±.68
ADX	(5)	132.2±17.8 <sup>^</sup>	271.0± 7.19 <sup>*</sup>	1.015±.03 <sup>*</sup>	7.07±0.94 <sup>^</sup>	3.66±.50
5B LEAN						
Control	(4)	66.5± 5.46	128.3±2.06	0.477±.09	7.02±1.2	3.70±.63
Suppl	(6)	100.9±33.2 <sup>#</sup>	199.9±13.6 <sup>#</sup>	0.589±.12	5.74±0.85	2.90±.51

The experiments were described in detail in the methods for (5A) sham operated (Sham) and adrenalectomized (ADX) obese mice and (5B) control and corticosterone supplemented (Suppl) lean mice. Values are means±SEM for the number of animals given in parentheses.

<sup>\*</sup> Statistically significant effect of ADX.

<sup>#</sup> Statistically significant effect of corticosterone supplementation.

## 5. BAT Mn (Table 5A and 5B)

The first premise of this thesis is that the lower BAT Mn content of the obese mouse (112) may be related to the high circulating levels of corticosterone also seen in the obese mouse. BAT Mn content was therefore measured in order to 1) confirm that BAT Mn is lower in the obese mouse 2) test the effect of ADX in restoring BAT Mn, and 3) observe the change (if any) in BAT Mn of lean mice upon corticosterone administration. Expressed per g tissue, ADX significantly increased BAT Mn content in the obese mouse. As the composition of BAT (very high lipid content) of the obese mouse might influence these results, Mn content is also expressed per mg DNA. As DNA content is indicative of cell number the latter units better reflect Mn content/cell. When reported on this basis the Mn content of BAT of the ADX obese mouse was still higher than that of the sham, but the difference did not reach statistical significance. It is interesting to note that both ADX of obese mice and corticosterone supplementation of lean mice caused a significant increase in BAT cell number. Here is another indication that hypercorticism in lean and obese mice has very different effects (also see epididymal fat pad weight, Table 3). Also note that BAT weight data listed on Table 3 differs from that on Table 5. This is because BAT of only those mice of each group which were

used for experiments determining Mn content are included in data for Table 5. On the other hand, BAT of all mice/group were included in the calculation of mean BAT weight shown on Table 3.

Using corticosterone supplementation in lean mice to mimic the GC state of the obese animal, did lower BAT Mn toward levels found in the obese animal, as compared to that existing in the lean control, however the difference between supplemented and control was not significant.

For control lean mice, the value of Mn/unit wet weight (7.024 ppm) was much higher than those reported previously for other tissues (<2 ppm),(83,85). However, as mitochondria are rich in Mn and active BAT contains many mitochondria, the concept that higher levels of Mn exist in this rapidly respiring tissue than in other tissues more commonly assayed (eg. liver, bone), is certainly plausible. Lower values for Mn content in BAT have also been published previously but the methods employed for Mn analysis (flame atomic absorption (112), neutron activation (84)) are much less sensitive (119,131) and required more sample preparation than procedures used for this thesis (flameless atomic absorption of tissue homogenates with a controlled temperature furnace). In employing the older methods Mn is bound to be lost by sample preparation procedures (eg. ashing, centrifuging). Also, only a small portion of the injected sample is actually atomized in flame atomic absorption, whereas using flameless atomic absorption

		nmoles DCPIP reduced/ min/mg protein
<hr/>		
6A		
OBESE		
Sham	(5)	289.6±11.5
ADX	(5)	395.4±26.3*
<hr/>		
6B		
Lean		
Control	(5)	464.0±38.0
Suppl	(5)	526.0±18.3
<hr/>		

The experiments were described in detail in Materials and Methods. Values are means±SEM for the number of animals given in parentheses.

\* Statistically significant (P<0.05) effect of ADX.

# Statistically significant (P<0.05) effect of corticosterone supplementation.

allows the entire aliquot injected to be atomized in a furnace. The latter procedure then yields a more valid result.

#### 6. Mitochondrial SDH (Table 6A and 6B)

SDH activity was used as a measure of potential flux through the TCA cycle. The low thermogenic state of obese mice (19,47) is reflected in lower SDH activity in obese versus lean mice. It was expected that treatments beneficial to BAT (or the contrary) might also affect SDH activity. The experiments on obese mice show that ADX did indeed restore SDH activity to higher levels, close to those observed in BAT of control lean mice. Normalized SDH activity upon ADX then indicates restoration of at least partial functioning to brown fat. While removal of GC via ADX had the expected effect in obese mice, GC supplementation of intact lean mice had no significant effect on SDH. SDH activity was if anything higher in corticosterone supplemented animals than in controls. One expected that if corticosterone impaired BAT function via Mn redistribution, thermogenesis would be depressed as in the obese mouse. This result then is surprising.

#### 7. Mitochondrial MnSOD

MnSOD activity responds very quickly to Mn deficient states (84,99,105). Thus, if BAT Mn content were altered by either of the two treatments tested here, MnSOD activity

TABLE 7 - MITOCHONDRIAL MnSOD

		ug 50% Inhibition	Total Protein ug	U/mg protein
7A OBESE				
Sham	(5)	8.27±0.62	1004.4± 64.9	123.5±19.7
ADX	(5)	9.73±1.20	1663.6±101.5 <sup>*</sup>	110.3±15.1
7B LEAN				
Control	(5)	5.46±0.48	730.6±118.5	188.7±15.9
Suppl	(5)	4.81±0.35	1580.0±214.5 <sup>#</sup>	212.3±14.9

One unit of enzyme is defined as the amount needed to cause 50% inhibition of colour formation. For each group total units per BAT pool were calculated by dividing total protein by the value of one unit. Specific activity was then determined by division of total protein into total units.

Mitochondrial MnSOD was measured as described in detail in the methods section in (5A) sham operated (Sham) and adrenalectomized (ADX) and in (5B) control and corticosterone supplemented (Suppl) lean mice. Values are means±SEM for the number of animals given in parentheses.

\* Statistically significant effect of ADX.

# Statistically significant effect of corticosterone supplementation.

may be compromised. The obese mouse has lower BAT Mn/g tissue and reduced MnSOD activity compared to lean (112). However, ADX did not appear to restore MnSOD activity in obese mice (Table 7A) even though as previously mentioned the Mn content of BAT, (Table 5A) was elevated to levels comparable to those observed in lean mice. The superoxide anion is a by-product of electron transport. The slight elevation in MnSOD activity upon corticosterone supplementation in lean mice versus control (Table 6B) is in keeping with the slight elevation also observed in SDH activity in this group as the superoxide anion is a by-product of electron transport.

#### 8. Lipid Peroxidation

The SOD enzyme activity represents one of the major cell defence mechanisms against free radical attack. Initiation of lipid peroxidation should therefore be more frequent when SOD activity is compromised. It was therefore of interest to determine whether lipid peroxidation was different in BAT of obese and lean mice and whether it was altered by the respective treatments. Lipid peroxidation was measured in whole homogenates and in isolated mitochondria. A higher rate of lipid peroxide formation was found in BAT homogenate of obese than of lean mice. ADX significantly decreased homogenate lipid peroxidation (Table 8A) but no effect of GC supplementation

TABLE 8 - LIPID PEROXIDATION

		Homogenates	Mitochondria
		nmol MDA/mg protein	
<hr/>			
8A			
OBESE			
Sham	(5)	96.78 $\pm$ 3.04	135.9 $\pm$ 5.09
ADX	(5)	67.58 $\pm$ 5.53*	159.3 $\pm$ 8.76
<hr/>			
7B			
LEAN			
Control	(5)	56.88 $\pm$ 1.32	169.6 $\pm$ 3.83
Suppl	(5)	58.84 $\pm$ 3.45	182.9 $\pm$ 10.0
<hr/>			

Lipid peroxidation was measured as described in detail in the methods section in (7A) sham operated (Sham) and adrenalectomized (ADX) obese mice and in (7B) control and corticosterone supplemented lean mice. Values are means $\pm$ SEM for the number on animals given in parentheses.

\* Statistically significant effect of ADX.

# Statistically significant effect of corticosterone supplementation.

was found (Table 8B). Specific activity of lipid peroxide formation was higher in the mitochondria than in the homogenates reflecting the fact that most of the radicals are produced by the mitochondrial electron transport chain and the fact that in the cytosol Cu/ZnSOD can sequester superoxide.

The expected pattern of higher lipid peroxide production in the obese mouse than that in the lean is more evident in the homogenates. This is likely due to the much larger store of lipid substrate available in the obese LAT homogenate. Superoxide does not necessarily act near its site of formation, but rather near its site of conversion to hydroxyl radical. The intracellular location of such sites is not known.

## DISCUSSION

### Experiment A - ADX of The Obese (ob/ob) Mouse

#### 1.) Age of Onset of Hypercorticism in The Obese Mouse

Central to research exploring the cause and effects of hypercorticism in the obese mouse, is the question of sequence: are elevated levels of serum corticosterone secondary to obesity or a primary etiological factor in the obese syndrome? Dubuc (14) produced a longitudinal profile of basal plasma corticosterone in obese mice between 17-56 days of age and found only minimal elevations at 17 days of age despite an increase in body fat, hyperinsulinemia and hypoglycemia at this stage. Dubuc points out that if the primary genotypic abnormality involved was hyperadrenocorticism, the greatest difference between the two genotypes would be in the weaning period (<17 days of age). However, careful scrutiny of Dubuc's data reveals a reported serum corticosterone in 17 day old lean C57Bl/6J mice of 28 ug/dl. This is very high - in fact all serum corticosterone values in lean mice reported in Dubuc's paper are much higher than those reported by: Dubuc himself in an earlier paper (13),  $4.8 \pm 0.9$  ug/dl; Herberg et al (15),  $3.0 \pm 0.3$  ug/dl; Smith et al (18),  $\approx 3$  ug/dl; and this thesis  $4.84 \pm 0.3$  ug/dl. There are two possible explanations for the high corticosterone levels

obtained by this author. First, he did not mention at which point during the diurnal cycle the samples were collected. Second and most likely, the crucial importance of minimizing stress during blood collection for corticosterone measurements was not widely appreciated in 1977. Dubuc's procedure of anesthesia and bleeding from the retroorbital plexus could have increased the corticosterone levels by as much as 3 or 4 times (17). Stress in obese mice also increases the corticosterone level, but since basal levels are already much higher, the response is blunted and the proportional increase much lower. Reliable, sensitive, affordable methods for corticosterone radioimmunoassay have only recently become widely available and applied. Even so, there is little consistency in source and specificity of antibody, buffer system, and separation methods from one laboratory to another. Therefore the reported non-significant difference in serum corticosterone in lean versus obese mice at 17 days may be in error and the hypothesis that elevated corticosterone plays a primary role in the development of the obese syndrome is still a worthwhile one. Unfortunately most investigators refer to the aforementioned results of Dubuc when reflecting on the sequence of metabolic abnormalities in the ob/ob mouse. No subsequent longitudinal profile of corticosterone levels, including the pre-weaning period, has since been published. Yukimura and Bray (29) have however demonstrated that ADX during the pre-weaning period

ameliorates many of the symptoms of the obese syndrome.

## 2.) Direct or Indirect Effect of GCs on BAT

As one of the prominent features of the obese syndrome is impaired thermogenesis by BAT it is worth investigating the possible effects of hypercorticism on BAT. GC-receptors have been reported in brown fat (132) therefore a direct effect of GCs on BAT thermogenesis is conceivable. Certainly GCs are required in permissive amounts for the normal thermogenic responses of BAT to cold and NE (133). Thus, the apparent inhibitory action of hypercorticism on thermogenesis (134,135) may reflect an indirect effect. The possibility was therefore investigated that redistribution of Mn under the influence of GC may cause a relative Mn deficiency in BAT. Mn deficiency is known to result in lower than normal MnSOD and higher than normal levels of mitochondrial lipid peroxidation (106).

## 3.) GC Excess and Mn Distribution

Initially several possible mechanisms were envisaged to explain why GC excess might result in a disparate Mn distribution amongst tissues. Hughes et al (114,115) demonstrated that administration of exogenous GCs in mice markedly affected the tissue distribution of the radioisotope <sup>54</sup>Mn. Removal of radiomanganese from the liver was accompanied by a percentile increase in the carcass as a consequence of these treatments. GCs have also been implicated in disparate distribution of other

trace minerals in the ob/ob mouse, such as zinc, via altering concentrations of metal binding proteins (136). It is conceivable then that transmanganin, the blood Mn transporter, is affected by hypercorticism. Another possibility is that elevated corticosterone levels (and accompanying high insulin levels) increase the activity of many oxidative enzymes and pathways thus increasing mitochondrial superoxide production and MnSOD requirements. Nishigori et al (116) found that GCs caused the elevation of hepatic lipid peroxides. It may be that under conditions of high GC, Mn is rerouted to tissues more essential to the survival of the animal (eg. liver, kidney) or to damage (ie. lens of the eye) at the expense of others such as BAT. At present, there is no firm evidence for any of these mechanisms. In fact ADX of normal mice was found not to evoke the opposite effect to that of GC administration, on the Mn radioisotope distribution (114). It has very recently been demonstrated that ADX had no effect on metallothionein the zinc binding protein, nor zinc levels in either the lean or the ob/ob mouse (130). Data presented in this thesis show that alteration of BAT Mn content/cell by ADX of obese mice or by GC administration to lean mice is not statistically significant. Nevertheless, as serum corticosterone levels increase, a trend toward lower BAT Mn content/cell is evident. Perhaps a larger sample size would show whether or not this trend indicates a redistribution process.

Also, due to implantation methodology, these experiments could only be carried out for 18 days (see page 62). It is likely that changes in tissue Mn concentration require a longer treatment period. The findings of Hughes et al (115) of altered tissue Mn concentrations after corticosterone supplementation resulted from experiments involving treatment periods of two months.

#### 4.) ADX of the ob/ob Mouse

Previous studies of the response of ob/ob mice to ADX have given variable results. Some authors (16,17) report no changes in the obesity that had developed prior to surgery despite post-surgical reductions in weight gain and food intake. Others (63,137) find that ADX results in the maintenance of food intake, body weight and body density at the same level as in the lean controls. The variability may be associated with the age of the mice. In suckling ob/ob mice, a larger proportion of body fat has not yet developed whereas in post-weaning ob/ob mice body energy density is already very different from that of their lean littermates. This suggests that GCs may be necessary for achieving, but not maintaining obesity in the obese mouse (17). ADX of five week old mice did not alter obesity (body weight) which had existed prior to the surgery. Only the post-surgery rate of weight gain was reduced compared to shams. In very young mice, GC-induced Mn-deficiency of BAT could result in peroxidative damage, compromising the

function of this energy buffering tissue. In older mice, restoration of appropriate Mn levels by ADX might not be able to reverse cell damage which has already been sustained in BAT.

If GC were important in Mn distribution, ADX of obese mice should have resulted in restoration of BAT Mn levels to those found in the lean control animal. A corresponding increase in MnSOD activity and decrease in lipid peroxidation were also expected. Instead while SDH activity and lipid peroxidation of homogenates were normalized, MnSOD, and Mn content per cell were not significantly altered by ADX, however, as mentioned on page 59, a trend toward expected changes in these latter parameters was observed. Either GC-induced Mn redistributions are not reversible (at least not in post-weaning mice), or observation of statistically significant changes in these parameters requires a longer treatment period, or some other mechanism is responsible for the low Mn contents of BAT observed in obese mice. Mitochondrial lipid peroxidation in obese sham mice was not different from that in lean controls. Thus, although MnSOD activity was also lower in the BAT of obese mice (compared to lean) it was probably sufficient to protect the mitochondria from superoxide attack at least at ambient temperatures ( $\sim 24^{\circ}\text{C}$ ). A longer treatment period may result in a lowering of Mn levels, and thus MnSOD activity, below a critical value where it could no longer protect against superoxide attack.

Further experiments are necessary before we can recognize the significance of the trends observed in BAT Mn content/cell, BAT MnSOD activities, and BAT lipid peroxidation data.

#### Experiment B - GC Administration to Lean Mice

##### 1.) Choice of Methodology

Subcutaneous implantation of corticosterone pellets (129) was the chosen method of GC supplementation in view of its simplicity, low cost, the establishment of stable circulating concentrations of steroid and minimum stress on the animal. Alternative methods include daily injections (16,135,138) and inclusion of corticosterone in drinking water (71) or chow (134). Limitations of such methods include the handling and stress associated with injections, the lack of control associated with incorporation in feed or water and most importantly the phasic signal accompanying all three of these methods (128). Most previous studies used these latter methods. Furthermore, in none of the published studies of the effect of administered GC, using any method, has the resulting concentration of corticosterone in the blood been measured. Here we have validated, by corticosterone RIA, that the blood levels achieved during GC administration by implantation are within the range of those observed in non-implanted obese mice of the same age. Pellet implantation however does have one major limitation. For reasons not clearly

understood circulating blood levels of corticosterone slowly begin to decline with time after approximately 2 1/2 weeks (128). For that reason experiments for this thesis were carried out for only 18 days.

## 2.) Glucocorticoid Excess in Lean Mice

No characteristic for the obese syndrome was manifested in the lean GC supplemented mice. These mice did not become hyperphagic, demonstrated no accelerated weight gain or change in metabolic efficiency, and showed no changes in Mn status or metabolism in BAT. With the exception of the Mn data, which are reported here for the first time, these findings support those reported previously (16,20) in the lean C57Bl/6J +/+ mouse. Some authors have demonstrated that implanting corticosterone pellets does induce adiposity in non-inbred obese mice of the Swedish colony (63), C57/6J mice (139), and C57BL10ScSn mice (134). Obviously there is a variable response amongst mice of different strains. It is well documented that treatment of adrenalectomized obese mice with corticosterone restores the phenotypic features of the ob/ob syndrome (16,20). Still such treatment does not produce comparable changes in the lean mice (16,20) of the C57BL/6 strain. It has recently been suggested that an enhanced responsiveness of the ob/ob mouse to blood corticosterone could explain some of the phenotypic changes occurring in obese mice, but not lean mice, when both groups have equal blood corticosterone levels (20,21) which

would explain the failure to duplicate the metabolic anomalies of the obese mouse by supplementation in the lean mouse.

### III. Enhanced Responsiveness of the Obese Mouse to GCs

#### 1.) Biologically Active Corticosterone

The hypersensitivity to GCs observed in the obese mouse may be related to differential proportions of free and protein-bound corticosterone, in the serum. Most studies demonstrate that both CBG-bound and albumin-bound corticosteroids are biologically inactive. The most compelling evidence for this comes from an inability of CBG-cortisol to evoke a GC response in vivo (glycogen deposition (140) or in vitro in GC responsive cells (induction of glycogen synthetase (141) and inhibition of carbon dioxide production (142))). Naeser et al (143) report that the plasma corticosterone binding capacity of obese and lean mice are not different. However these data were derived from obese mice of the Swedish colony not the C57BL/6 ob/ob mouse discussed in this thesis. The only reference to corticosterone distributions between free and bound fractions (15) in the ob/ob mouse, shows that free endogenous corticosterone levels in ob/ob mice (0.68 ug/dl plasma) were 5-6 times higher than those found in lean +/+ mice (0.12 ug/dl plasma). Thus, the obese mouse may appear more responsive to corticosterone due to a higher amount of biologically active steroid. However the accuracy of these

data are questionable. The authors used the only available method for obtaining such data, equilibrium dialysis, a procedure fraught with difficulty. Equilibrium dialysis can be used for the assay of binding capacity only by performing a series of experiments at different concentrations of steroid. In Westphal's (144) opinion, there is no simple, satisfactory method available to measure the CBG concentration in a serum or plasma. Among the reasons cited for the difficulties are the very low CBG concentrations in the blood, possible interferences with other binding proteins and competitive as well as non-competitive inhibition of binding by other steroid hormones. Nevertheless, by equilibrium dialysis one can approximate the number of binding sites per unit of protein, and the association constant for the steroid-protein complex. Using this information and a set of theoretical formulas designed by Westphal, the proportions of free and bound steroid can be derived. Unfortunately the amount of free corticosterone found in the serum of lean +/+ mice as reported by Herberg and Kley (15) tells us nothing about the amount of free corticosterone that might be found in the serum of a lean corticosterone supplemented mouse. The latter would be more valuable information to assess the apparent GC hypersensitivity of the obese mouse.

## 2.) Glucocorticoid Receptors

A further explanation for differential sensitivity between lean and obese C57BL/6 mice may be found by

examining what happens to GCs at their target cells. GC receptors are found in essentially every mammalian tissue, including BAT (145). However the binding of GC to its receptor seems to elicit a different set of responses in BAT as compared to other tissues. Fain (146) found that GCs did not inhibit glucose oxidation by BAT in the rat whereas in all white fat pad depots and muscle, inhibition occurred upon GC binding by cellular receptors. Phosphoenolpyruvate carboxykinase (PEPCK) is a major regulatory enzyme of gluconeogenesis; GCs affect the activity of this enzyme in white fat but not BAT (147). Furthermore GCs have been found to inhibit the proton conductance pathway of BAT significantly (20,62,134). Of course as no other tissue has a proton conductance pathway this action is unique to BAT. Thus if a change in GC receptor status, efficiency of GC binding by that receptor, or alterations in the interaction of the GC-receptor complex with DNA, were to exist in the obese mouse, a possible explanation for its differential sensitivity to GCs would be provided. Unfortunately no investigations of this sort have been performed on the ob/ob mouse. Shirwany et al (138) have reported a refractoriness of liver cells to GCs following a single large dose of corticosterone in rats. The authors suggest that regeneration of GC receptors is responsible. Perhaps in the obese mouse, chronically high levels of corticosterone stimulate higher

levels of GC receptors in tissues than is the case in lean mice. Upon GC replacement following ADX, this increased number of receptors could be responsible for the differential response in obese versus lean mice.

Finally on the question of GC sensitivity it is worth noting that the spleen weights were reduced to an equal extent in both sham obese and lean corticosterone supplemented mice. Obviously the lean and obese mouse are equally sensitive to GCs at least at the immunological level.

### 3.) Hyperinsulinemia

GC excess can induce other important changes not examined closely in our experiments, which may act simultaneously to affect phenotypic changes characteristic of the obese syndrome. One important example is insulin. In sham-operated obese mice plasma insulin concentration was 16 times that of lean mice, while plasma corticosterone was roughly 4 times that of lean mice. In the corticosterone supplemented lean mice insulin levels were only 3-4 times higher of the controls. Perhaps it is an abnormal insulin secretory response to GC excess in the obese mouse which is the differential factor. Many instances of abnormal insulin secretion in response to a given stimulus have been reported for the obese mouse (2,148).

### IV.) Summary

If GCs do play a primary role in the obese syndrome,

it does not appear to occur via effects on Mn distribution. Lower concentrations of Mn in BAT and liver (112) of the obese mouse do not appear to be a consequence of hypercortism as supplementation of lean mice with corticosterone did not alter significantly Mn content of BAT. However, further experiments, particularly longer treatment periods are needed to confirm this conclusion. Hypercortism alone cannot duplicate the traits of the obese syndrome as intact lean mice with levels of total corticosterone equivalent to that found in the obese mouse, did not become hyperphagic, obese, or severely hyperinsulemic. GCs then could only act as primary effectors by an ability to overstimulate another metabolic process(es) in obese mice, that remains stable in lean mice. Hypersecretion of insulin is a likely candidate for such a process. This anabolic hormone stimulates lipogenesis and the phenomenon of insulin resistance has been suggested to play a role in inhibition of BAT thermogenesis (149). On the other hand, as previously shown and confirmed here, ADX alone can normalize some aspects of the obese syndrome. This points to a primary role for GCs. Therefore, the two treatments, ADX of obese mice, and GC supplementation of lean, seem to lead to opposite conclusions. Resolution of this puzzle may be provided by evoking the recent proposal that the obese mouse is more sensitive to the action of GCs than is the

lean mouse (20,21). However, much more information in the areas of 1) the GC receptor interaction with the hormone and post receptor binding events, 2) total versus free (active) corticosterone, and 3) the role of the adrenal gland in the development of abnormal insulin homeostasis in ob/ob mice must be provided before such a proposal can be substantiated.

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