CONTROL OF BROWN ADIPOSE TISSUE THERMOGENESIS BY PITUITARY-ADRENAL HORMONES IN THE OBESE ZUCKER (fa/fa) RAT

by

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ABSTRACT

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CONTROL OF BROWN ADIPOSE TISSUE THERMOGENESIS BY PITUITARY-ADRENAL HORMONES IN THE OBESE ZUCKER (fa/fa) RAT

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The specific binding of [^{3}H] GDP to isolated brown fat mitochondria has been used extensively to assess the activity of the thermogenic function of the tissue. BAT GDP binding is reduced in obese fa/fa rats in comparison to their lean littermate controls. Corticotropin (ACTH) stimulated BAT mitochondrial GDP binding in young obese fa/fa rats to the levels observed in lean rats. This effect was maximal at 24 hrs and attenuated by chronic treatment. The stimulatory response to ACTH was only observed in lean rats at high dose levels of ACTH or at lower doses when endogenous secretion of corticosterone was absent in adrenalectomised rats maintained on exogenous corticosterone. Scatchard analysis of GDP binding showed that ACTH increased maximum binding (B_{max}) for both high and low affinity sites, without a major change in their dissociation constants (K_d). ACTH increased serum triiodothyronine levels in the obese but not lean rat, but increased serum insulin in both lean and obese rats. Chronic treatment with ACTH reduced food intake and also reduced weight gain and fat deposition in obese fa/fa rats.

Treatment with propranolol, a β-antagonist, suppressed the stimulatory effect of ACTH, observed in obese fa/fa rats. This suggested that the acute ACTH stimulatory effect was sympathetically mediated. Further experiments demonstrated that metopirone, a corticosterone biosynthesis inhibitor, restored BAT mitochondrial GDP binding in obese fa/fa rats to control values.

Together with other published data, these results suggest that the pituitary-adrenal system may be important in the regulation of BAT thermogenesis, corticosterone having an inhibitory effect and ACTH a stimulatory effect, possibly being mediated through control of the sympathetic nervous system.

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To My Mother, With Love

ABBREVIATIONS

Adx	adrenalectomy
ACTH	adrenocorticotropic hormone
BAT	brown adipose tissue
B_{max}	maximum binding
BSA	bovine serum albumen
DIT	diet induced thermogenesis
EDTA	ethylenediaminetetraacetic acid
GDP	guanosine diphosphate
HEPES	2-(N-2-hydroxyethylpiperazine-N'-2) ethane sulphonic acid
IBAT	interscapular brown adipose tissue
Kd	dissociation constant
NST	non shivering thermogenesis
T_3	triiodothyronine
T_4	thyroxine
TRIS	tris (hydroxymethyl) aminomethane
VMH	ventromedial hypothalamus
WAT	white adipose tissue

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Obesity

Obesity has been defined as an excessive accumulation of fat in the body. Obesity, at present, is one of the major health problems in Western Societies. Statistics show that men who are overweight by about 20% have a 30% greater mortality rate, mostly from hypertension and heart disease, than those of desirable weight *(Curtis-Prior, 1983)*. In the Eastern Societies, particularly in the Middle East, obesity seems to have received very little attention, although a number of diseases and health disorders in close association with obesity have been reported.

However, the first law of thermodynamics is important in any discussion of obesity. It can be summarized by the equation below:

Energy intake = Resting metabolic rate + Work done + Thermogenesis ± Energy stored

1.1.1 Energy intake

All energy intake occured as food or infused nutrients. However, not all the gross energy of food is available to the animal, as some is lost in the faeces and urine. The remaining energy, the metabolisable energy, is available to the rat.

In order to find whether the increase in food intake can cause the overweight, experiments have been done on some adult rats at the age of 4-7 months. They were fed a highly palatable cafeteria diet in addition to their chow diet, and they increased their body weight by about 50% *(Sclafani and Springer, 1976; Rothwell and Stock, 1983b)*. However, while such weight gain may be typical of adult rats, younger rats (4-5 weeks old) do not show any significant increase in body weight after allowing them to have a cafeteria diet, despite increasing energy intake, as an increase in energy expenditure occurred instead *(Rothwell and Stock, 1982b)*.

1.1.2 Resting metabolic rate

Resting metabolic rate is the basal part of all energy expenditure. It is defined as the oxygen consumed under very precise experimental conditions, i.e. at rest and postabsorptive.

The value will be lower during sleep and is variable during

the waking day. It might be changed according to weight, age and sex, and is often expressed in the same term as food intake, i. e. kilo-calories per kg per day *(Miller, 1983)*.

1.1.3 Physical activity

The energy required for physical activity is also important in energy balance. Exercising young rats (7 weeks old) gained less weight than free eating sedentary controls. The increase in energy expenditure associated with exercise also resulted in a significant decrease in the percentage of fat in the carcass *(Crews et al., 1969)*. Similarly, wheel running rats (4 months old) have a lower body weight than do their non-running littermates *(Sclafani and Springer, 1976)*. Active animals always maintain lower body weight than do sedentary ones. For instance, when exercise accompanies hyperphagia, weight gain will be reduced significantly, but not prevented. Four month-old rats having a cafeteria diet in addition to their chow, and housed in the running wheel, still gain weight more than their control littermates *(Sclafani and Springer, 1976)*.

1.1.4 Thermogenesis and energy balance

The metabolized energy from food is utilized for maintenance, work, growth and heat production. Energy utilization which is not associated with net work will result in heat production.

a) Heat production in response to cold

There are two types of adaptive heat production in response to cold:

i) Shivering thermogenesis: The mechanism responsible for heat production in shivering involves rapid contraction and relaxation of the muscles when acutely exposed to cold. Shivering thermogenesis usually predominates, but as the duration of exposure increases, this is gradually replaced by non-shivering thermogenesis (NST). Cold acclimated animals will exhibit a high rate of heat production without any visible signs of shivering. NST is more effective at maintaining body temperature, while shivering interferes with locomotion and coordination *(James and Trayhurn, 1981)*.

ii) Non-shivering thermogenesis: This takes place without muscular contraction. NST occurs not only during chronic cold exposure, but it also plays an important role in thermoregulation of the new-born of many species and the arousal of hibernating animals, such as the golden hamster *(Trayhurn and James, 1981)*. It is also important in maintaining a 'set' body temperature in homeothermic animals. NST is under the control of hypothalamic thermoregulatory effector pathways which mediate their control principally through the sympathetic nervous system *(Jansky, 1973)*.
However, the main site responsible for NST is brown adipose tissue (More details in Section 1.4).

b) Dietary-induced thermogenesis (DIT)

This is the increase in metabolic rate which follows the ingestion of food, and it consists of two quite separate obligatory and adaptive components.
The energy costs of digestion, absorption and also the cost of synthesizing body fat and protein is considered as obligatory DIT. However, if variation in the level of intake (the plane of nutrition) results in disproportionate increases in heat production, and with low energy gains, this could be considered as an adaptive DIT. This mechanism occurs to resist weight gain and obesity during overfeeding. Young (4-5 weeks old) Sprague-Dawley rats show a good example of DIT when they are overfed. Overfeeding by giving them cafeteria diets may cause a rise in heat production sufficient to prevent any excess weight gain *(Rothwell and Stock, 1980a)*. Similarly, when young rats fed a high fat diet or high carbohydrate cafeteria diet increase their energy intake, energy expenditure (thermogenesis) is also increased with both kinds of diet *(Rothwell and Stock, 1983b)*. However, this increase in thermogenesis is dependent on age and strain of rat. When rats of different ages (3.5 and 6.5 months old) were fed cafeteria diets, an increase in energy intake occurred in both groups by about 30%. Although an increase in body weight was observed in both groups, it was significantly higher in the older rats *(Rothwell and Stock, 1983b)*, while

energy expenditure was significantly higher in the younger 3.5 month-old rats *(Rothwell and Stock, 1983b)*. These results suggested that rats aged 3.5 months can exhibit DIT in response to overfeeding, but the capacity for thermogenesis is decreased with age and nearly absent in 6.5 month-old rats. DIT results from the sympathetic activation of brown adipose tissue, and is therefore very similar to NST *(Rothwell and Stock, 1979)*. More details are given in Section 1.4.

Thus, in order to maintain steady body weight and maintain energy balance, any increase in food intake must be compensated by increased energy expenditure. Although many investigators have suggested that overweight results from overfeeding and this has been discussed recently - for review see *Miller (1983), Miller and Parsonage (1975) and James and Trayhurn (1981)*.

However, obesity could also be the result of a defect in energy expenditure, particularly thermogenesis *(Jung et al., 1979)*, as a reduction in thermogenesis has been found in some genetically obese animals *(Holt et al., 1983; Himms Hagen and Desautels, 1978; Bazin et al., 1983)*.

1.2 Animal Models of Obesity

The great difficulty in identifying the factor responsible for the development of obesity in man has led to considerable interest in animal models. A wide range of such models is now used in experimental studies on regulation of energy metabolism *(Bray and York, 1979)*.

They can be divided into:

1. Experimentally induced obesity.
2. Genetically inherited obesity.

1.2.1 Experimental obesity

a) Hypothalamic obesity: The syndrome of hypothalamic obesity has been produced by a wide range of techniques. It can be produced by electrical lesion or knife cuts in the region of the ventromedial hypothalamus, or by chemical methods e.g. injection of monosodium glutamate or gold thioglucose *(Bray and York, 1979; Powley and Opsahl, 1974)*. Ventromedial hypothalamic lesions are associated with increased food intake in adult rats, as a region

of the brain that inhibited feeding, the so-called 'satiety centre' had been destroyed. Hypothalamic hyperphagia was followed by rapid gain in body weight, associated with the accumulation of extra lipid deposits and the appearance of obesity *(Bray and York, 1979; York, 1983)*. In weaning rats ventromedial hypothalamic lesions do not induce hyperphagia. The obesity in these rats results solely from the reduction in energy expenditure *(Frohman, 1978; Frohman et al., 1969)*. However, at both ages ventromedial hypothalamic obesity is associated with excess secretion of insulin (hyperinsulinemia) *(Frohman, 1978)*.

b) Dietary induced obesity

There are a number of different methods for inducing a dietary obesity, the degree of obesity showing great variation with animal strain and diet type.

i) High fat diets. Both rats and mice exhibit excessive weight gains when fed a high fat diet. The weight gain is mainly due to extra fat deposition, although a small increase in body protein has also been reported *(Schemmel et al., 1970; Lemonnier, 1972)*.
High fat diets generally produce large increases in body weight and obesity, but there are exceptions. For example, in studying seven strains of rats, *Schemmel et al. (1970)*, found that only very small weight gain occurred in some strains, while others, such as Osborne-Mendel, became massively obese after having a high fat diet.

ii) High carbohydrate diet. This diet may be used to induce a mild form of obesity in rats. Most high carbohydrate diets have included sucrose as a major component of the dietary energy - the sucrose being included either in the food or as a solution in the drinking water, as a supplement to the normal chow diet *(Kanarek and Hirsch, 1977)*. Energy intake is increased by the addition of sucrose to the diet. However, the increase in body weight may vary from a small or even negative effect to a substantial increase in body fat after sucrose feeding *(Kanarek and Marks-Kaufman, 1979)*.

iii) The cafeteria snack food diet. The cafeteria feeding regimen first introduced by *Sclafani and Springer (1976)*, involves offering rats various palatable foods, e.g. chocolate, bananas, cheese, biscuits, in addition to their normal chow.

As a result, the rat fails to regulate its energy intake to the normal amount, since it eats more food each day and the food has a higher energy density, energy intake may be increased by over 50% *(Rothwell and Stock, 1982b)*, and there may be a considerable weight gain. It has been reported that over 90% of any excess weight gain consists of fat deposition with a consequent large increase in body energy store *(Sclafani and Gorman, 1977; Sclafani and Springer, 1976)*. However, the response of rats to cafeteria feeding is very variable and many animals are able to compensate for the further excess energy intake by increasing energy expenditure, so that there is no increase in body energy stores *(Rothwell et al., 1982b)*. Young rats in particular often fail to gain any weight despite increasing dietary intake by over 50% *(Rothwell and Stock, 1982b; Rothwell et al., 1982a)*. The capacity for compensatory diet induced thermogenesis appears to decrease with age *(Rothwell and Stock, 1979, 1980a)*. The mechanism involved in this diet related thermogenesis will be discussed in a later section (Section 1.4).

Another form of dietary-induced obesity results from meal feeding in which the availability of food is restricted to 1 or 2 short periods each day. Rats normally 'nibble' throughout the day, eating up to 12 meals in each 24 hour period. Meal-feeding results in an increase in the percentage of fat in the body, although body weight often remains normal *(Le Magnen and Devos, 1970; Le Veille, 1970; Cohn et al., 1965)*.

1.2.2 Genetically inherited obesity

There are now several animal models of genetically inherited obesity available for research *(Bray and York, 1971, 1979)*. In a number of these models, the obesity is transmitted through a single gene defect which may be of either a dominant (yellow obese mice AY/a)

or recessive nature, some have polygenic inherited, e.g. New Zealand mouse NZO, Japanese/Toronto mouse KK *(Dulin and Wyse, 1970)*. The most common of these models are those resulting from a single recessive gene defect, e.g. the obese (ob/ob) and diabetic (db/db) mice, and the fatty (fa/fa) rat. The genetically obese fa/fa Zucker rat is a good rodent model for early onset type obesity. It has been used in the studies described in this thesis.

1.3 The Zucker 'fatty' fa/fa Rats

This mutation was first described in 1961 by *Zucker and Zucker*. It arose from a cross between Sherman and Merck Stock M rats *(Zucker and Zucker, 1961)*. The single recessive mutant gene, which causes the obesity, is called the 'fa' (fatty) gene. Obese rats are designated fa/fa genotype. Both the homozygous dominant rat (Fa/Fa) and the hetrozygote Fa/fa are lean *(Zucker and Zucker, 1963)*, although the presence of the recessive gene can be shown in the hetrozygote for a number of metabolic changes, e.g. diet-related stimulation of oxygen consumption and brown adipose tissue thermogenesis *(York et al., 1984)*.

1.3.1 Characteristic of obesity in obese (fa/fa) rats

a) Increased adiposity. Obesity in obese fa/fa rats is characterised by a marked increase in fat cell size (hypertrophy) with an accompanying increase in the number of fat cells (hyperplasia) in comparison with their lean littermates *(Johnson et al., 1971; Cleary et al., 1979)*. Hypertrophy of fat cells can be detected at an early age (7 days) whereas hyperplasia varies from site to site and with age *(Cleary et al., 1979)*. Both hypertrophy and hyperplasia continues to increase at an accelerated rate beyond week 14 of the rat's life *(Cleary et al., 1979)*. As a result of both hyperplasia and hypertrophy, an enlargement in the adipose depots occur, the depots ultimately becoming several fold larger than those seen in normal rats *(Johnson et al., 1971)*. The greatest accumulation is observed in the abdominal pads, i.e. the retroperitoneal area and the gonadal pad *(Johnson et al., 1971)*. The increase in fat cell size and number is accompanied by a marked increase in the activities of thymidine kinase and DNA polymrase, enzymes associated with proliferate activity of pre-adipocytes *(Cleary et al., 1976; 1979)*. These enzymes remain

elevated in fa/fa adipose tissue beyond the age at which the enzyme activity declines in tissues derived from lean rats *(Cleary et al., 1976)*. Therefore it is possible that the continued adipocyte proliferation in obese fa/fa rats occurs in response to excessive demand for lipid storage, which results from the enhanced lipogenesis *(Johnson et al., 1978)*.

Adipose tissue lipoprotein lipase activity, an enzyme associated with storage of circulating triglyceride, also shows an increase in obese fa/fa rats *(De Gasquet et al., 1973)*. Plasma triglyceride is elevated in obese fa/fa rats *(Barry and Bray, 1969)*. So it appears that the increase in lipoprotein lipase activity is not enough to decrease the concentration of circulating triglyceride *(De Gasquet et al., 1973)*. The body weight of obese fa/fa rats is significantly higher than that of the lean rats. At 26 weeks of age, obese fa/fa rats are about 200 g heavier then their lean littermates *(Bell and Stern, 1977)*.

The somatic growth of obese fa/fa rats is stunted, skeletal muscles are found to be smaller in the obese than in the lean *(Reeds et al., 1982)*. A reduction in deposition of carcass protein was also shown in fa/fa rats, particularly in older ones *(Radcliffe and Webster, 1976)*.

b) Energy imbalance

i) Food intake. Hyperphagia is a characteristic of the obese fa/fa rats. Total food intake of the obese fa/fa rats exceeded that in the lean by about 40% *(Bray and York, 1972)*. The meal pattern of obese fa/fa rats included enlarged meal size and decreased meal frequency *(Becker and Grinker, 1977)*.

Studying the diurnal feeding pattern shows that the largest proportion of excess food intake is during the light period, but they also eat more than the lean rats during the dark period *(Haberey et al., 1980; Becker and Grinker, 1977)*.

Studying the regulation of energy intake in obese fa/fa rats shows that when obese fa/fa and lean rats were fed diets varying in composition, such as a high protein, high carbohydrate and high fat diets and after dilution of the diet with indigestible bulk, while lean rats attempt to adjust

their food intake such that the calories ingested remained constant, obese fa/fa rats showed some responsiveness to caloric density, for instance they decreased their food intake as the percentage of fat in the diet increased, but they did not show any response to diluted food *(Bray and York, 1972; Wangsness et al., 1978)*. However, obese fa/fa rats always consumed more calories than the lean rats. Indeed, when obese fa/fa rats were allowed to select their own diets from a variety of diets differing in protein/fat content, they always selected a high fat/low protein diet *(Castonguay et al., 1982)*, while lean weaning and adult rats have the ability to regulate their protein intake, when they were given the opportunity to balance both protein intake and energy intake. These observations again suggest that obese fa/fa rats may have a defect in the regulation of protein intake.

The effects of drugs such as amphetamine and monoamine oxidase inhibitors have also been studied. While amphetamine completely suppressed food intake in lean rats, similar doses of amphetamine only reduced food intake of obese fa/fa rats by 50% *(Bray and York, 1972)*. In contrast, monoamine oxidase inhibitors suppressed feeding to a similar degree in both lean and obese fa/fa rats *(Bray and York, 1972)*.

ii) Energy expenditure

Oxygen consumption is reduced in obese fa/fa rats compared with their lean littermates *(Bray, 1969; Planche et al., 1983)*. They also show an increased energetic efficiency, since the gain in body fat per gm of food eaten and the energy deposition per metabolisable energy intake are higher in fa/fa than in lean rats *(Deb et al., 1976; Marchington et al., 1983)*, indicating an increase in energetic efficiency. Obese fa/fa rats are also characterised by their lower spontaneous activity *(Stern and Johnson, 1975)*, although this reduction in activity does not appear in young obese rats. However, the decrease in physical activity may make a small contribution to the energy imbalance in older rats. In contrast, the fall in the metabolic rate and in brown adipose tissue thermogenesis (more details in Section 1.4) are more important in reducing energy

balance and the consequent rapid development of obesity in the young fa/fa rats.

1.3.2 Endocrine status

a) Insulin. Obese fa/fa rats are characterised by hyperinsulinemia *(Zucker and Antoniades, 1972; York et al., 1972)*. The initial elevation in serum insulin levels has been observed by weaning. It rises to peak values at 15 weeks of age, but is followed by a considerable drop at later ages *(Jeanrenaud et al., 1983; Zucker and Antoniades, 1972)*. This increase in serum insulin is accompanied by an increase in endocrine pancreatic tissue, resulting primarily from β-cell hypertrophy and hyperplasia *(Shino et al., 1973)*. Although the excessive food intake of the obese fa/fa rats will not restore insulin levels to normal values *(Stern et al., 1975)*. Similarly a restrictive carbohydrate and fat intake after weaning will not prevent hyperinsulinemia, since the pancreatic islets of obese fa/fa being fed a high carbohydrate or high fat diet still release more insulin than do those of lean *(Zucker and Antoniades, 1972; Stern et al., 1975)*.

Insulin is known to be the major endocrine stimulation of lipogenesis. Correction of hyperinsulinemia of fa/fa rats by streptozotocin treatment reduced hepatic and adipose tissue fatty acid synthesis close to the normal levels observed in lean littermates *(Godbole et al., 1978)*.

However, hyperinsulinemia is not essential for the excessive lipid deposition observed in suckling obese fa/fa rats, since adipocyte hypertrophy appears at 7 days of age, before the appearance of hyperinsulinemia *(Boulange et al., 1979)*. However, the role of insulin status in the development of obesity in weaned fa/fa rats was examined by both *Stolz and Martin (1982) and Chan et al. (1982)*. They equalised circulating insulin concentration in both lean and obese fa/fa rats by initially inducing diabetes in both (either by single injection of streptozotocin or an intracardiac injection of alloxan), and then replacing the same level of exogenous insulin. These workers found that obese fa/fa rats continued to partition more energy towards carcass lipid deposition despite normal serum insulin. This occurred in spite of the fact that total body weight gain and food intake per body weight were similar in both lean and obese fa/fa rats *(Stolz and Martin, 1982)*. The hyper-

insulinemia of fa/fa rats is associated with a gradual development of insulin resistance. Thus, the obese fa/fa rats present a high degree of chronic insulin resistance *(Cushman et al., 1978)*. However, during the initial hypersecretion of insulin all tissues demonstrates the same expected responses to insulin stimulation. As at 5 weeks of age fa/fa rat adipose tissue and liver metabolism are sensitive to insulin, whereas by 12 weeks, adipose tissue and muscle, but not liver, show a major loss of insulin sensitivity *(Godbole et al., 1978; Kemmer et al., 1979)*. Insulin resistance has been well documented in obese animals. It has been reviewed extensively by *Olefsky (1981, 1976); Kahn and Neville, 1973 and Crettaz and Jenrenaud, 1980*.

b) Glucagon. Plasma glucagon levels are in obese fa/fa rats is significantly lower than those of lean control rats *(Nishikawa et al. 1981; Nosadini et al., 1980; Bruce et al., 1977)*. The release of glucagon in response to arginine and decreasing glucose concentration have been studied in obese fa/fa rats. *Eaton et al. (1976)* reported an impaired glucagon release in response to arginine, which was associated with hypertrophy of islets in the pancreas of obese fa/fa rats by the age of 16 weeks. However, *Nishikawa et al. (1981)* found that obese rats released more glucagon than lean rats in response to arginine infusion. The differing results might be due to the differing ages of the animals used in these studies, as younger fatty rats were used in the latter experiments (7-8 weeks of age). The hypertrophy of islets in fatty rats is age dependent *(Shino et al., 1973)*.

c) Thyroid function. Genetically obese fa/fa rats have an impairment in the formation or release of thyrotropin releasing hormone (TRH) from the hypothalamus and an impaired thyroid response to circulating thyroid stimulating hormone (TSH) *(Bray and York, 1971; York et al., 1972)*.

Serum thyroxine (T_4) concentration is significantly lower in obese fa/fa rats compared with lean *(Autissier et al., 1980)*. The level of triiodothyronine (T_3) - the active hormone - is also reduced in obese fa/fa rats, and showed a relationship to fa gene dosage *(York et al., 1984)*. A reduction by over 50% has been found in fa/fa compared with the homozygous Fa/Fa lean groups, with the hetrozygous Fa/fa, having an intermediate serum triiodothyronine concentration *(York et al., 1984)*. Treatment of obese (3-4 month old)

fa/fa rats with thyroid powder, produced a decrease in weight gain and carcass lipid content, but without reduction in food intake *(Levin et al., 1982)*.

Rats treated with thyroid powder also show a reduction in brown adipose tissue GDP binding, markedly at lower environmental temperature ($5^{o}C$), compared with their control rats, who showed an increase in GDP binding at $5^{o}C$ *(Levin et al., 1982; Sundin, 1981)*.

d) Adrenal Corticosteroids

Serum corticosterone level has been reported to be the same in lean and obese fa/fa rats in a number of research papers *(Yukimura et al., 1978; Shargill et al., 1983; York et al., 1984)*. However, *Martin et al. (1978)* have suggested that serum corticosterone concentration is increased in obese fa/fa rats at certain times of the diurnal cycle. Their measurements were made on older fa/fa rats. Adrenalectomy prevents the obesity of the obese fa/fa rats *(Yukimura et al., 1978)*. It reduced weight gain, abolished the hyperphagia and normalised energetic efficiency *(Marchington et al., 1983)*. In addition, adrenalectomy decreased serum insulin and hepatic lipogenesis in obese rats to values close to those in lean rats *(York and Godbole, 1979)*.

Replacement of corticosterone to adrenalectomized fa/fa rats, restored the hyperphagia and the obesity while it had little effect on food intake of adrenalectomized lean rats *(Yukimura et al., 1978)*. It is unlikely that normalization of body weight gain after adrenalectomy was due to the reduction in food intake, since food restriction in fa/fa rats does not prevent obesity *(Bray et al., 1973; Radcliff and Webster, 1976)*

1.3.3 Lipogenesis

In vivo and in vitro studies of lipogenesis have indicated a marked increase in both liver and adipose tissue fatty acid synthesis in young (4-5 weeks) obese fa/fa rats, compared to their lean littermates *(Godbole and York, 1978; Bray, 1968)*. The total hepatic lipogenesis was increased with age, whereas the total adipose tissue

lipogenesis falls. These changes and the development of insulin resistance in the adipose tissue of obese rats might reflect the increase in insulin secretion *(York and Bray, 1973a; Godbole and York, 1978)*. However, the total amount of fatty acid synthesis in adipose tissue remains elevated in fa/fa rats in comparison with lean rats because of the increased mass of adipose tissue in obese fa/fa rats *(Godbole and York, 1978)*. Lipogenesis enzymes also have an increased activity in obese fa/fa rats at 4-8 weeks of age, which falls to normal in 4-7 month-old fa/fa rats *(Taketomi et al., 1975)*. The increased rate of fatty acid synthesis is also associated with an enhanced conversion of glycerol to triglyceride in young, but not in older obese fa/fa rats *(Martin and Lamprey, 1975)*. The liver rather than adipose tissue is the major site of enhanced lipogenesis. Study of the rates of lipogenesis using $^{3}H_2O$, either in vitro with hepatocytes, or in vivo, have shown that liver is responsible for about 90% of total fatty acid synthesis *(Bloxham et al., 1977; Godbole and York, 1978)*, suggesting that the liver is the major site of the increased lipogenesis *(Godbole and York, 1978)*. This is consistent with the demonstration of increased hapatic secretion of VLDL *(Bloxham et al., 1977; Schonfeld and Pfleger, 1971)*, increased levels of serum triglyceride and by increased turnover rate of serum triglyceride *(Shargill et al., 1983)*. However, the increase in serum triglyceride represents the imbalance between secretion into and removal from the blood, which is found despite the increased level of lipoprotein lipase *(Gruen et al., 1978; De Gasquet et al., 1973)*.

Study of lipogenesis in suckling preobese fa/fa and lean rats shows that hepatic lipogenesis and lipogenesis enzyme activities are similar in both lean and preobese fa/fa rats *(York et al., 1981; Thenen et al., 1984)*. The suppression of lipogenesis in preobese suckling fa/fa rats has been attributed to the high fat milk diet *(Godbole et al., 1981)*. Indeed, an increase in lipogenesis in both liver and adipose tissue has been observed in lean and preobese fa/fa pups, after allowing them to have access to solid food (high carbohydrate chow diets) before weaning *(York et al., 1981)*. However, adipose tissue lipogenesis and lipogenic enzyme activities were significantly increased in preobese suckling pups *(York et al., 1981; Bazin and Lavau, 1982)* in the absence of any early consumption of solid diet.

The adipose tissue hyperlipogenesis in suckling fa/fa rats has been found, in spite of the fact that the high fat content of rat milk would be expected to suppress lipogenesis *(Hahn, 1972)*.

In order to determine the effect of the energy composition of early diet on lipogenesis, *Thenen et al. (1984)* fed obese and lean pups artificially with high carbohydrate and high fat diets from day 10 to 20, and measured lipogenesis in vivo. A similar response was shown in both lean and obese fa/fa pups. While high carbohydrate feeding enhanced hepatic lipogenesis in both lean and obese pups, there was a significantly increased rate of total fatty acid synthesis in the liver of preobese fa/fa rats compared to lean rats. These results support previous observation that the liver is the main site of lipogenesis *(Godbole and York, 1978)*. The change from the high fat to a high carbohydrate diet at weaning was a major stimulus to the enhanced lipogenesis of fa/fa rats *(Thenen et al., 1984)*.

Similarly, it has been found, when weaning was delayed that there was no enhancement in lipogenesis or serum insulin in preobese fa/fa rats *(Godbole et al., 1978)*. These data might suggest that the enhancement in hepatic fatty acid synthesis might result from the hyperphagia and hyperinsulinemia first observed in obese fa/fa rats immediately after weaning. Thus the enhancement rate of lipogenesis in fa/fa rats was abolished by pair feeding *(Godbole et al., 1978)*. Treatment of the obese fa/fa rats with streptozotocin also reduces both hepatic and adipose tissue lipogenesis to values close to normal *(Godbole and York, 1978)*.

However, studies of lipogenesis in hepatocyte cultures derived from Zucker foetuses bearing fa/fa gene, showed that lipogenesis in fa/fa gene cultures is significantly less than the other cultures which were derived from homozygous Fa/Fa lean foetuses *(Goldestien et al, 1980)*. This suggests that the development of obesity in obese fa/fa rats cannot be attributed to elevated hepatic lipogenesis in foetuses *(Goldestien et al, 1980)*.

1.3.4 Development of obesity in obese (fa/fa) rats

Preobese suckling fa/fa rats can be recognized by their adipocyte hypertrophy, and heavier inguinal fat pads, both of which can be detected by 7 days of age *(Boulange et al., 1979)*. Lipoprotein lipase also shows an increase in activity in the first few days of life. This increase occurs even before hyperinsulinemia and hyperphagia *(Gruen, 1979)*. Adipose tissue thymidine kinase and DNA polymraze also show marked increase in preobese phase *(Cleary et al., 1979)*. Studies of lipogenic capacity in the preobese fa/fa rats which were denied early access to dietary chow have shown it to be either normal *(Godbole and York, 1978)* or enhanced *(Bazin and Lavau, 1982)*.

Hyperphagia is absent before weaning, energy intake is the same in suckling preobese fa/fa and lean pups, as indicated by accumulation of ^{3}H from maternal milk labelled with $^{3}H_2O$ *(Godbole et al., 1981)*. So it appears that the energy imbalance at this age results from a reduction of energy expenditure. Indeed, a reduction in oxygen consumption was noticed at 7 days of age in suckling preobese fa/fa rats *(Kaplan, 1979)*. A significant hypothermia was also detected in preobese fa/fa rats, and this has been used as an early test for the genotype, as every animal which became obese had a low rectal temperature *(Godbole et al., 1978)*. Preobese fa/fa rats are also more sensitive to cold. Obese fa/fa rats were less capable of defending their body temperature than lean rats, as they showed greater decrease in core temperature, after exposure to a low temperature environment. This suggested that preobese fa/fa rats have a defect in thermoregulatory thermogenesis *(Planche et al., 1983)*. Interscapular brown adipose tissue is hypertrophied in preobese fa/fa rats after the first week of life *Bazin et al., 1983)*. In older animals (14 days of age) a reduced level of brown adipose tissue thermogenesis has been observed *(York et al., 1984)*. This impairment is maintained throughout adulthood *(Holt and York, 1982)*.

An increase in serum insulin level in obese fa/fa pups has been detected prior to weaning by 17 days of age *(Bazin and Lavau, 1982)*. Preobese fa/fa rats show a greater insulin output than lean in response to an acute glucose stimulation. This suggests that an increase in insulin secretory capacity is already present before weaning *(Jeanrenaud*

et al., 1983; York et al., 1981; Shargill and York, 1983). However, this increase in insulin secretory response can be prevented by acute atropine treatment *(Jeanrenaud et al., 1983).* While in vitro experiments with perfused pancreas preparation from suckling fa/fa rats did not show hypersecretion of insulin in response to glucose *(Chan et al., 1983)*, despite this enlarged pancreatic islets were present in suckling fa/fa rats.

However, an increase in food intake appears in the fa/fa rats after weaning, when they start to eat solid food *(Stern and Johnson, 1977).* Hyperphagia is not necessary for the development of obesity, since fa/fa rats pair-fed to the food intake of their lean littermates, still deposit excess fat, which again indicates the defective energy expenditure. Body protein content is similar in obese fa/fa and lean rats at 16 days of age. The reduction in protein deposition in the body and skeletal muscle mass appears after weaning *(Reeds et al., 1982).*

At the age of 4 weeks, when fa/fa rats can be visually detected as obese, body weight begins to increase rapidly. This rapid excess weight gain has been generally ascribed to their high level of food intake *(Dilettuso and Wangsness, 1977; Bray and York, 1972)* in combination with their reduced energy expenditure. At this age (after weaning) obese fa/fa rats also show a lower level of spontaneous activity *(Stern and Johnson 1977).* However, spontaneous activity is not important for the development of obesity, since obesity was developing even before weaning *(Planche et al., 1983).* Furthermore, when fa/fa rats were made to exercise by swimming, it delayed but did not prevent, the full development of obesity *(Walberg et al., 1982).* Food restriction along with exercise resulted in more permanent effects on adipose cellularity than exercise alone *(Walberg et al., 1982).*

1.4 Brown Adipose Tissue

It has generally been accepted that brown adipose tissue is the main site of NST *(Jansky, 1973; Smith and Horwitz, 1969).*

a) Location and morphology of brown adipose tissue. Brown adipose tissue is distributed, as small deposits, throughout the body, particularly in the interscapular, subscapular and axillary regions *(Afzellius, 1970).* In small rodents, BAT constitutes

in the region of 1 - 2% of the body weight *(Himms-Hagen, 1983)*. The largest deposit of the tissue, and easiest to remove and to identify, has been found in the interscapular area *(Trayhurn and James, 1983)*. Brown adipose tissue is developed in newborn animals (including human infants) in hibernators and in some cold acclimatised non-hibernators *(Jansky, 1973; Himms-Hagen, 1979)*. In the human adult, BAT is found in the neck, axillae, mediastinum and around the kidney *(Hull, 1966)*. Brown fat is morphologically distinguished from white fat in that brown adipose tissue cells usually contain several small drops of triglyceride. They are described as multilocular, unlike white adipose tissue cells, which have one large drop of lipid *(Afzelius, 1970)*. However, the main function of white adipose tissue is as a store of triglyceride (energy store), while the primary function of BAT is heat production *(Hull and Segal, 1966)*.

b) Brown adipose tissue appearance. The colour of brown fat ranges from pale buff to dark reddish brown, depending largely on environmental and nutritional conditions. The colour derives largely from the high concentration of mitochondria which contain high levels of cytochromes *(Trayhurn and James, 1981)*. BAT cells possess a high concentration of mitochondria which are distributed throughout the cytoplasm and vary in shape and size *(Afzelius, 1970)*. The mitochondria are more abundant and have more cristae than those of white adipose tissue *(Afzelius, 1970)*.

c) Blood supply. BAT is well vascularised with a very rich capillary network which surrounds each cell *(Hull, 1966)*. The flow of blood through brown adipose tissue during thermogenesis is one of the highest for any tissue; in spite of its small mass, it can receive 34% of cardiac output. It can extract and utilize all the oxygen supplied by this high blood flow *(Foster and Frydman, 1978)*.

d) Innervation. BAT has an abundant sympathetic innervation with adrenergic fibres, which form nest-like networks around every fat cell *(Daniel and Derry, 1969)*, unlike white adipose tissue which contains few adrenergic fibres, except those related to blood vessels, and contain much less noradrenaline than does

BAT *(Schimazu and Takahashi, 1980)*. BAT contains a high concentration of noradrenaline *(Barnard et al., 1970)*. Indeed, when BAT is in the thermogenic state (cold or overfeeding), noradrenaline turnover is increased in BAT *(Young et al., 1982)*.

1.4.1 Brown adipose tissue thermogenesis in normal animals

In cold acclimated rats, and in cafeteria-fed rats, thermogenesis in BAT is quantitatively established as an important component of overall energy expenditure *(Himms-Hagen, 1983)*. The cold acclimated rat - one that has lived at $4^{\circ}C$ for two or three weeks - remains lean despite its extreme hyperphagia, because of the large increase in metabolic rate, referred to as cold-induced NST *(Himms-Hagen, 1976; 1983)*. In cafeteria-fed rats, an increase in their energy intake occurs, with a reduction in their efficiency of energy gain, as a greater proportion of their energy intake is lost as heat. This gives an example of animal-exhibited DIT

Indeed, in overfeeding rats, the size of the interscapular brown adipose tissue was significantly greater than in normally-eating rats, and its wet weight increased two fold while body fat and body weight increased only moderately *(Tulp, 1981)* This thermogenic response induced by overfeeding could be blocked by the administration of the beta-adrenergic blocker, propanolol, which is known to block the thermogenic activity of BAT

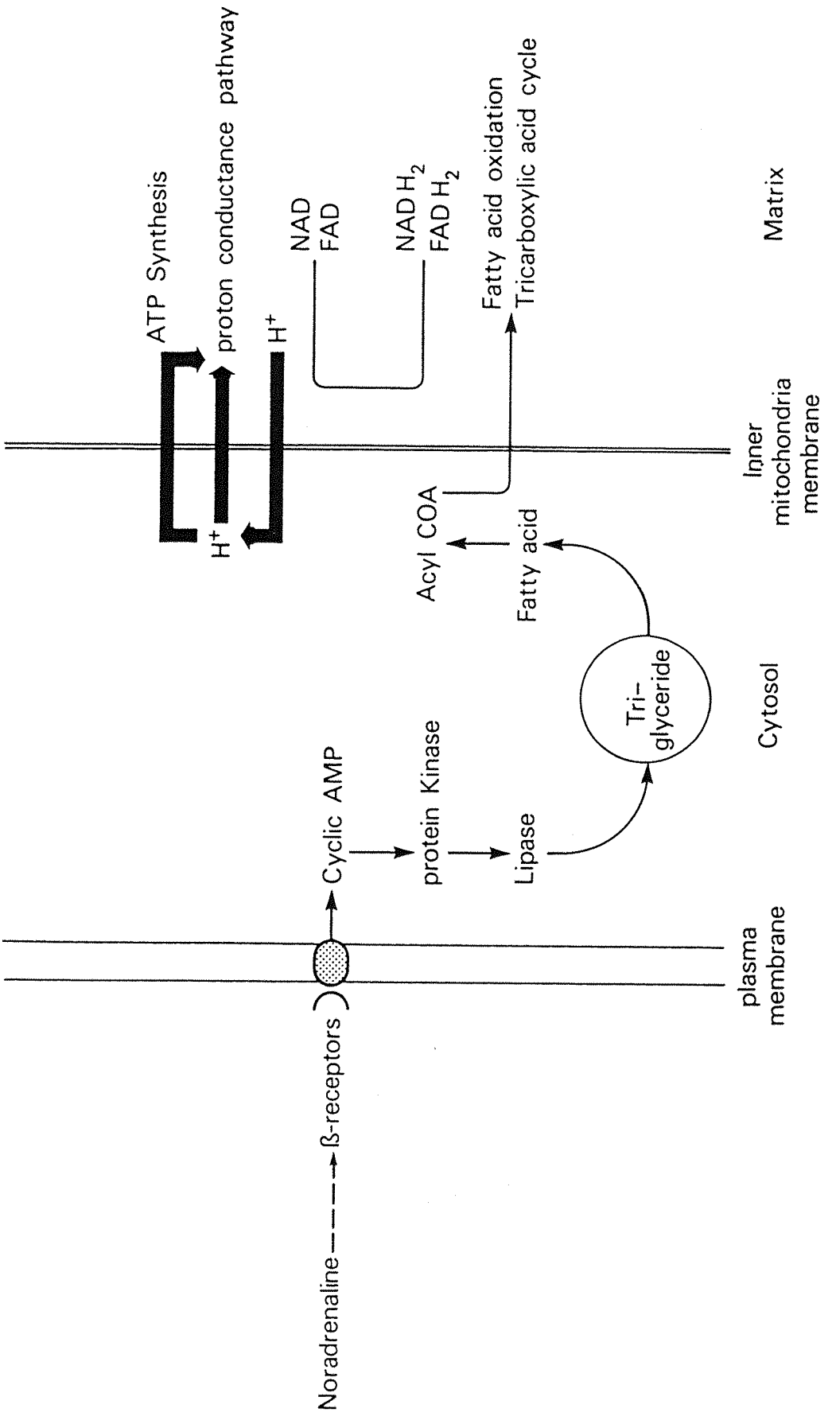
However, there is a similarity between NST exhibited in cold adapted animals *(Himms-Hagen,1976)* and DIT seen in cafeteria fed rats *(Rothwell and Stock, 1979;1980b)*. In both there is an enhanced capacity to respond to thermogenic effects of noradrenaline, an increase in BAT mass, and thermogenesis activity, and an increase in noradrenaline turnover *(Young and Landsberg, 1979)*. The similarities between diet and cold-induced thermogenesis suggest that both have a common metabolic origin residing in BAT, and that DIT may be important in the maintenance of body temperature as well as in energy balance regulation *(Rothwell and Stock, 1980b)*.

1.4.2 Mechanism of thermogenesis in brown adipose tissue mitochondria

Brown adipose tissue mitochondria are unique in that they can become physiologically uncoupled and produce heat instead of synthesising adenosinetriphosphate (ATP) *(Himms-Hagen, 1983)*. Thermogenesis is initiated by the release of noradrenaline from sympathetic nerve endings, which binds to a β_1-receptor on the plasma membrane of the brown adipocyte. Adenyl cyclase is then activated with the production of cyclic AMP, which in turn increases lipolysis by activating the rate-limiting triglyceride lipase (responsible for hydrolysing triglycerol to diglyceride and fatty acids). The released fatty acids are oxidized in the mitochondria, and protons are pumped across the inner mitochondria membrane in the same manner as in other tissues. However, brown adipose tissue mitochondria differ fundamentally from those in other tissues because of the presence of a proton conductance, or leakage, pathway. In normal mitochondria, respiration produces a proton gradient ($\Delta\mu H^+$ proton motive force), across the inner mitochondria membrane and the passage of protons back through the membrane is linked to the synthesis of ATP by ATP synthetase. But in BAT, the proton gradient is dissipated by the movement of the protons through the proton conductance pathway. This prevents the development of an electrochemical gradient of sufficient potential to sustain ATP synthesis. ATP synthesis is bypassed and the dissipation of the gradient uncouples oxidation phosphorylation and allows uncontrolled oxidation of fatty acids. The general scheme for thermogenesis in BAT is shown in Figure 1. The proton conductance pathway is associated with a specific protein of molecular weight 32,000K dalton, which is located in the inner mitochondrial membrane *(Cannon et al., 1982)*. This protein appears to be unique to BAT and has a particular affinity for purine nucleotides, such as guanosine-diphosphate (GDP). Nucleotide diphosphates bind to the 32,000 molecular weight protein and close the proton leak. This couples the mitochondria, allowing the generation of a sufficient electrochemical gradient to support ATP synthesis. In contrast, fatty acids (or fatty acyl CoA thioesters) have also been shown to bind to the 32,000 molecular weight protein and result in opening of the proton leak channel *(Nicholls, 1979)*. This property of binding purine nucleotides has been used as a monitor (indicator) for an assay for the thermogenesis

FIGURE 1 MECHANISM OF THERMOGENESIS IN BROWN ADIPOSE TISSUE

Arrows in bold print indicate movement of protons across the inner mitochondrial membrane, being pumped out by the respiratory chain and re-entering via the 32K protein channel and the ATP synthesis.


ATP Synthesis
proton conductance pathway
H+
H+
NAD
FAD
NAD H2
FAD H2
Fatty acid oxidation
Tricarboxylic acid cycle
Matrix
Inner mitochondria membrane
Acyl COA
Fatty acid
Tri-glyceride
Cytosol
Cyclic AMP
protein Kinase
Lipase
plasma membrane
Noradrenaline
ß-receptors

state of BAT. By incubating BAT mitochondria with [^{3}H] GDP, the amount of radioactivity which is bound reflects the degree of thermogenesis *(Nicholls, 1979; Trayhurn and James, 1981)*.

1.4.3 Brown adipose tissue function in animal obesity

a) Brown adipose tissue thermogenesis in obese animals. It has become evident from a wide range of studies that the regulation of BAT thermogenesis is an important mechanism for controlling energy balance and preventing obesity *(Rothwell and Stock, 1983)*. Obesity in genetically obese animals is associated with a failure to activate thermogenesis in BAT. BAT thermogenesis indicated by the binding of purine nucleotide (GDP) to BAT mitochondria, was reduced by 25% in 14 day old suckling fa/fa rats *(York et al., 1984)*. This impairment progressively increases with age throughout adulthood *(Holt and York, 1982)*. Obese ob/ob and diabetes db/db mice are cold sensitive, dying of hypothermia in a few hours if exposed to 4^{o}C, as they fail to switch on NST in BAT *(Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980)*.
However, fa/fa rats are not cold sensitive. They are able to switch on BAT thermogenesis, when exposed to cold *(Holt et al., 1983, Triandafillou and Himms-Hagen, 1983)*. Acute (24 h at 4^{o}C) and chronic (7 days) exposure to cold, activate BAT thermogenesis in fa/fa rats, as it did in lean rats *(Triandafillou and Himms-Hagen, 1983; Holt et al., 1983)*. In contrast, feeding a cafeteria diet to young fa/fa rats fails to activate BAT thermogenesis as it does in lean rats. These rats accumulate more extra fat expressed by an increase in inguinal white adipose tissue weight *(Triandafillou and Himms-Hagen, 1983)*. Confirming *Holt et al. (1983)*, overfeeding rats with additional sucrose solution did not increase BAT thermogenesis in fa/fa rats, while a typical increase in BAT thermogenesis was evident in lean rats.
The increase in mitochondrial GDP binding in overfed rats, enhanced sympathetic activity and increased the noradrenaline turnover in BAT *(Landsberg and Young, 1982)*. In contrast with the lean rats, fa/fa rats appear to have a defect in the central mechanism which normally switches on thermogenesis in BAT in

response to food. Since they failed to respond to sucrose overfeeding with any increase in BAT GDP binding, but could increase BAT thermogenesis in response to environmental cold.
Recently, it has been found that the sympathetic stimulation of BAT is impaired in obese fa/fa rats *(Levin et al., 1982; York et al., 1985)*, as a reduction in noradrenaline concentration and turnover was found in BAT of obese fa/fa rats, compared with their lean littermates *(York et al., 1985)*.

b) Brown adipose tissue in development of obesity. It was shown that, by 9 days of age, inguinal adipose tissue fatty acid synthase activity was 50% higher in obese fa/fa rats than in lean rats *(Bazin and Lavau, 1982)*. Brown adipose tissue was also a very early site of expression of obesity in fa/fa genotype *(Bazin and Lavau, 1983)*. By 7 days of age, the fat content of the tissue was already increased by 30% in obese fa/fa pups, compared with their lean littermates. These data agree with the increase in interscapular BAT weight observed in 7 day-old fa/fa pups *(Boulounge et al., 1981)*. Several altered metabolic fluxes could contribute to the increased fat accretion observed in BAT in obese Zucker pups, such as, an increased uptake of circulating triglyceride through the action of lipoprotein lipase, a decreased lipid mobilization and oxidation, an enhanced fatty acid synthesis activity. The last hypothesis was proved by the finding that fatty acid synthesis in vivo was markedly increased in BAT of obese fa/fa pups within the first day of life *(Bazin and Lavau, 1983)*. However, hyperlipogenesis appears in BAT in preobese fa/fa rats *(Lavau et al., 1982)* and continues throughout adulthood.

1.4.4 Neuroendocrine control of brown adipose tissue

a) Central control of thermogenesis. The hypothalamus plays an important part in BAT thermogenesis *(Jansky, 1973)*. Two areas of the hypothalamus are specially involved with food intake: the ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH). Destruction of the LH results in aphagia, weight loss and increased metabolism *(Von de Porten and Davis, 1979, Yoshida et al., 1983)*

while lesion of the VMH induces hyperphagia and obesity *(Bray and York, 1979)*. However, the VMH also appears to be involved in the control of BAT thermogenesis, since the electrical stimulation of the VMH causes an enhancement in fatty acid synthesis in BAT, but not in white adipose tissue, or the liver (hepatic lipogenesis) *(Schimazau and Takahashi, 1980)*. *Perkins et al. (1981)* showed that electrical stimulation of the VMH caused an increase in the temperature of the interscapular BAT depot.

b) Sympathetic control of thermogenesis. The primary stimulant to BAT thermogenesis is either noradrenaline, which can be released from sympathetic nerves within the tissue, or adrenaline released from the adrenal medulla during extreme cold. Sympathetic activity in BAT was estimated from the rate of noradrenaline turnover within interscapular BAT *(Young et al., 1982)*. Cold acclimated rats show an increase in sympathetic activity in BAT, indicated by an increase in BAT noradrenaline turnover *(Young et al., 1982; York et al., 1985)*. Cold acclimated obese fa/fa rats show an increase in BAT noradrenaline turnover, which is similar to levels observed in cold adapted lean rats *(York et al., 1985)*.

However, although overfeeding with sucrose produces an increased noradrenaline turnover in the lean Zucker rat, this response was absent in the obese fa/fa rats.

The effects of noradrenaline on BAT are mediated by interaction with β-receptors *(Svobada et al., 1979)*. Binding studies and experiments on respiration of isolated brown adipocytes indicate that noradrenaline acts mainly on the β_1-receptors *(Svobada et al., 1979; Buckoweicki et al., 1980)* although β_2-receptors may also play a minor role *(Rothwell et al., 1982)*. There are some questions over the involvement of α_1, α_2 receptors in BAT thermogenesis *(Rothwell et al., 1982)*. *Mohell (1984)* has suggested that only 80% of the noradrenaline-induced BAT thermogenesis can be blocked by the β antagonist propranolol, while 20% of the effect is mediated through α-receptors mechanism. It should be stated, however, that the α-receptor mediated thermogenesis function is not mediated at the mitochondrial thermogenic motor level.

c) Endocrine control of brown adipose tissue. Thyroid hormones, insulin, glucagon and adrenal cortical hormones, have all been implicated as having a role to play in thermogenesis. However, it is most likely that these hormones are all either permissive or inhibitory in their action, and noradrenaline is still the hormone of major importance for the peripheral activation of thermogenesis *(Stribling, 1983)*.

i) Noradrenaline. The rapid activation of BAT in response to acute stimuli appears to be entirely due to sympathetic release of noradrenaline *(Nedergaard and Lindberg, 1982)*. Noradrenaline injection (25-50 µg/100 g body wt), 40 minutes before killing caused an increase in BAT mitochondria GDP binding in lean and in obese fa/fa rats *(Brooks et al, 1982; Holt 1984)*. This finding is consistent with the enhanced response to noradrenaline in terms of blood flow *(Rothwell and Stock, 1981)* and oxygen consumption *(Rothwell and Stock, 1979)* in these animals.

Noradrenaline is assumed to exert a dual influence on BAT by rapidly stimulating substrate mobilization, allowing oxidation and heat production and , by inducing a chronic increase in the mass of BAT which is usually due to both hypertrophy and hyperplasia of fat cells *(Le Blanc and Villemaire, 1970)*. BAT hypertrophy also occurs after chronic treatment with noradrenaline *(Desautels and Himms-Hagen, 1979)*. Mory et al (1984) have recently shown that noradrenaline injections result in all the characteristic changes in BAT morphology and mitochondrial function which have been reported in cold acclimated rats (Mory et al., 1984).

ii) Thyroid hormones. Thyroid hormones seem to be an important mediator of thermogenesis, since cold exposure and overfeeding which cause a rise in BAT thermogenesis in lean rats, produce a rise in plasma thyroid hormones *(Rothwell and Stock, 1979; Tulp et al., 1980)*, particularly in triiodothyronine (T_3) *(Holt et al., 1983)*. Indeed, hypothyroidism and hyperthyroidism cause a decrease and an increase in metabolic rate respectively *(Girardier, 1977; Le Blanc and Villemaire, 1970)*.

Thyroidectomized rats cannot survive in the cold, as they do not show the normal increase in BAT mitochondrial GDP binding *(Triandafillou et al., 1982)*. The noradrenaline content of BAT in adult thyroidectomized rats is normal *(Kennedy et al., 1977)*. However, it seems likely that the failure of thyroidectomized rats to respond to cold exposure is due to a reduced affinity of the β-adrenergic receptor for noradrenaline *(Gibson 1982; Girardier, 1981)*. Small replacement doses of thyroid hormone are sufficient to restore normal thermogenesis *(Triandafillou et al., 1982)*. Chronic treatment of fa/fa rats with thyroid powder, impaired noradrenaline turnover in BAT and restricted weight gain *(Levin, 1982)*. However, many biochemical and physiological mechanisms have been advanced to explain the thermogenic actions of T_3 and T_4 (For review, see *Himms-Hagen, 1976)*. One suggestion is that these hormones may act on protein synthesis, to increase the β-adrenoreceptors population there by increasing the maximum response to noradrenaline *(Rothwell et al., 1982)*.

Brown adipose tissue thermogenesis function may, however, be depressed in hyperthyroid rats. Indeed, *Sundin (1981)* showed that BAT mitochondrial GDP binding was reduced by thyroid hormone treatment of rats housed at a range of environmental temperatures. These results presumably reflect the ability of thyroid hormones to enhance other thermogenic metabolic processes, thus reducing the requirement for BAT thermogenesis to maintain temperature.

iii) Corticosterone. Corticosterone stimulates lipogenesis and glycogenesis in BAT *(Lachance and Page, 1953)*. Specific corticoid receptors have been identified in BAT *(Feldman, 1978)*, which indicates that BAT may be a target organ for these hormones. Adrenalectomised rats will not survive in cold exposure, but replacement with corticosterone permits the normal thermogenic response. Thus, like thyroid hormones, corticosterone appears to be required in a permissive manner for normal BAT thermogenesis. Chronic corticosterone treatment of lean rats or lean mice, suppresses BAT mitochondrial

GDP binding *(Galpin et al, 1983; Holt et al., 1983)*. Corticosterone suppresses diet induced thermogenesis, but has no inhibitory effect on non-shivering thermogenesis *(Galpin et al., 1983; York et al., 1985)*.

Adrenalectomy has been shown to prevent excess weight gain and decrease food intake in obese Zucker fa/fa rats *(Yukimura et al., 1978)*. Adrenalectomy of obese fa/fa rats also corrects the impairment in BAT GDP binding to control value *(Holt and York, 1982)*. Indeed, a rapid increase in BAT GDP binding of obese fa/fa rats occurs within 24 hours after adrenalectomy, and this is followed by a slower rate of increase over the next six days *(Holt et al., 1983)*. The increased fat content which is usually present in BAT of obese fa/fa rats *(Lavau et al., 1982)* is changed after adrenalectomy, the appearance of BAT of obese fa/fa rats becomes similar to that of the lean controls *(Holt et al., 1983)*. Corticosterone replacement prevents the normalization of BAT GDP binding of obese rats. Similarly, while corticosterone suppresses BAT thermogenesis of lean Zucker rats down to the value of obese fa/fa rats, it has no effect on intact obese rats. Adrenalectomy of fa/fa rats also normalizes the defective thermogenesis response to food *(Holt et al., 1983)*, since sucrose-fed adrenalectomized fa/fa rats show an increase in BAT mitochondrial GDP binding *(Holt et al., 1983)*. The evidence suggests that the obese fa/fa rat is characterised by a corticosterone dependent inhibition of BAT thermogenic response to diet.

The mechanism of the corticosterone inhibitory effect on diet induced BAT thermogenesis has recently been studied by *York* and his co-workers. The BAT noradrenaline turnover is suppressed in BAT of obese fa/fa rats and does not respond to dietary signals *(York et al., 1985)*. After adrenalectomy, BAT noradrenaline turnover, as an index of sympathetic activity, is normalised in the obese fa/fa rat and again responds to dietary signals. Thus, the evidence suggests that corticosterone inhibits the sympathetic responses to dietary input signals.

iv) ACTH. Chronic treatment with ACTH can cause an increase in blood flow throughout BAT and also an increase in metabolic rate (25%) *(Kuroshima et al., 1968; Jansky, 1973)*. Hypophysectomy prevents the excessive weight gain in obese fa/fa rats *(Powely and Morton, 1976)*. It also reduces thermogenic response to cold *(Fellenz et al., 1982)*. Hypophysectomized rats cannot survive when exposed to cold *(Fellenz et al., 1982)*.

It has been found, also, that cold exposure which causes an increase in BAT thermogenesis in the rat *(Holt et al., 1983)*, leads to an increase in plasma ACTH level *(Usategui et al., 1977)*. However, it seems likely that pituitary hormones (including ACTH) are essential for survival of rats in the cold, which is probably due to their trophic effects on the thyroid and adrenal cortex *(Fellenz et al., 1982)*, since rats without thyroid hormones or glucocorticoids do not survive in the cold *(Fellenz et al., 1982)*.

1.5 Aim of the Project

Obesity in obese fa/fa rats is associated with an impaired BAT thermogenesis *(Holt and York, 1982)*. Obese fa/fa rats are able to survive cold exposure, as an increase in BAT thermogenesis occurs, but they do not increase thermogenesis in response to dietary signals, either extra sucrose or a cafeteria diet *(Triandafillou & Himms-Hagen,1983; Holt et al,1983)*. This suggests that obese fa/fa rats do not have the ability to regulate thermogenesis in response to dietary intake, which might be the primary reason for the impaired thermogenesis and the obesity.

The development of the obesity in fa/fa rats is prevented by adrenalectomy, which results in normalization of BAT thermogenesis and a restoration of thermogenic response to dietary signals *(Holt and York, 1982; Holt et al., 1983)*. Corticosterone has been shown to suppress the normal increase in brown adipose tissue thermogenesis, but has no effect on the increase in thermogenesis after cold acclimation *(Galpin et al., 1983)*. Adrenalectomy is followed by an increase in ACTH secretion, and the administration of exogenous corticosterone suppresses the ACTH release *(Yukimura et al., 1978)*.

So the aim of this project is:

1. To investigate the effect of exogenous ACTH on brown adipose tissue.

2. To see whether the normalization of brown adipose tissue, after adrenalectomy of obese fa/fa rats, is due to the hypersection of ACTH.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Adrenocorticotropic hormone (ACTH) Synachten Depot	Ciba Laboratories, Horsham, Sussex, U.K.
Atractyloside, potassium salt	Sigma Chemicals, Poole, Dorset, U.K.
Bovine serum albumin fraction V	Sigma Chemicals, Poole, Dorset, U.K.
Charcoal (Norit GSX)	Hopkin and William Ltd., from BDH, Poole, Dorset, U.K.
Corticosterone	Sigma Chemicals, Poole, Dorset, U.K.
1,2,6,7-^{3}[H]-corticosterone	Radiochemicals Centre, Amersham, U.K.
Coomasie Brilliant Blue G	Sigma Chemicals, Poole, Dorset, U.K.
Cytochrome C Type III	Sigma Chemicals, Poole, Dorset, U.K.
Dextran T-70	Pharmacia Fine Chemicals Ltd., Milton Keynes, U.K.
Fentanyl-Fluanisone (Hypnorm)	Crown Chemicals Co., Kent, U.K.
Gelatin	Sigma Chemicals, Poole, Dorset, U.K.
Guanosine diphosphate, sodium salt	Sigma Chemicals, Poole, Dorset, U.K.
8-^{3}[H] Guanosine diphosphate sodium salt	Radiochemical Centre, Amersham, U.K.
Insulin radioimmunoassay kit	Wellcome Reagent Ltd., Amersham, U.K.
Metopirone	Aldrich Chemical Company
NCS tissue solubilizer	Radiochemical Centre, Amersham, U.K.
Propranolol	Sigma Chemicals, Poole, Dorset, U.K.

Rat Insulin Standard	Novo Laboratories, Denmark
U-^{14}[C]-sucrose	Radiochemical Centre, Amersham, U.K.
Triiodothyronine radioimmuno-assay kit	RIA (U.K.) Ltd., Newcastle, U.K.
Valium	Roche Products, Herts, U.K.
Scintillation cocktails: Beckman ready-solv NA and Tritoscint	G & G Chemicals, Ascot, Berks, U.K.

Chemicals which are not listed above were purchased either from BDH Chemicals, Poole, Dorset, U.K., or from Sigma Chemicals, Poole, Dorset, U.K.

2.2 Experimental Animals

Zucker lean (Fa/Fa and Fa/fa) and obese (fa/fa) male rats were bred from hetrozygote (Fa/fa) parents in the University animal facility. All pups were weaned at 3 weeks of age on to chow diet (Christopher Hill Ltd., Poole, Dorset, U.K.) and fed ad libitum. They were housed at 24°C with a 12 hr-light/12 hr-dark cycle (08.00-20.00 hrs). Rats aged between 28 and 35 days of age were used routinely in the experiments described in this thesis.

2.3 Sucrose Feeding

Over feeding was induced in lean and obese (fa/fa) rats by giving them 35% (w/v) sucrose solution, in addition to the chow and drinking water.

2.4 Measurement of Food Intake

Food intake was measured by placing the rats in individual wire mesh cages. The weight of chow was measured every day at 10.00 a.m. Allowance was made for any spilled food. The consumption of sucrose drinking solution was assessed by the daily change in weight of the drinking bottle.

2.5 Adrenalectomy

Adrenalectomies and sham adrenalectomies were performed from the dorsal approach under valium (0.25 mg/rat intraperitoneally), fentanyl-fluanisome (1 mg/rat intramuscularly) anaesthesia. Animals recovered within 2 - 3 hours. They were maintained at a warm temperature, 27°C, during the first 24 hours. All operative procedures were performed by research colleagues. Adrenalectomized rats were maintained on 0.9% (w/v) saline drinking solution.

2.6 Hormones and Propanolol Treatments

2.6.1 ACTH injection

Lean (Fa/ ?) and obese fa/fa rats were injected with corticotropin (Synachten Depot, Corticotropin -(1-24)-peptide absorbed to a zinc phosphate complex) intramuscularly at doses ranging from 25-100 µg/100 g body weight as indicated in the text.

2.6.2 Corticosterone

Lean (Fa/?) and obese (fa/fa) adrenalectomized rats were injected with corticosterone (1 mg/day) subcutaneously in an ethanol/ dimethyl formamide/0.9 (w/v) saline (2:1:7 by volume) vehicle. Control animals were injected with vehicle only.

2.6.3 Propranolol

Obese fa/fa rats were injected subcutaneously with propranolol (2 mg/100 g) body weight prepared freshly in 0.9% (w/v) saline solution. Control animals received an equivalent volume of saline vehicle.

2.6.4 Metopirone

Obese fa/fa rats were injected subcutaneously with metopirone (25 mg/100 g) body weight prepared in ethanol, 0.9% (w/v) saline (1:3 by volume) vehicle. Control animals were injected with vehicle only.

2.7 Preparation of Brown Adipose Tissue Mitochondria

Brown adipose tissue mitochondria were prepared by a method modified from that described by *Cannon and Lindberg (1979)*.

Homogenization Buffer: 0.25 M sucrose
1 mM Hepes buffer 1 pH 7.2 at $4^{o}C$
0.2 mM EDTA

Rats were killed by decapitation. Interscapular brown adipose tissue was removed and dissected free from surrounding white adipose tissue and connective tissue and placed in cold ($4^{o}C$) sucrose buffer.

The tissue was dried out and weighed, then homogenized in sucrose buffer with 5% (w/v) dilution. A glass homogenizer and teflon pestle was used with 3-5 strokes. Homogenates were centrifuged at low speed (700 g) for 10 minutes at $4^{o}C$ in an MSE centrifuge. The fat layer was removed and the supernatant was transferred carefully to another dry, clean centrifuge tube. The pellet was resuspended in 5 mls buffer, and centrifuged again at the same speed. The two supernatant fractions were mixed together and centrifuged at 8500 g for 15 min in order to sediment the mitochondria. The mitochondria pellet was washed by resuspending in sucrose buffer, containing 2% (w/v) bovine serum

albumin (fatty acid-free) and centrifuged again at 8500 g to sediment the mitochondria.

Finally, the mitochondrial pellet was washed with sucrose buffer in a similar manner. The final mitochondrial pellet was resuspended in 0.25 M sucrose at a protein concentration of 1.5 - 2 mg/ml and stored on ice (4°C) for not longer than 1 hr before use.

2.8 Measurement of ^{3}[H] Guanosine Diphosphate (GDP) Binding to Brown Adipose Tissue Mitochondria

The method was based upon that described by *Nichols (1976)*, with some modifications.

Incubation Buffer: 20 mM Tris, pH 7.1 at 23°C
100 mM Sucrose
1 mM EDTA
10 mM Choline Chloride
5 µM Rotenone
100 µM Atractyloside
0.2 µCi [U-^{14}C] Sucrose (specific activity 584 Ci/mol)

Assay Procedure

0.3 - 0.4 mg of mitochondria protein was incubated in 500 µl of incubation buffer containing 10 µM 8-^{3}[H] GDP (1.25 µCi) (specific activity 10 Ci/mmol), in the presence and absence of excess (100 µM) cold GDP. The mitochondria were incubated at 23°C for 10 mins. The reaction was stopped by sedimenting the mitochondria in a Beckman microfuge, at full speed for 2 mins.

Supernatant was removed by vacuum, and the base end of the microfuge tube containing the pellet was cut and placed in a scintillation vial. 500 µl NCS-tissue solublizer was added and the vials were incubated at 45°C for 1 hr with regular vortexing to dissolve the mitochondria. The tissue solublizer was then neutralised with glacial acetic acid (20 µl). 5 mls of Beckman Ready-Solv scintillation fluid was added. ^{3}[H] and ^{14}[C] radioactivity was determined for each sample by counting them in a Phillips Scintillation Counter for 5 mins/vial at an efficiency of 63% for ^{14}[C] and 28% for ^{3}[H]. The specific binding of GDP was calculated as the difference in binding of ^{3}[H] GDP

in the presence and absence of excess (100 µM) cold GDP. [U-^{14}C] sucrose radioactivity was used to determine the water space in the mitochondrial pellet.

2.9 Protein Determination

The method of *Bradford (1976)* was used. This method is based upon the binding between Coomassie Brilliant Blue and the protein, which causes a shift in the absorption at 595 nm.

Reagents: 200 mg Coomassie Blue was dissolved in 100 mls of 95% (w/v) ethanol. 200 mls of orthophosphotic acid was added and the volume was made up to two litres with distilled water.

Assay

5 mls of the above reagent was added to about 30-60 µg of mitochondrial protein and to different amounts of bovine serum albumin (range from o - 70 µg) as a standard curve. Samples were left for 10 mins for colour development and the absorbance was read in a dual beam Unicam SP8 - 400 Spectrophotometer, against a blank tube at 595 nm wave length.

2.10 Measurement of Mitochondrial Succinate-Cytochrome C Oxidoreductase Activity

The assay was based on the method of *Tisdale (1967)*. The principle of this method is the reduction of cytochrom C by succinate, the reduced cytochrom C giving an increase in absorbancy at 550 nm.

Assay

A Pye Unicam SP8-400 dual beam spectrophotometer was used. The assay was carried out at 37^{o}C. 1 ml cuvette was used, which contained:

10 mM potassium phosphate buffer pH 7.4

1 mM NaN_3

0.2 M EDTA

0.1% (w/v) Cytochrom C

10% (w/v) Bovine Serum Albumin Fraction V

30 - 50 µg of mitochondrial protein was preincubated for 3 mins at 37^{o}C.

The reaction was initiated by adding 100 µl of 5 mM sodium succinate. Distilled water was added to the blank cuvette to adjust the volume. The deviation in optical density was measured over the first two minutes. The extinction coefficient which was used for Cytochrom C (reduced-oxidized) was 18.5 x $10^{-6} M^{-1} Cm^{-1}$ and the enzyme activity was calculated as follows:

$$\frac{\Delta \text{in O.D./min/mg protein}}{18.5} \quad \mu\text{mol/min/mg protein}$$

2.11 Removal of Fatty Acid from Bovine Serum Albumin by Charcoal

The method of *Chen (1967)* was used. Charcoal treatment of bovine serum albumin at low pH removes all the fatty acids bound to bovine serum albumin.

Procedure

30 gm of charcoal was washed first with distilled water and left overnight. Then the crude charcoal residue, which remained on the surface of the water, was removed by vacuum. 70 gm of bovine serum albumin (fraction V) was added and dissolved with stirring. The pH was lowered to 3.0 by the slow addition of 0.2 M Hcl over a 1 hr period. The solution was placed on ice and left stirring for one hr. Charcoal was removed by centrifugation at 20,000 g for 1 hr at 4°C. The supernatant was decanted quickly and filtered, using a millipore filter, and after that the pH was adjusted to pH 7.0 by adding 0.2 M NaOH. The final solution was adjusted to 10% (w/v) and aliquots were stored at -20°C until required. The protein concentration was determined using the *Bradford* method.

2.12 Measurement of Serum Corticosterone

A radioimmunoassay method was used to measure total serum corticosterone *(Fahmy et al., 1975)*. Blood samples were collected from rats by decapitation, using a guillotine. Blood was allowed to clot at room temperature for about 10 min, then centrifuged at 700 g for 10 min to collect serum. All serums were frozen at -20°C and stored until required. Antiserum to corticosterone was kindly supplied by Dr. A. Thomas from the Department of Physiology, Southampton University.

Assay buffer and dextran coated charcoal

0.15 M sodium chloride was dissolved in 0.01 M phosphate buffer pH 7.4, containing 1% (w/v) gelatin. Dextran-coated charcoal was prepared by dissolving 25 mg dextran-T70 in 100 mls buffer, 250 mgs charcoal was then added. This mixture was prepared and left on ice with very slow stirring for at least 1 hr before use.

Corticosterone solution

10 mgs of corticosterone was dissolved in 100 mls ethanol and left at 4°C as stock solution. 100 µl of 1,2,6.7-^{3}[H]-corticosterone (specific activity 93 Ci/mmol, 10 µCi/ml) was evaporated under nitrogen and re-dissolved in 10 mls ethanol to give a final activity of 10 µCi/ml. This solution was stored at 4°C. The working ^{3}[H]-corticosterone solution was prepared for each assay immediately before use, by evaporating off the ethanol under nitrogen and re-dissolving the ^{3}[H]-corticosterone in 15 mls of assay buffer to end with radioactivity of approximately 30,000 dpm/100 µl.

Assay Procedure

Standards were prepared by diluting the stock corticosterone solution (100 µg/ml) in ethanol in a series of dilutions to give 0.1; 0.2; 0.2; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9; 1; 3; 5; 7 ng/ml. 100 µl of ethanol was added to a further three tubes:

1. A 'Zero' standard, allowing the measurement of the absolute amount of ^{3}[H]-corticosterone bound to the antisera.
2. A 'blank' to check the efficiency of the charcoal system for removal of unbound ^{3}[H]-corticosterone. No antiserum was added.
3. This tube contained ^{3}[H]-corticosterone and buffer only, so the total amount of radioactivity could be assessed

Serum samples were extracted in five times the volume of ethanol, vortexed for 10 seconds, and centrifuged for 10 mins at 4°C. 20 µl aliquots were taken for assay.

Ethanol from all assay tubes was removed by evaporation under a stream of nitrogen. Samples were placed in water bath at 4°C during

evaporation. Antiserum was diluted 3 to 5 fold with buffer immediately before use, the precise dilution depending upon the antiserum titre. 100 µl of diluted antiserum was added to all tubes except tubes 2 and 3 above, to which 100 µl of buffer was added. Tubes were mixed for 5 sec. on a vortex mixer and then left for 30 mins at room temperature. 100 µl of [^{3}H]-corticosterone were added, the tubes were vortexed for 5 sec. each, and then incubated in a water bath at 30^{o}C for 1 hr. After the incubation, all tubes were placed on ice for 15 min, before the addition of 500 µl dextran coated charcoal solution. Samples were mixed and left for another 15 min on ice.

All tubes were then centrifuged at 4^{o}C 500 g for 20 min. The supernatant was decanted into a scintillation vial, 5 mls Tritoscint scintillation fluid was added. The bound radioactivity in the samples was determined in a Phillips Scintillation Counter for 2 min/sample. Total corticosterone concentration in the unknown samples were calculated from the standard curve, in which the counts bound expressed as % of total counts added was plotted against cold corticosterone concentration. A typical standard curve is shown in Figure 2.

2.13 Measurement of Serum Free Corticosterone

The percentage of free corticosterone was determined by the method of *Martin et al. (1977)*.

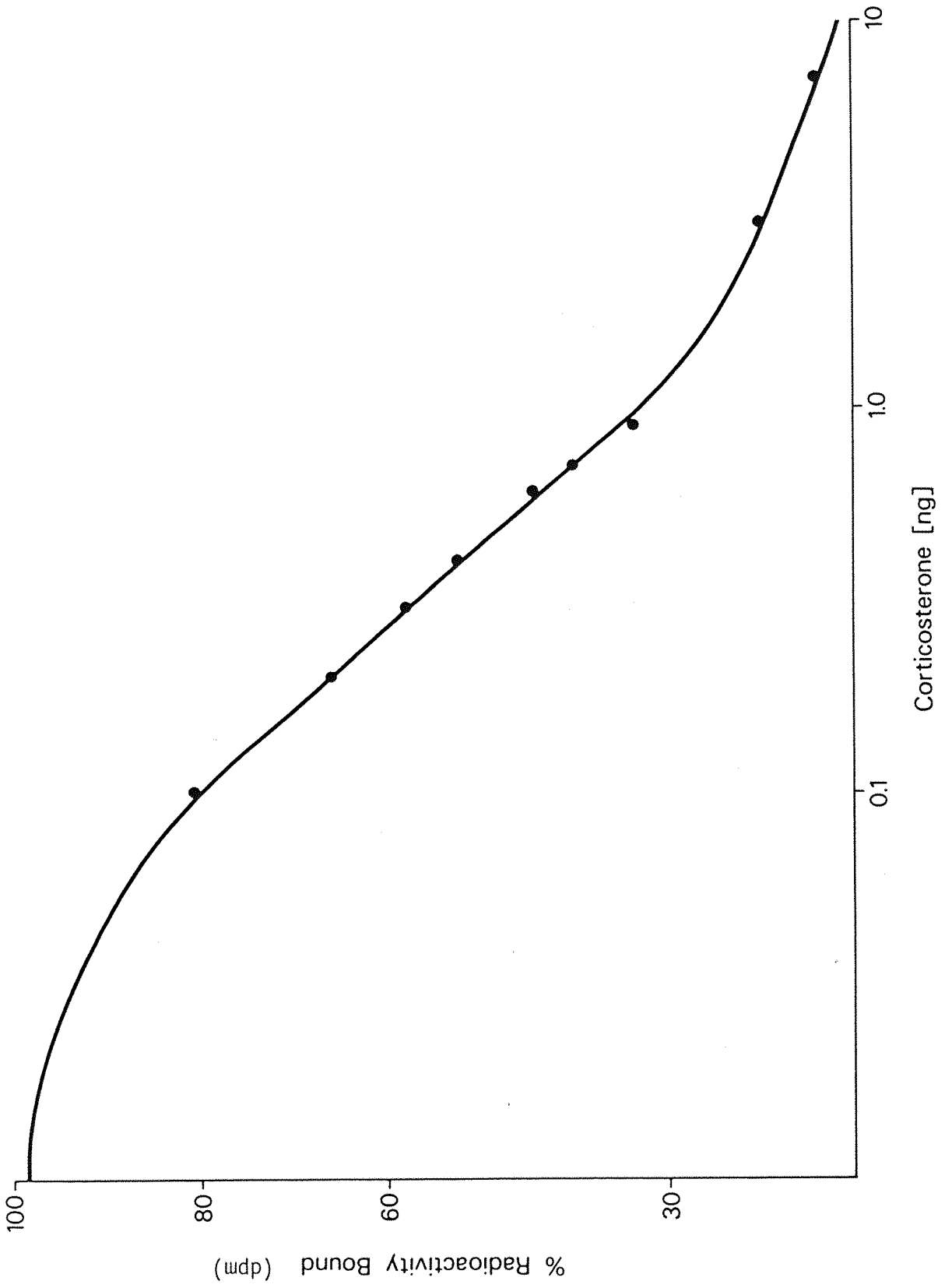
Dextran coated charcoal

0.375 gm Dextran was dissolved in 100 ml of 50 mM phosphate buffer pH 7.4. 3.75 gm activated charcoal was then added, and the mixture was kept stirring magnetically until used.

Assay Procedure

A trace amount of [^{3}H]-corticosterone (50 nCi, approximately 30,000 dpm) was equilibrated with the corticosterone present in 200 µl serum by incubating at 37^{o}C for 30 min. After incubation, 20 µl samples were taken for determination of total radioactivity. The remaining mixture was placed in ice for 30 min. 30 µl of Dextran coated charcoal was then added to remove the unbound hormone. Samples were left for a further 10 min, then centrifuged at 500 g for 30 mins at 4^{o}C.

FIGURE 2 A TYPICAL STANDARD CURVE FOR CORTICOSTERONE ASSAY



A 50 µl aliquot of supernatant was taken, 5 mls of Tritoscint added and the radioactivity counted in a Phillips scintillation counter. This count determined the radioactivity bound to corticosterone binding globulin (CBG). The concentration of the free corticosterone in serum was then calculated as follows:

$$\text{Free corticosterone} = \frac{\text{Total dpm} - (\text{CBG Bound dpm})}{\text{Total dpm}} \times \text{Total corticosterone concentration}$$

2.14 Measurement of Corticosterone Binding Globulin

Serum samples were assayed for corticosterone binding globulin by the method of *Martin et al. (1977)*.

Procedure

1. The endogenous corticosterone was removed from serum by adding 30 µl of dextran coated charcoal C (prepared as described in Section 2.13) to 100 µl serum (in duplicate) and incubated at 37°C with gentle shaking for 90 mins.
2. Samples were then centrifuged at 500 g for 20 mins at 4°C.
3. Supernatant was diluted 1:15 (v/v) in 50 mM phosphate buffer (pH 7.2).
4. Diluted serum (300 µl) containing 74 nM ^{3}[H]-corticosterone in the presence and absence of excess 7.4 µM cold corticosterone, was incubated at 0 - 4°C for 90 mins.
5. Free and bound steroid was separated by adding 50 µl of dextran coated charcoal, and left for 10 mins, then centrifuged as in Step 2.
6. To 200 µl supernatant 5 mls of Tritoscint was added and the radioactivity counted in a Phillips scintillation counter. The concentration of corticosterone binding globulin, expressed as µg bound corticosterone per 100 ml serum, was calculated from the difference between total binding and non-specific binding.

2.15 Measurement of Serum Insulin Concentration Using Radioimmunoassay

Serum insulin was measured using the Wellcome Kit, with some modification of the *Hales and Randle (1963)* method, in which the separation of antibody bound from the free insulin was done by using the double antibody procedure.

Insulin binding reagent

The freeze dried contents of the mixture of guinea pig anti-insulin serum, and rabbit anti-guinea pig globulin serum, in 40 mM sodium phosphate, pH 7.2, containing 20 mM EDTA, 0.1% sodium azide and 0.5% bovine serum albumin, were reconstituted with 8 mls deionised water and mixed by gentle inversion.

Buffers

A. 50 mM phosphate buffer pH 7.4, containing 0.5% (w/v) bovine serum albumin and 0.025% (w/v) thiomersal.

B. 50 mM phosphate buffer pH 7.4, containing 0.5% bovine serum albumin (w/v), 0.025% thiomersal (w/v), and 0.9% sodium chloride (w/v).

Standard insulin solution

The rat insulin standard used in the assay was diluted serially in buffer (B), to give a range from 6.25 μU/ml to 100 μU/ml. The assay was performed in triplicate for each sample in 2 ml glass tubes. The following tubes were set up:

1. 'Blank' to which 100 μl buffer B was added to serve as a control of the washing procedure.
2. 'Zero' to which no unlabelled insulin was added.
3. The standard insulin solutions.
4. The unknown serum samples.

100 μl of buffer (A) was added to tube 1. While tubes 2, 3 and 4 received 100 μl of reconstituted binding reagent. The contents of all tubes were vortexed and left at $4^{o}C$ for 6 hrs. 100 μl of the working solution of the iodinated (^{125}I) insulin (0.25 μCi/ml) was then added to all tubes plus three other tubes

for 'total' counts. All tubes were mixed and left at 4°C for 18 hrs. Then 1 ml of buffer (B) was added to all tubes except 'total'. At the end of the incubation, tubes were centrifuged at 2000 g for 20 min, and supernatant decanted. Each tube was then counted for 1 min in a Beckman gamma counter, with an efficiency for ^{125}I of 80%. Radioactivity bound was expressed as a percentage of the total radioactivity added after subtraction of the background count.

$$\text{Radioactivity bound (\% added)} = \frac{\text{Bound cpm - Blank cpm}}{\text{Total cpm - Blank cpm}}$$

The means of each set of triplicate were determined, and plotted as mean percentage bound versus concentration of standard insulin. The curve was used to determine the insulin concentration in the unknown samples.

2.16 Measurement of Serum Triiodothyronine (T_3) using Radioimmunoassay

Serum triiodothyronine concentration was measured by using Gamma-B T_3 Kit radiommunoassay. A standard curve was prepared using a set of ten T_3 standard ranging from 0 - 12.8 nmoles/litre (provided with the kit).

Procedure

1. 500 µl of the antibody complex was added to 50 µl serum, vortexed, and incubated at 37°C for 30 min.
2. 200 µl of ^{125}I-T_3 was then added to all tubes, vortexed and incubated for 30 min at 37°C.
3. The tubes were then centrifuged at 700 g for 20 min at 9°C.
4. The supernatant was decanted and tubes allowed to drain on a pad of absorbant tissue, blotted gently to remove remaining drops of liquid.
5. Tubes were then counted in a Beckman biogamma counter for 1 min each.

Non-specific binding was calculated by including tubes which contained 550 µl of 0.4% BSA-borate buffer instead of the serum and antibody.

Radioactivity bound was expressed as a percentage of the total radioactivity added:

$$\frac{\text{Bound - Blank}}{\text{Total - Blank}} \times 100$$

Triiodothyronine concentration in the unknown samples was determined from the standard curve in which percentage bound was plotted versus concentration of standard T_3.

2.17 Determination of Radioactivity

Radioactivity in samples was measured by liquid scintillation counting, using Phillips liquid scintillation analysers, models PW 451G. Counting efficiency was 63% for ^{14}[C], and 28% for ^{3}[H]. Variation in quenching were corrected by external standardisation, using preprogrammed quench curves. The scintillants used were either Beckman Ready-Solv. or Tritoscint (dimerthyl POPOP and Triton X-100 in Xylene).

2.18 Statistics

Results are expressed as means ± standard error of the mean (S.E.M.). All results were also assessed for statistical significance by student's 't' test.

CHAPTER 3

RESULTS

A defect in BAT thermogenesis has been demonstrated in obese fa/fa rats, which can be corrected by adrenalectomy *(Holt and York, 1982)*. Adrenalectomy prevents the development of obesity in fa/fa rats, it reduces weight gain and food intake to the levels observed in the lean rat and restores energetic efficiency to normal *(Marchington et al., 1983)*. The effects of adrenalectomy on fa/fa rats were prevented by corticosterone replacement *(Holt et al., 1983; Yukimura et al., 1978)*. However, it would be anticipated that changes in plasma corticosterone level after adrenalectomy or corticosterone administration would be accompanied by reciprocal changes in ACTH secretion. Indeed, adrenalectomy raised plasma ACTH level in obese fa/fa rats, as would be expected *(Yukimura et al., 1978)*. Thus, it is unclear whether the restoration of BAT function which followed adrenalectomy of fa/fa rats reflected the removal of corticosterone inhibition or a response to excessive ACTH stimulation.

In the following section, the effect of exogenous ACTH on brown adipose tissue mitochondrial GDP binding in fa/fa rats is reported.

3a. Characteristics of lean and obese fa/fa rats at the age of 5 weeks

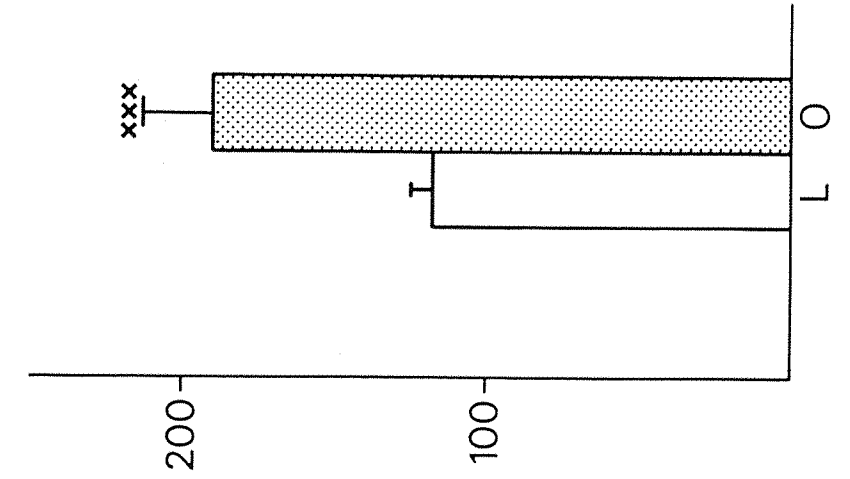
Measurement of food intake for obese fa/fa and lean rats showed that obese fa/fa rats were hyperphagic; they ate 40% more than lean rats (Figure 3). At 5 weeks of age the obese fa/fa rats were significantly heavier than the lean rats. The wet weight of the BAT was increased in the fa/fa rat (Table 1). This probably reflected an increase in the fat content of BAT, as the total protein content was significantly lower in obese fa/fa than in the lean rats, confirming previous reports *(Holt and York, 1982; Triandafillou and Himms-Hagen, 1983)*. Total tissue succinate cytochrome C oxidoreductase activity, a mitochondrial marker enzyme, was also reduced in BAT of obese fa/fa rats. In contrast, the activity of succinate cytochrome C oxidoreductase in the isolated mitochondria was similar in lean and obese fa/fa rats. This data indicated that mitochondrial content of BAT in obese fa/fa rats was decreased. The specific binding of ^{3}[H]-GDP to isolated brown fat mitochondria has been used extensively to assess the activity of the thermogenic proton conductance pathway unique to these mitochondria *(Rothwell et al., 1982)*. Table 1,

FIGURE 3 FOOD INTAKE AND BODY WEIGHT OF LEAN AND OBESE fa/fa RATS

5 week old lean (☐) and fa/fa (▦) rats were housed individually. Food intake was measured daily as described in Section 2.4, for 7 days. Food intake is expressed as KJoules/day (KJ/day) as: 1 g chow food ≡ 9.41 K Joules.

Values represent means ± S.E.M. for 4 rats in each group.

** $p < 0.01$; *** $p < 0.001$ compared with lean group.


Food intake [kJ/d]
200
100
xxx
L
O

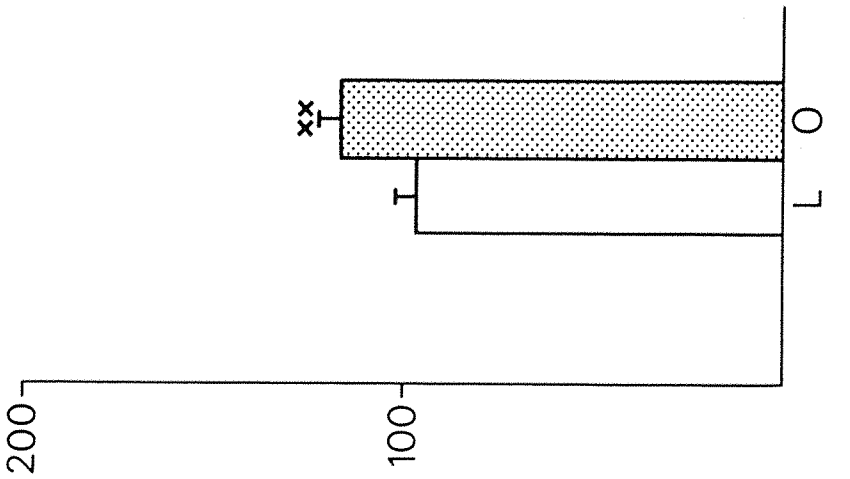

Body weight [g]
200
100
xx
L
O

TABLE 1

CHARACTERISTICS OF BROWN ADIPOSE TISSUE IN LEAN AND OBESE fa/fa RATS AT THE AGE OF 5 WEEKS

	Lean	Obese fa/fa
Brown adipose tissue:		
Wet weight (g)	0.27 ± 0.02	0.50 ± 0.05**
Protein (mg)	22.7 ± 1.6	17.2 ± 1.7*
Succinate cytochrome C oxidoreductase (µmol/min/tissue)	2.16 ± 0.3	1.20 ± 0.1**
GDP binding (pmole/mg protein)	231 ± 6	86 ± 2***
Total binding (pmole/tissue)	1411 ± 83	716 ± 52***
Mitochondrial recovery (%)	22.4 ± 0.9	15.2 ± 1.0***

Brown adipose tissue mitochondria were prepared as described in Section 2.7. BAT protein content, succinate cytochrome C oxidoreductase activity and mitochondrial GDP binding were measured as described in Section 2.9, 2.10 and 2.8 respectively. Recovery of mitochondria was measured as % recovery of succinate cytochrome C oxidoreductase activity from the whole tissue homogenate. Total tissue binding was calculated based on 100% recovery of mitochondria. Values represent means ± S.E.M. for 4 rats in each group.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to lean group.

also shows that the specific binding of ^{3}[H]-GDP to BAT mitochondria was decreased by 40% in obese fa/fa rats, despite their hyperphagia. The reduction in GDP binding did not result from differing purity of mitochondrial preparations, since the specific activity of succinate-cytochrome C oxidoreductase was similar in mitochondria preparation from both lean and obese rats (0.25 ± 0.01 µmol/min/mg protein and 0.24 ± 0.01 µmol/min/mg protein for lean and obese rats respectively). Total tissue GDP binding, expressed for the whole brown fat depot was decreased in the fa/fa rats when compared to the lean rat. This was due to both the reduced GDP binding/mg mitochondrial protein and the decreased mitochondrial population. The yield of mitochondria (% recovery) from brown adipose tissue mitochondria preparation was less in the obese fa/fa rats compared to the lean.

3b. The acute effect of ACTH on brown adipose tissue function and serum corticosterone in lean and obese fa/fa rats

The acute response of BAT in lean and obese fa/fa rats to 50 µg ACTH (i.m.) are shown in Table 2. ACTH had no significant effects on either BAT wet weight or protein content or BAT mitochondrial content (as indicated by succinate cytochrome C oxidoreductase activity) in lean or in obese fa/fa rats. Also, no changes were observed in BAT mitochondrial GDP binding in lean rats after ACTH treatment. In contrast, the impaired GDP binding observed in obese fa/fa rats was increased more than 2 fold after a single injection of (50 µg) ACTH, to reach levels similar to those observed in control lean rats. Serum corticosterone level was similar in lean and obese fa/fa rats, confirming the previous data of *Yukimura et al. (1978); Shargill and York (1983) and York et al. (1984)*. Serum corticosterone concentration in response to ACTH was significantly increased in both lean and obese fa/fa rats after ACTH treatment.

3c. Serum free corticosterone concentration and corticosterone-binding globulin in lean and obese fa/fa rats

Corticosterone exists in serum in both unbound (free) and protein bound form. A large proportion of circulating corticosterone is bound to corticosteroid-binding globulin (CBG) *(Ballard, 1979)*. Since it is generally believed that biological activity is limited to the unbound form of this hormone, it was important to know if the reduced BAT GDP-

TABLE 2

THE ACUTE EFFECT OF ACTH ON BROWN ADIPOSE TISSUE FUNCTION AND SERUM CORTICOSTERONE IN LEAN AND OBESE fa/fa RATS

	Control		+ ACTH	
	Lean	Obese fa/fa	Lean	Obese fa/fa
Brown adipose tissue:				
Wet weight (g)	0.20 ± 0.02	0.29 ± 0.03†	0.25 ± 0.02	0.31 ± 0.1
Protein (mg)	17.7 ± 0.3	13.5 ± 1.1††	19.5 ± 2.5	14.9 ± 0.9
Succinate cytochrome C oxidoreductase (µmol/min/tissue)	2.5 ± 0.45	1.16 ± 0.07†	2.8 ± 0.36	1.65 ± 0.13
GDP binding (pmole/mg protein)	267 ± 18	98 ± 14†††	251 ± 6	227 ± 16***
Serum corticosterone (µg/dl)	21.6 ± 0.9	22.6 ± 4.2	39.0 ± 7.1	34.5 ± 2.0*

Lean and obese fa/fa rats (4 weeks old) were injected with ACTH (50 µg of synachten - Depot/rat) 24 hrs before killing. Brown adipose tissue mitochondrial GDP binding was determined as described in Section 2.8, and serum corticosterone concentration was determined as described in Section 2.12. Values represented means ± S.E.M. for four animals in each group. *$p < 0.05$, ***$p < 0.001$ compared with respective control group; †$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$ compared with lean group.

binding of fa/fa rats could reflect an increased serum free corticosterone concentration since corticosterone is known to suppress BAT function *(Holt et al., 1983; Galpin et al., 1983)*. It has been shown previously that the concentration of both free and bound corticosterone was increased in obese (ob/ob) mice *(Herberg and Kley, 1975)*. Thus the high level of the hormone reflects the hyperadrenocorticism in genetically obese mice, which has also been described by *Naeser (1979)*. However, the total serum corticosterone concentration was similar in obese and lean rats in the present study (Table 3). The results in this table also show that both the percentage and the concentration of free corticosterone were similar in lean and obese fa/fa rats. By 10 weeks of age, total serum corticosterone concentration had increased in both lean and obese rats to similar values and the percentage of free corticosterone had decreased in both groups, but once again to similar values.

The concentration of corticosterone binding globulin was assessed from the maximum binding of ^{3}[H]-corticosterone described in Section 2.19. These results (Figure 4) suggest that corticosterone-binding globulin concentration was nearly 2 fold higher in the obese than lean rats. The level of corticosterone binding globulin observed in the lean controls (24 µg/dl) was similar to that previously reported for Sprague-Dawley rats *(D'Agostino et al., 1982)*.

3d. Time course of the response of brown adipose tissue GDP binding and serum corticosterone to ACTH in lean and obese fa/fa rats

In order to study the effect of ACTH further, the effect of daily administration of ACTH on BAT GDP binding was investigated. The results of this experiment are shown in Figure 5. No changes in BAT GDP binding were observed in lean rats in the first 24 hours after ACTH injection (50 µg/rat/day). However, by 72 hours there was a significant fall in GDP binding which continued until it reached levels of only 90 ± 7 pmoles/mg protein by 7 days. The time course of the response to ACTH was quite different in the obese group, ACTH had no significant effect on GDP binding in the initial 12 hours, but a maximal stimulation of GDP binding was observed after 24 hours. At this time the BAT mitochondrial GDP binding was similar to that of the lean group and close to the levels observed in untreated lean control rats. Subsequently

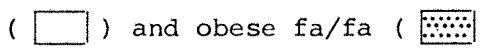
TABLE 3

SERUM FREE CORTICOSTERONE IN LEAN AND OBESE fa/fa RAT

	Age (week)	Sex	No. of Animals	Serum Corticosterone		
				Total (µg/dl)	Free (µg/dl)	% Free
Lean	4	F	8	23.9 ± 4.9	6.2 ± 1.4	26.6 ± 1.7
Obese fa/fa	4	F	8	24.7 ± 2.7	6.6 ± 1.2	27.4 ± 1.8
Lean	10	F	5	64.0 ± 5.5	12.4 ± 1.5	19.3 ± 1.4
Obese fa/fa	10	F	5	64.7 ± 8.2	10.4 ± 1.5	16.2 ± 1.2

Total serum corticosterone and free corticosterone were measured as described in Section 2.12, 2.13 respectively. Values represent means ± S.E.M. for the number of rats shown in the Table.

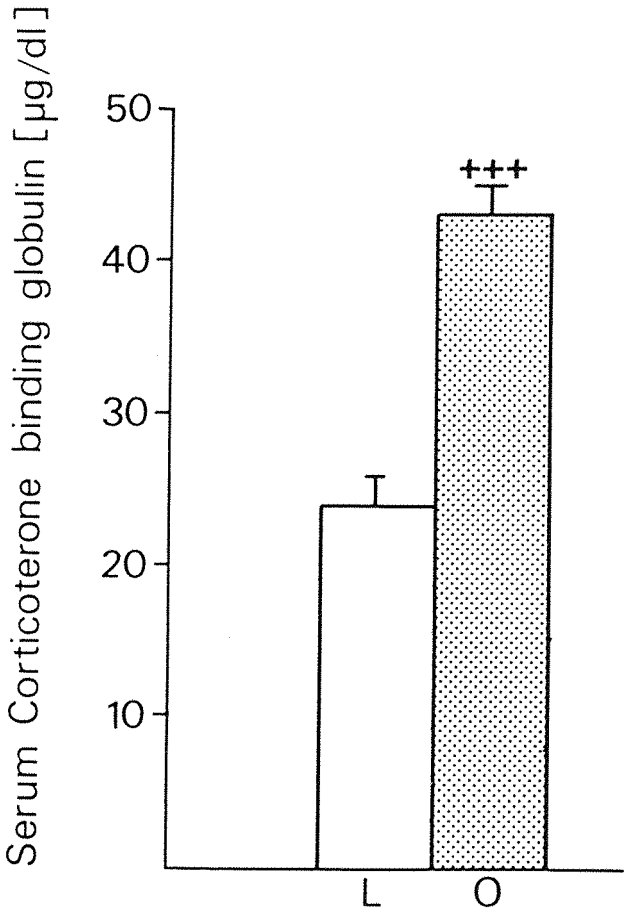
FIGURE 4 SERUM CORTICOSTERONE-BINDING GLOBULIN IN LEAN AND OBESE fa/fa RATS



Serum corticosterone binding globulin (CBG) was measured in male lean ( ) and obese fa/fa ( ) rats as described in Section 2.14

Values represent means ± S.E.M. for seven animals in each group.

$^{\dagger\dagger\dagger}p < 0.001$ compared with lean group.


Serum Corticoterone binding globulin [µg/dl]
50
40
30
20
10
+++
L
O

there was a steady fall in GDP binding level in ACTH treated obese rats back towards the initial control values.

Figure 6 shows the time course of the response of BAT total GDP binding to depot ACTH in lean and obese fa/fa rats. These were essentially similar to those previously reported for GDP binding/mg protein, although the changes were slightly exaggerated by a small increase in total binding in both groups which observed after 72 hours, and this reflected the increase in BAT mitochondrial population.

Initial Serum corticosterone concentrations were similar in lean and obese fa/fa rats. The time course of the response of serum corticosterone to ACTH (Figure 7) appeared to differ in the two groups of rats. After 12 hours there was no increase in serum corticosterone in the lean rats (no measurements were made at any earlier times), but at 24 hours (1 hour after the second ACTH injection) serum corticosterone was increased. As ACTH administration continued, serum corticosterone was increased further in the lean group. In contrast, in the obese group, serum corticosterone was elevated after 12 and 24 hours. This, apperent difference in response in the lean group could reflect a difference in absorption of the ACTH or a very much slower response to ACTH. However, by 120 hours, serum corticosterone level rises to higher values in the obese group than in the lean group. In both groups there was a progressive increase in serum corticosterone from 24 hours onwards, at a time when BAT mitochondrial GDP binding was falling at similar rates in both lean and obese fa/fa rats.

3e. Brown adipose tissue in lean and obese fa/fa rats treated with varying doses of ACTH

The effects of different doses of ACTH on brown adipose tissue in lean and obese fa/fa rats are shown in Table 4. ACTH had no significant effect on BAT wet weight or protein content in lean and obese fa/fa rats at the dose of 25 or 50 µg, but a significant increase in BAT protein content was observed in lean rats at the highest ACTH dose (100 µg). However, no significant changes were observed in tissue succinate cytochrome C oxidoreductase activity with ACTH treatment in these experiments, suggesting that the mitochondria content of brown fat was not altered acutely by ACTH treatment.

Figure 8 shows the effect of different doses of ACTH on brown

FIGURE 5 TIME COURSE OF THE RESPONSE OF BROWN ADIPOSE TISSUE GDP BINDING TO ACTH IN LEAN AND OBESE fa/fa RATS

Lean (o----o) and obese (●——●) fa/fa rats were injected with ACTH 50 µg/day. Animals were killed at the times indicated. BAT mitochondrial GDP binding was determined as described in Section 2.8. Values represent means ± S.E.M. for four rats in each group.

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control zero time group; $^{\dagger}p < 0.05$; $^{\dagger\dagger\dagger}p < 0.001$ compared with lean group.

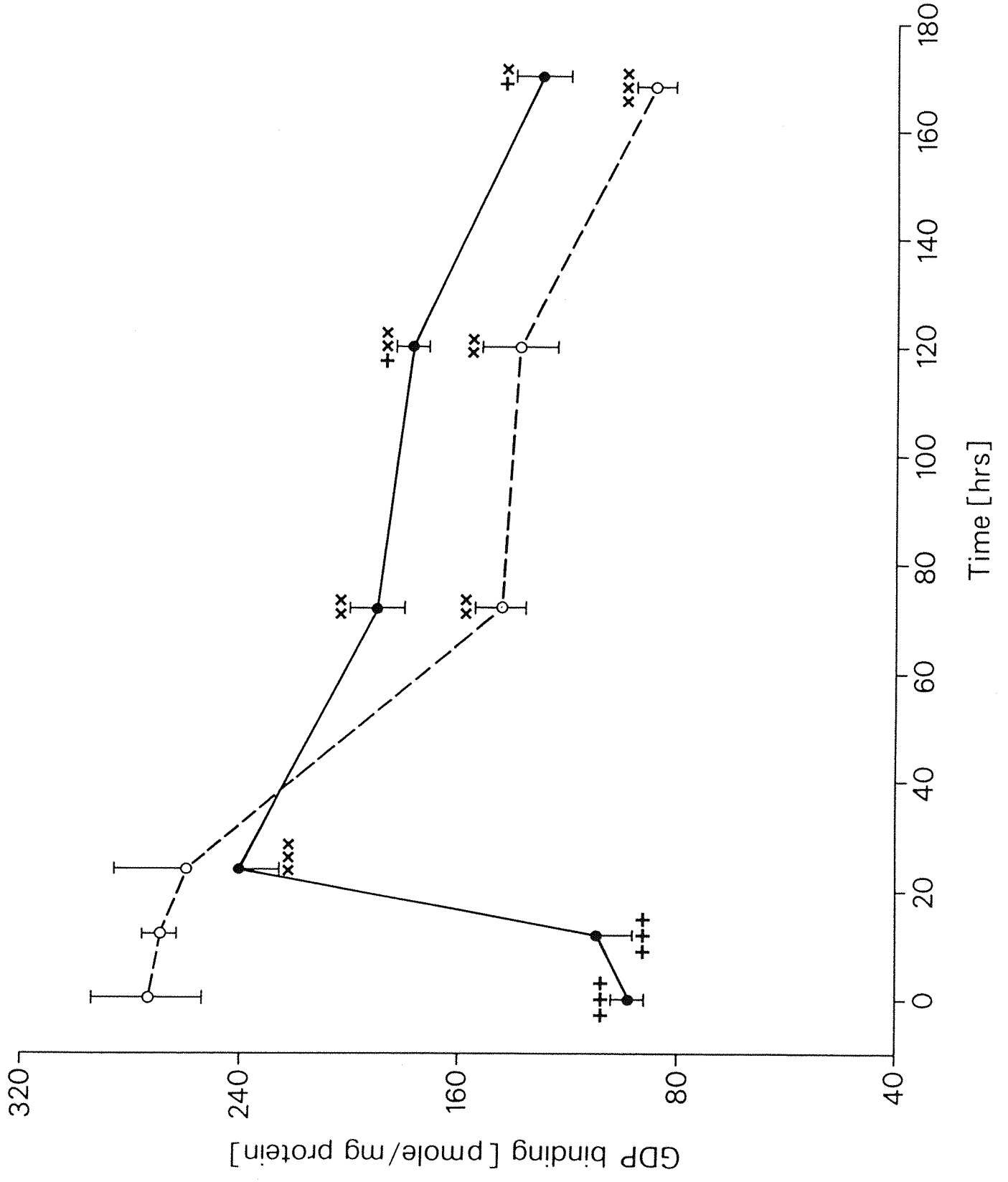

GDP binding [pmole/mg protein]
320
240
160
80
40
0
20
40
60
80
100
120
140
160
180
Time [hrs]
+++
+++
xxx
xx
xx
+xxx
xx
xxx
+x

FIGURE 6 TIME COURSE OF THE RESPONSE OF THE TOTAL BROWN ADIPOSE TISSUE GDP BINDING TO ACTH IN LEAN AND OBESE fa/fa RATS

Lean (o----o) and obese fa/fa (●——●) rats were injected with ACTH as described previously (Fig. 5). Total tissue binding was calculated based on 100% recovery of mitochondria.

Values represent means ± S.E.M. for four rats in each group.

$^{***}p < 0.001$ compared with zero time control group; $^{\dagger\dagger\dagger}p < 0.001$ compared with lean group.

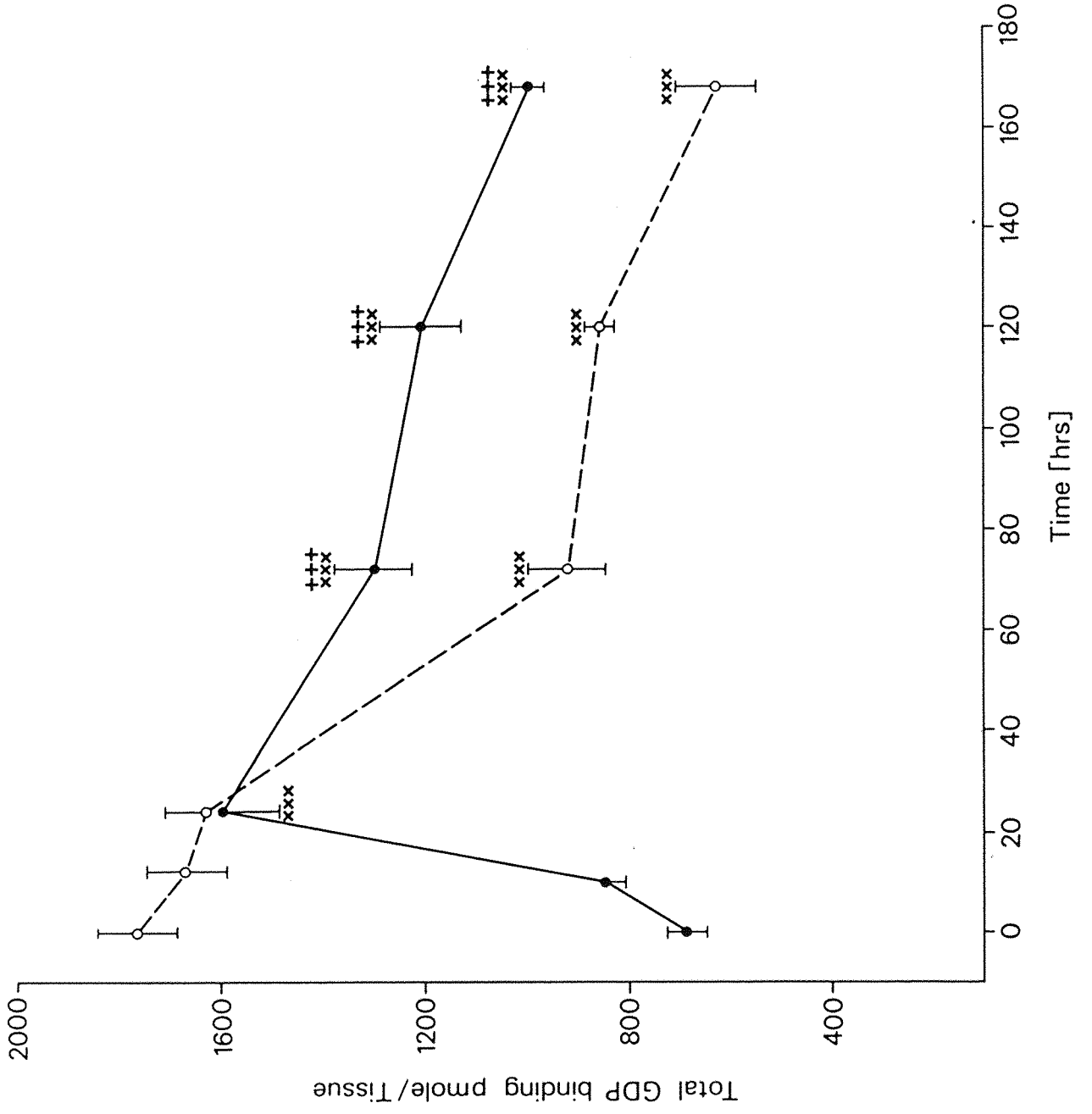
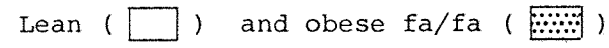
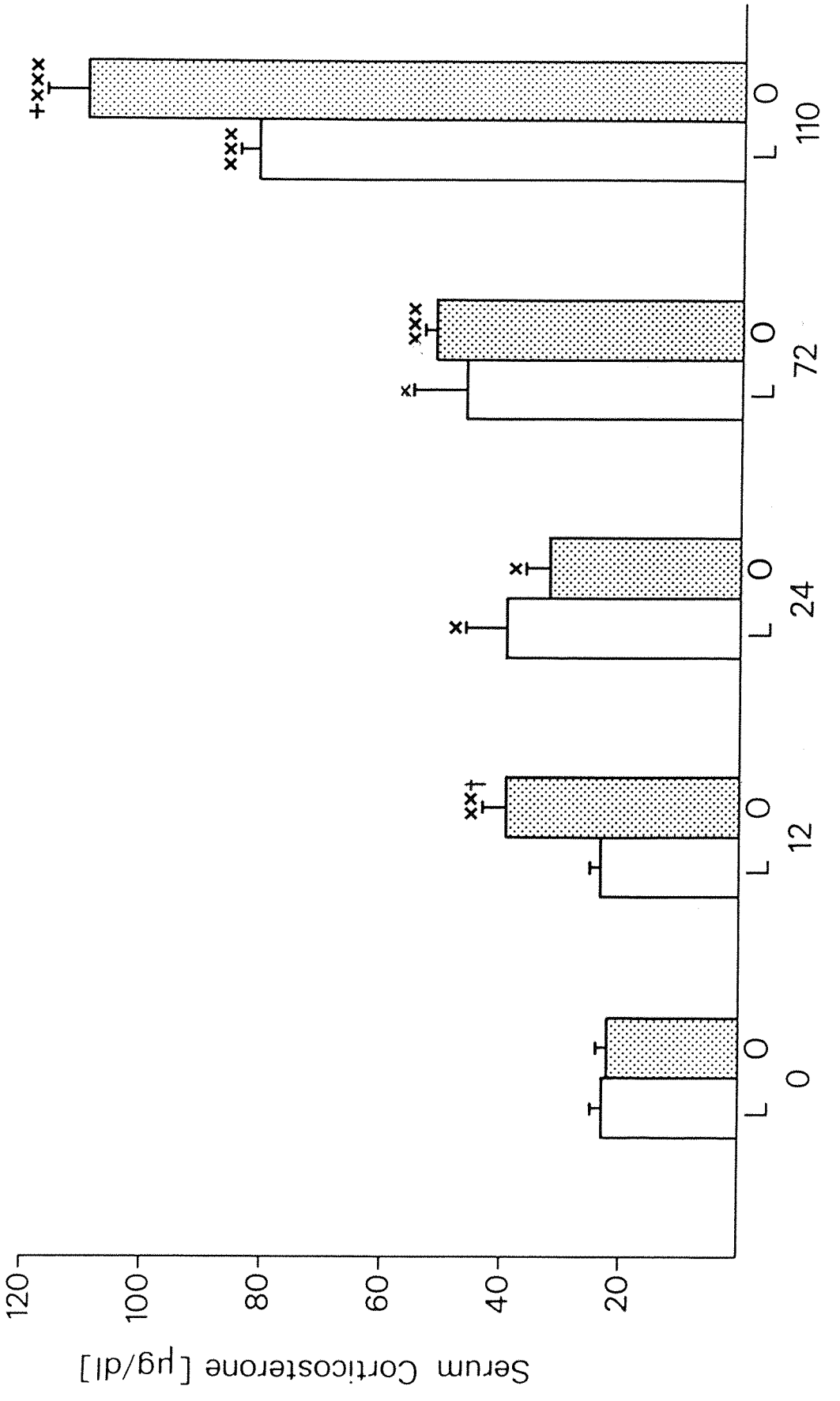

Total GDP binding pmole/Tissue
2000
1600
1200
800
400
0
20
40
60
80
100
120
140
160
180
Time [hrs]

FIGURE 7 TIME COURSE OF THE RESPONSE OF SERUM CORTICOSTERONE TO ACTH IN LEAN AND OBESE fa/fa RATS



Lean ( ) and obese fa/fa ( ) rats were injected with ACTH (50 µg/rats) intramuscularly. Animals were killed at the time indicated. Serum corticosterone determined as described in Section 2.12. Values represent means ± S.E.M. for four rats in each group. *$p < 0.05$

**$p < 0.01$; ***$p < 0.001$ compared with zero time group; †$p < 0.05$ compared with lean group.


Serum Corticosterone [µg/dl]
120
100
80
60
40
20
L O
0
12
24
72
110

adipose tissue GDP binding. In lean rats, an increase in GDP binding was only observed in rats given the highest treatment dose of ACTH (100 µg) when there was a 25% increase. No changes were observed in GDP binding in animals given either 25 or 50 µg ACTH. In obese fa/fa rats an increase of more than 2 fold in GDP binding was observed after a single injection of 50 µg ACTH, restoring GDP binding to the same level observed in control lean rats. At the highest doses of ACTH (100µg) the increase was smaller (only 39%).

Table 5, shows the effect of different doses of ACTH on serum corticosterone, insulin and triiodothyronine in lean and obese fa/fa rats. A significant increase in serum corticosterone concentration was observed in lean rats treated with either 50 µg or 100 µg ACTH. Indeed serum corticosterone level was elevated nearly 2 fold in rats given 100 µg ACTH. In contrast, in obese fa/fa rats, a significant elevation in serum corticosterone level was observed after treatment with 50 and 100 µg ACTH, but still it did not reach the level attained in the lean group. Serum insulin was elevated in the fa/fa rats when compared to the lean rats. Table 5 also shows the characteristic hyperinsulinemia of fa/fa rats aged 4 - 5 weeks. A significant increase in serum insulin concentration was observed in both lean and obese fa/fa rats following the administration of both 50 and 100 µg ACTH. Although the increase was of similar order in both lean (163%) and obese (210%) groups after 100 µg ACTH, the serum insulin level was still about 10 fold higher in the obese fa/fa rats than in the lean. Serum T_3 levels were lower in 5 week old fa/fa rats than in lean animals. A significant increase was observed in obese fa/fa rats after a single injection of 50 µg ACTH, but the level remained lower than that observed in either the lean control or lean ACTH group.

3f. Scatchard analysis of brown adipose tissue mitochondrial GDP binding in obese fa/fa rats treated with ACTH

Scatchard analysis of BAT mitochondrial GDP binding for lean and obese fa/fa rats was reported by *Holt and York (1982); French et al. (1985); Himms-Hagen (1976).* Recently they have shown the presence of two binding sites, a low capacity high affinity and a low affinity-high capacity site. The number of low affinity sites was reduced in obese rats but normalised after adrenalectomy. Obese fa/fa rats showed an

TABLE 4

EFFECT OF DIFFERENT DOSES OF ACTH ON BROWN ADIPOSE TISSUE IN LEAN AND OBESE (fa/fa) RATS

	Rats	Control	ACTH (µg/rat)		
			25 µg	50 µg	100 µg
Brown adipose tissue					
Wet weight (g)	Lean	0.18 ± 0.01	0.17 ± 0.02	0.20 ± 0.01	0.19 ± 0.01
	Obese	0.35 ± 0.60	0.39 ± 0.03	0.32 ± 0.03	0.36 ± 0.03
Protein (mg)	Lean	20.9 ± 2.11	21.0 ± 1.80	21.1 ± 2.3	28.6 ± 2.8*
	Obese	13.25 ± 1.61	15.8 ± 0.97	14.6 ± 1.70	15.6 ± 0.8
Succinate cytochrome C oxido-	Lean	2.31 ± 0.15	-	2.63 ± 0.23	2.48 ± 0.18
reductase (µmol/min/tissue)	Obese	1.45 ± 0.10	-	1.65 ± 0.21	1.62 ± 0.17

Lean and obese fa/fa rats (4-5 weeks old) received a single dose of ACTH or saline intramuscularly 24 hrs before killing. BAT protein content, succinate cytochrome C oxidoreductase were determined as described in Section 2.9 and 2.10 respectively. Values represent means ± S.E.M. for 8 animals in each experimental group with 4 animals for control. *$p < 0.5$ compared with control (no ACTH) group.

FIGURE 8 BROWN ADIPOSE TISSUE GDP BINDING IN LEAN AND OBESE fa/fa RATS TREATED WITH DIFFERENT DOSES OF ACTH

Lean (o----o) and obese (●——●) fa/fa rats (4-5 weeks old) received a single dose of ACTH or saline intramuscularly 24 hrs before killing. BAT mitochondrial GDP binding was determined as described in Section 2.8. Values represent means ± S.E.M. for 8 animals in each group and 4 for control.

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control group.

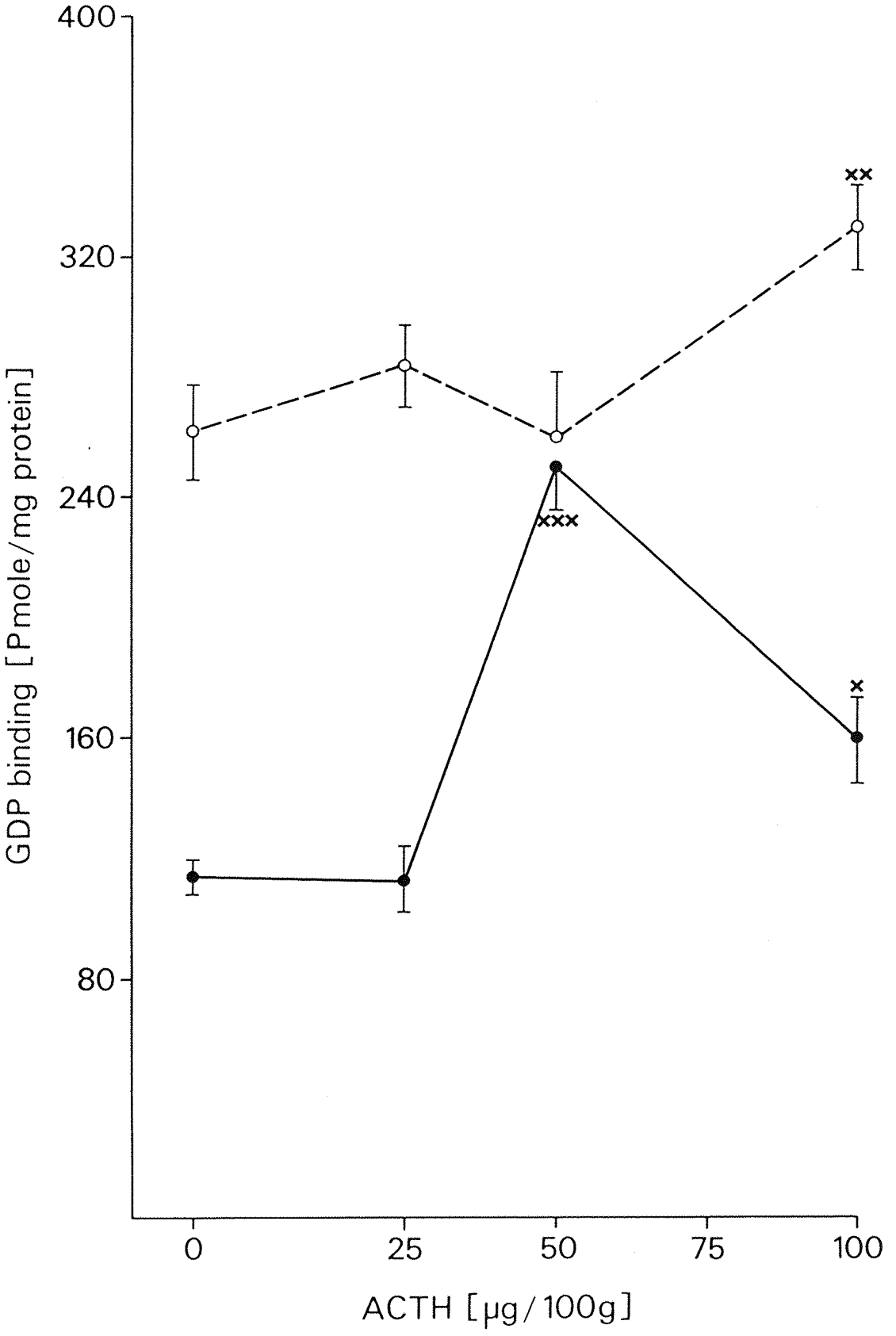

400
320
240
160
80
GDP binding [Pmole/mg protein]
0
25
50
75
100
ACTH [µg/100g]
xx
xxx
x

TABLE 5

EFFECT OF ACTH ON SERUM CORTICOSTERONE, INSULIN AND TRIIODO-THYRONINE IN LEAN AND OBESE fa/fa RATS

	Corticosterone (µg/dl)		Insulin (ng/ml)		Triiodothyronine (nmoles/litre)	
	Lean	Obese fa/fa	Lean	Obese fa/fa	Lean	Obese fa/fa
Control - saline	23 ± 2	22 ± 2	0.84 ± 0.1	8.06 ± 1.1†††	1.73 ± 0.10	0.6 ± 0.10†††
50 µg ACTH	39 ± 7*	34 ± 4*	2.60 ± 0.6*	16.61 ± 1.9***†††	1.95 ± 0.20	0.9 ± 0.10 **†
100 µg ACTH	48 ± 7***	40 ± 5*	2.21 ± 0.8	25.52 ± 1.2**†††		

Lean and obese fa/fa rats (4-5 weeks old) received a single dose of ACTH or saline intramuscularly 24 hrs before killing. Serum corticosterone, insulin and triiodothyronine were determined as described in Section 2.12, 2.15 and 2.16 respectively. Values represent means ± S.E.M. for four rats in each group/ *p < 0.05; **p < 0.01; ***p < 0.001, compared with control (no ACTH group); †p < 0.05; ††† p < 0.001, compared with lean group.

increase in BAT mitochondrial GDP binding after being treated with ACTH. So, in order to see whether this increase in GDP binding is due to an increase in the number of binding sites or a change in affinity for GDP, binding was studied using Scatchard analysis. Scatchard analysis of ^{3}[H]-GDP binding to BAT mitochondria for obese control and obese (50 µg ACTH treated 24 hours before killing) is shown in Figure 9. Data were analysed for either a single straight line plot or for 2 lines plots which would summate to give the observed experimental points by the method used by *Bryant et al. (1983)*. In each case the error for a 2 line plot was considerably less than for a single site plot as shown in Table 6, which also summarises the data for maximum binding (B_{max}) and apparent dissociation constant (K_d). The B_{max} and K_d values for both high and low affinity sites in the obese control rats were similar to that previously observed *(Holt, 1984)*. The affinity of GDP for the high affinity binding site was changed slightly in ACTH treated obese rats, as shown by the change in K_d (0.183; 0.110 µM for the obese control and obese/ACTH respectively. B_{max} for the high affinity site was also increased after ACTH injection from 51 to 82 pmole/mg protein. While no change was observed in K_d for the low affinity site (3.07; 3.3 µM for the obese control and obese/ACTH respectively). B_{max} was increased from 137 to 305 pmole/mg protein in the ACTH-treated group.

3g. Effect of treatment with ACTH (for 3 days) on lean and obese (fa/fa) rats

Tables 7, 8 and 9, show the results of an experiment in which lean and obese fa/fa rats were treated with ACTH (50 µg/day) for 3 days. At 4 weeks of age obese fa/fa rats were slightly, although not significantly, heavier than their lean littermates (Table 7). ACTH impaired body weight gain of lean rats, although the reduction was not statistically significantly after 3 days, and prevented the excessive weight gain of obese rats (Table 7). Indeed the weight gain of ACTH treated obese and control lean rats was similar. Food intake of lean rats was not affected by ACTH treatment. In contrast, the hyperphagia which was apparent in control obese rats was abolished by ACTH treatment, which reduced food intake of the fa/fa rats down to the level observed in lean control and lean ACTH groups (as it is shown in Table 7).

FIGURE 9 SCATCHARD ANALYSIS OF GDP BINDING TO BROWN ADIPOSE TISSUE IN OBESE FA/FA RATS INJECTED WITH ACTH

8 obese fa/fa (●——●) were injected with ACTH (50 µg/rat 24 hrs before killing) and 8 fa/fa (o----o) were used as control. Rats were sacrificed and BAT pooled for each group, mitochondria was prepared as described in Section 2.7. Scatchard analysis was performed by using various concentrations of GDP, ranging from 10 µM to 0.1 µM. GDP binding was determined as described in Section 2.8.

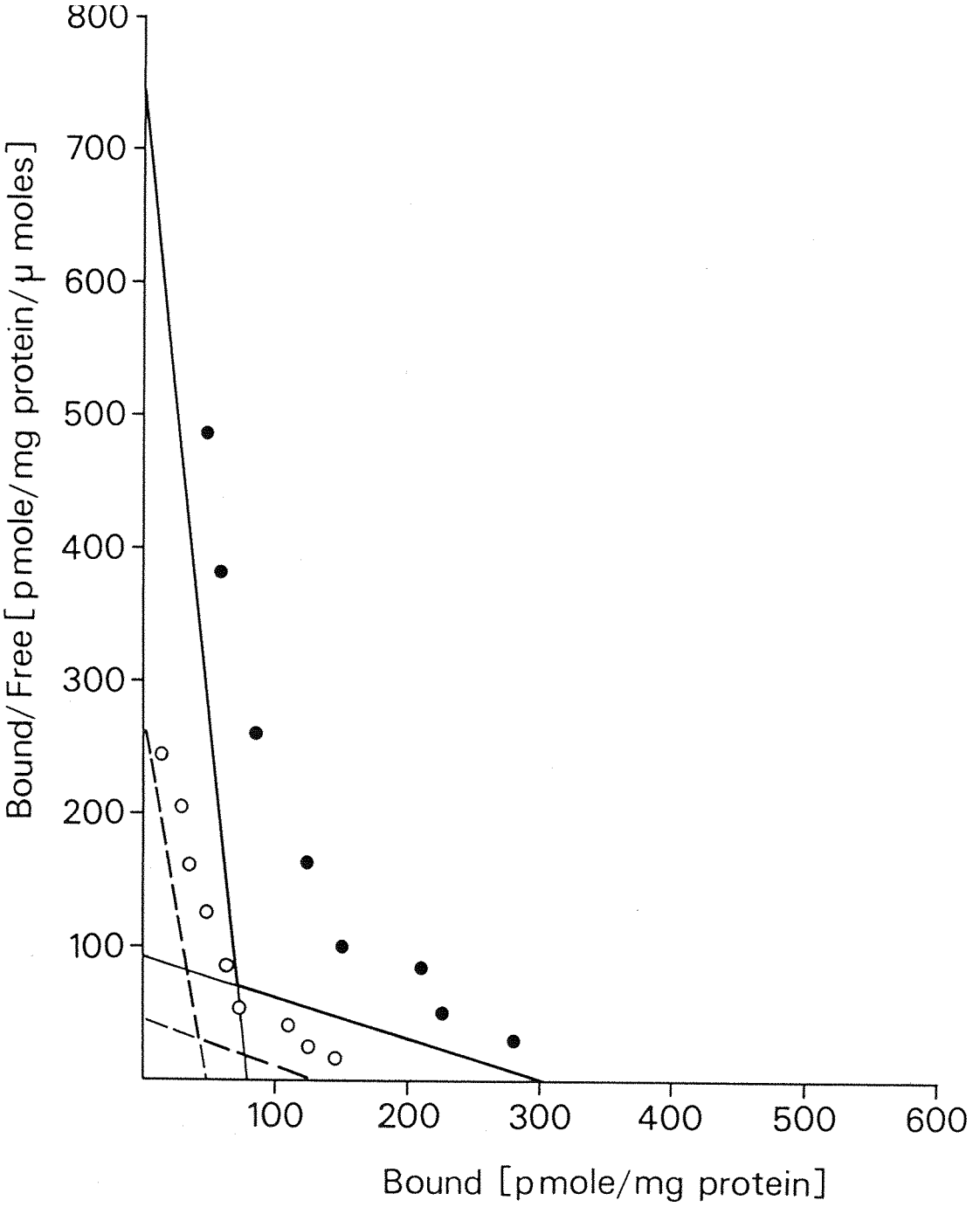

800
700
600
500
400
300
200
100
Bound/ Free [pmole/mg protein/μ moles]
100
200
300
400
500
600
Bound [pmole/mg protein]

TABLE 6

GDP BINDING TO BROWN ADIPOSE TISSUE IN fa/fa RATS INJECTED WITH ACTH

	Site 1		Site 2		
	Kd (μM)	B_{max} (pmole/mg prot.)	Kd (μM)	B_{max} (pmole/mg prot.)	Error
Obese Control	0.183	51	3.07	137	0.96 (3.9)
Obese + ACTH	0.110	82	3.30	305	0.10 (6.5)

Error values (1-r)% represent the error for the Scatchard plots as 2 lines with the error for the data as single line plots given in brackets.

Table 8, shows that in obese fa/fa rats no change was observed in brown adipose tissue wet weight after treatment with ACTH, although a tendency for an increase in both BAT, total protein content and in succinate cytochrome C oxidoreductase activity was observed in the obese-ACTH group. In contrast, there was a significant increase in brown adipose tissue in lean rats treated with ACTH, although no significant changes were observed in either total protein content or succinate cytochrome C oxidoreductase activity.

The specific binding of ^{3}[H] GDP to brown adipose tissue mitochrondria showed a large increase in obese fa/fa rats after ACTH treatment, while a reduction by 40% in mitochondria GDP binding was observed in lean rats. These values are consistent with those previously reported (Section 3d). The net result of these changes was that BAT mitochondrial GDP binding was greater in ACTH treated obese rats than in ACTH treated lean rats. Indeed BAT GDP binding had increased in ACTH treated obese fa/fa rats despite their reduction in food intake. In contrast, GDP binding in lean rats was reduced.

Table 9, shows the effect of daily injection of ACTH on serum corticosterone and insulin concentrations. A significant increase in serum corticosterone level was observed in obese fa/fa rats. A similar increase was observed in serum corticosterone of lean rats, although in this case the increase was not significant. Serum insulin also increased significantly in both lean and obese fa/fa rats treated with ACTH. The increase in obese rats was similar to that previously reported (Section 3b), 24 hours after ACTH treatment, whereas the levels attained in lean rats after 3 days were greater than those observed after 24 hours of ACTH treatment.

3h. Effect of chronic (21 days) treatment with ACTH on body weight, food intake and brown adipose tissue in obese fa/fa rats

Since ACTH treatment increased BAT GDP binding, and reduced food intake and body weight of obese fa/fa rats, it was possible that the increase in ACTH which followed adrenalectomy was responsible for the prevention of obesity. In order to study this further, the effects of chronic treatment of ACTH on obesity in obese fa/fa rats were investigated. Obese fa/fa rats (4 weeks old) were injected with ACTH

TABLE 7

THE EFFECT OF TREATMENT WITH ACTH (FOR 3 DAYS) ON BODY WEIGHT, FOOD INTAKE OF 4 WEEK OLD LEAN AND OBESE fa/fa RATS

	Control		+ACTH	
	Lean	Obese (fa/fa)	Lean	Obese (fa/fa)
Food intake (KJ/day)	137.4 ± 2.7	194.7 ± 23.0†	136.4 ± 7.2	141.2 ± 14.4*
Initial body weight (g)	63.8 ± 3.4	85.5 ± 13.3	62.4 ± 2.4	81.0 ± 10.5
Gain in body weight (g)	7.8 ± 1.2	19.5 ± 1.1†††	4.9 ± 1.3	7.5 ± 1.5***

Lean and obese fa/fa rats were housed individually and treated with ACTH (50 µg/rat/24 hr) intramuscularly for 72 hrs. Food intake was measured daily as described in Section 2.4. Values represent means ± S.E.M. for four rats in each group. *$p < 0.05$; ***$p < 0.001$ compared with respective control group; †$p < 0.05$; ††† $p < 0.001$ compared with lean group.

TABLE 8

THE EFFECT OF TREATMENT WITH ACTH (FOR 3 DAYS) ON BROWN ADIPOSE TISSUE IN LEAN AND OBESE fa/fa RATS

	Control		+ ACTH	
	Lean	Obese fa/fa	Lean	Obese fa/fa
Brown adipose tissue:				
Wet weight (g)	0.22 ± 0.03	0.52 ± 0.02†††	0.36 ± 0.02**	0.54 ± 0.05††
Protein (mg)	25.3 ± 3.7	20.2 ± 2.1	29.6 ± 3.8	29.0 ± 2.6**
Succinate cytochrome C oxidoreductase (μmol/min/tissue)	2.2 ± 0.3	1.1 ± 0.1††	2.9 ± 0.7	1.5 ± 0.2†
GDP binding (pmol/mg protein)	239 ± 26	90 ± 4†††	144 ± 9**	191 ± 26$^{\dagger **}$
Total GDP binding (pmol/tissue)	1700 ± 60	685 ± 20†††	856 ± 68***	1299 ± 75$^{\dagger\dagger ***}$

4 week old lean and obese fa/fa rats were housed individually as described in Table 4. BAT, protein, succinate cytochrome C oxidoreductase and mitochondrial GDP binding were measured as described in Section 2.9, 2.10 and 2.8 respectively. Total tissue binding was calculated based on 100% recovery of mitochondria. Values represent means ± S.E.M. for four rats in each group. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control group; $^{\dagger} p < 0.05$; $^{\dagger\dagger} p < 0.01$; $^{\dagger\dagger\dagger} p < 0.001$ compared with lean group.

TABLE 9

EFFECT OF DAILY ACTH INJECTION ON SERUM CORTICOSTERONE AND INSULIN IN LEAN AND OBESE fa/fa RATS

	Control		+ ACTH	
	Lean	Obese fa/fa	Lean	Obese fa/fa
Serum corticosterone (µg/dl)	27 ± 5.0	26 ± 3.0	46 ± 9	51 ± 2***
Serum insulin (ng/ml)	0.8 ± 0.1	9.8 ± 0.2	5.3 ± 2.0*	20.1 ± 3.0**††

Lean and obese fa/fa rats were housed and treated as described previously in Table 7. Serum corticosterone and insulin were measured as described in Section 2.12, 2.15 respectively. Values represent means ± S.E.M. for four rats in each group/ *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with control; †† $p < 0.01$ compared with lean group.

(50 µg/rat/day) for 21 days. Results in Table 10 show that there was a significant reduction in body weight and food intake in ACTH treated obese fa/fa rats. However, these chronic changes were accompanied by a lack of linear growth. The length of rats treated with ACTH did not change significantly during the 21-day period. The Lee Index, a measure of obesity (Simson and Gold, 1982), was greater in ACTH treated obese rats than in the control obese group, suggesting an increase in obesity. However, this data contrasted with the reduced weight gain and the absence of any growth in the inguinal white fat pad in the obese ACTH treated group, whereas this fat depot increased in weight by nearly four fold in the control obese group. Similarly, the weight/length ratio did not increase significantly in the ACTH treated obese group, to the same extent as those of the control obese group. The effect of chronic ACTH treatment on BAT is shown in (Table 11). BAT wet weight increased two fold during the 21-day experimental period, in obese control rats. However, protein content did not change significantly during this period. Similarly, no change in succinate cytochrome C oxidoreductase activity was observed. ACTH treatment had no effect on tissue wet weight, BAT protein content or on mitochondrial content. In contrast, BAT mitochondrial GDP binding was reduced in the ACTH treated obese group by more than 50%. A reduction in the total tissue GDP binding was also observed. Serum corticosterone was increased significantly after chronic treatment with ACTH.

3i. Effect of ACTH on brown adipose tissue GDP binding in adrenalectomized lean and obese fa/fa rats maintained on corticosterone

It has previously been shown that corticosterone suppressed BAT mitochondrial GDP binding in mice and rats, while adrenalectomy enhanced GDP binding in both mice and rats (*Holt et al., 1982; Galpin et al., 1983; Holt, 1984).* In contrast, no stimulation of BAT GDP binding by ACTH could be demonstrated in lean rats, although a large stimulation was apparent in obese rats. In order to demonstrate an independent effect of ACTH it was necessary to study the response of ACTH in the absence of any secretion of endogenous corticosterone.

Thus, lean and obese fa/fa rats were adrenalectomised and maintained on either saline drinking solution (0.9% w/v) alone, or together

TABLE 10

EFFECT OF CHRONIC TREATMENT (21 DAYS) WITH ACTH ON BODY WEIGHT, FOOD INTAKE OF OBESE fa/fa RATS

	Zero time Control	Saline Vehicle	ACTH
Initial body wt (g)	68 ± 5.3	65 ± 4.8	66.7 ± 5.9
Change in body wt (g)	-	128 ± 23.0	24 ± 5.0***
Length (cm)	11.5 ± 0.2	17.1 ± 0.6	12.9 ± 0.7††*
$\frac{\text{Wt (g)}}{\text{Length (cm)}}$	5.9 ± 0.3	10.3 ± 0.9	7.5 ± 0.24**††
Lee index	353.5 ± 2.0	328 ± 16.6	357 ± 9.1††
Food intake (KJ/day)		197.6 ± 3.8	158.0 ± 9.0†††
Inguinal white adipose tissue (g)	0.78 ± 0.1	2.84 ± 0.2	0.70 ± 0.1†††

4 week old obese fa/fa rats were injected with ACTH 50 µg/rat/day for 21 days. Rats were housed individually, food intake was measured daily as described in Section 2.4. Lee index was calculated from the formula:

$$\text{Lee index} = \sqrt[3]{\text{Body wt (g)}} \times \frac{10^3}{\text{length (cm)}}$$

Values represent means ± S.E.M. for four animals in each group.
*$p < 0.05$; **$p < 0.01$ compared with zero time control group;
†$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$ compared with saline vehicle control.

TABLE 11

EFFECT OF CHRONIC TREATMENT (21 DAYS) WITH ACTH ON BROWN ADIPOSE TISSUE OF OBESE fa/fa RATS

	Zero time control	Saline Vehicle	ACTH
Brown adipose tissue:			
Wet weight (g)	0.38 ± 0.04	0.69 ± 0.04	0.74 ± 0.12
Protein (mg)	14.5 ± 1.23	17.6 ± 3.10	16.5 ± 1.02
Succinate cytochrome C oxidoreductase (μmol/min/tissue)	1.20 ± 0.1	1.01 ± 0.1	1.10 ± 0.2
GDP binding (pmol/mg protein)	86 ± 8	89 ± 4	38 ± 3***†††
Total GDP binding (pmole/tissue)	537 ± 11	554 ± 28	237 ± 14***†††
Serum corticosterone (μg/dl)	18.2 ± 4.5	24.2 ± 3.0	55.3 ± 12*†

4 week old obese fa/fa rats were injected with ACTH (50 μg/rat/day) for 21 days. Brown adipose tissue protein, succinate cytochrome C oxidoreductase activity and mitochondrial GDP binding determined as described in Section 2.9, 2.10 and 2.8 respectively. Total tissue binding was calculated based on 100% recovery of mitochondria. Values represent means ± S.E.M. for four rats in each group. *$p < 0.05$; ***$p < 0.001$ compared with control at zero time; †$p < 0.05$; †††$p < 0.001$ compared with (same vehicle) control.

with corticosterone replacement (1 mg/day for 7 days). This latter treatment gave serum corticosterone concentration of 2.5 ± 0.4 and 1.8 ± 0.3 µg/dl 24 hr after the last corticosterone injection, for 8 lean and 8 obese rats respectively. The acute response of GDP binding to ACTH (50 µg) in adrenalectomized lean and obese fa/fa rats maintained on corticosterone is shown in Table 12. Corticosterone replacement prevents the increase in GDP binding of adrenalectomized lean rats. These results confirm previous findings of *Holt et al. (1983)*. However, in both adrenalectomized lean and obese fa/fa rats maintained on corticosterone, ACTH stimulated GDP binding by a similar amount (71 pmoles/mg in both groups). This result suggests that ACTH stimulated GDP binding even in lean rats when the inhibitory effect of corticosterone was absent, as GDP binding in both lean and obese rats was increased by 29% and 42% respectively.

3j. Effect of feeding additional sucrose on food intake, body weight and brown adipose tissue in lean and obese fa/fa rats injected with ACTH

A reduction in BAT mitochondrial GDP binding has been shown in obese fa/fa rats. Since BAT in obese fa/fa rats respond normally to cold exposure, this suggests that obese fa/fa rats fail to increase BAT thermogenesis in response to dietary signals *(Holt et al., 1983)*. Lean rats overfed by either additional sucrose or cafeteria diet, showed an increase in GDP binding *(Holt et al., 1983; Triandafillou et al., 1983)*. This increase in BAT thermogenesis which appears after overfeeding could be prevented by corticosterone treatment *(York et al., 1985; Galpin, 1983)*. ACTH injection restored the level of BAT mitochondrial GDP binding observed in fa/fa rats to lean values. So it was of interest, therefore, to examine whether the increased thermogenesis seen in ACTH-treated rats was related to an improvement in the ability to regulate thermogenesis in response to dietary intake.

Results in Table 13 and 14, showed the result of experiments in which lean and obese fa/fa rats were injected with ACTH (50 µg/day), while fed with additional sucrose for 3 days. Table 13, shows that total food intake in lean rats, having additional sucrose, is significantly higher than in the control and lean/ACTH group. Despite the large increase in energy intake, there was no increase in body weight

TABLE 12

EFFECT OF ACTH ON BROWN ADIPOSE TISSUE GDP BINDING IN ADRENALECTOMISED LEAN AND OBESE fa/fa RATS MAINTAINED ON CORTICOSTERONE

	GDP binding (pmole/mg protein)		Total GDP binding (pmole/tissue)	
	Lean	Obese fa/fa	Lean	Obese fa/fa
Sham-operated	253 ± 8^{a}	104 ± 9^{c}	1294 ± 48^{x}	520 ± 73^{z}
Adrenalectomized	240 ± 20^{a}	238 ± 24^{a}	1300 ± 76^{x}	1190 ± 55^{x}
Adrenalectomized + corticosterone + saline	246 ± 10^{a}	161 ± 31^{c}	1202 ± 63^{x}	678 ± 80^{z}
Adrenalectomized + corticosterone + ACTH	317 ± 15^{b}	232 ± 15^{a}	1560 ± 65^{y}	1266 ± 100^{x}

Lean and obese fa/fa rats (4 weeks old) were adrenalectomized and sham operated, as described in Section 2.5. Adrenalectomized animals were maintained on saline drinking solution. They were maintained on corticosterone (1 mg/day); Subcutaneously or saline vehicle as described in Section 2.6.2, after 6 days rats were given either 50 µg ACTH or saline vehicle intramuscularly, 24 hrs before killing. Other details were as described in Table 12. Values represent means ± S.E.M. for five rats in each group. Values with different superscript letters are significantly different or $p < 0.05$ at least.

TABLE 13

EFFECT OF FEEDING ADDITIONAL SUCROSE ON FOOD INTAKE, BODY WEIGHT OF LEAN AND OBESE fa/fa RATS INJECTED WITH ACTH

	Lean			Obese (fa/fa)		
	Chow only	+ACTH	Additional Sucrose +ACTH	Chow only	+ACTH	Additional Sucrose +ACTH
Food intake						
Chow (KJ/day)	105 ± 2.2	108.1 ± 0.9	82.8 ± 2.0^{ec}	188.2 ± 2.7	172.8 ± 6.3	124.6 ± 1.8^{ecy}
Sucrose (KJ/day)			58.0 ± 4.2			63.0 ± 2.0^{y}
Total (KJ/day)	105 ± 2.2	108.1 ± 0.9	140.8 ± 3.1^{ec}	188.2 ± 2.7	172.8 ± 6.3	187.6 ± 2.0^{ycd}
Initial body wt (g)	61.4 ± 3.4	62.3 ± 2.2	64.3 ± 4.0	109.0 ± 4.0	99.0 ± 6.1	107.0 ± 5.0^{y}
Increase in body wt (g)	14.3 ± 0.9	4.6 ± 0.6	4.8 ± 0.6^{c}	20.2 ± 2.8	8.2 ± 0.5	12.5 ± 1.0^{by}

12 lean and 12 obese fa/fa rats were housed individually and offered chow ad libitum. 8 rats in each group were injected with ACTH (50 µg/rat/day) intramuscularly, 4 of those rats were allowed to drink a 35% (w/v) sucrose solution ad libitum, as described in Section 2.3. The intake of chow and sucrose were measured daily for 4 days (as described in Section 2.4). After 4 days all rats were killed. Values represent means ± S.E.M. for four rats in each group. $^{b}p < 0.01$; $^{c}p < 0.001$, compared with control group; $^{d}p < 0.05$; $^{e}p < 0.001$, compared with ACTH group; $^{y}p < 0.001$, compared with lean group.

TABLE 14

EFFECT OF FEEDING ADDITIONAL SUCROSE ON BROWN ADIPOSE TISSUE IN LEAN AND OBESE fa/fa RATS INJECTED WITH ACTH

	Lean			Obese (fa/fa)		
	Chow only	+ACTH	Additional Sucrose+ACTH	Chow only	+ACTH	Additional Sucrose+ACTH
Brown adipose tissue						
wet wt (g)	0.18 ± 0.01	0.39 ± 0.04	0.36± 0.01^{c}	0.40± 0.02	0.70 ± 0.06	0.56 ± 0.07^{az}
protein (mg)	14.3 ± 3.0	20.6 ± 2.2	23.8 ± 1.9^{a}	14.4 ± 0.5	23 ± 1.6	19.3 ± 2.5
Succinate cytochrome C oxido-reductase (µmol/min/tissue)	1.81 ± 0.05	2.0 ± 0.4	2.7 ± 0.71^{a}	1.04± 0.1	1.84 ± 0.3	1.98 ± 0.3^{a}
GDP binding (pmol/mg protein)	277 ± 6	136 ±15	358 ±30^{ae}	103 ± 9	190 ± 4	193 ± 3^{cy}
Total GDP binding (pmol/tissue)	1250 ±69	1148 ±64	1640 ±106^{eb}	720 ±72	1367 ±53	1349 ±34^{c}

12 lean and 12 obese fa/fa rats were housed and treated as described in Table 13. Brown adipose tissue, protein, succinate cytochrome C oxidoreductase and mitochondrial GDP binding were measured as described in Section 2.9, 2.10 and 2.8, respectively. Total binding was calculated based on 100% recovery of mitochondria. Values represent means ± S.E.M. for four rats in each group. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$, compared with control group; $^{e}p < 0.001$, compared with ACTH group; $^{z}p < 0.05$; $^{y}p < 0.001$, compared with lean group.

in the sucrose fed ACTH group when compared with the ACTH alone group. Both groups gained significantly less weight than the control (chow only) group.

In contrast with the obese fa/fa group, no increase in total food intake was observed in the obese/sucrose/ACTH group compared with the control group. In both groups, food intake was significantly higher than in the obese ACTH/group. The increase in body weight was significantly more in the obese ACTH group, but still did not reach the body weight of the control (chow only) obese group.

Table 14 shows the effect of feeding additional sucrose accompanied with ACTH injection, on BAT in lean and obese fa/fa rats. An increase in BAT wet weight, protein content and succinate cytochrome C oxidoreductase activity was observed in lean and obese fa/fa rats, compared with their respective control groups. BAT mitochondrial GDP binding was depressed in ACTH-treated lean group, but was increased significantly when these rats were given supplementary sucrose. However, the stimulation was less than that previously observed by *Holt et al. (1983)*, which might be due to ACTH treatment. In contrast, obese fa/fa rats did not show any further increase in GDP binding, as no increase in energy intake was observed.

3k. Effect of the β-antagonist propranolol on brown adipose tissue in obese fa/fa rats injected with ACTH

It has been shown that BAT thermogenesis could be blocked by β-antagonists, such as propranolol, and BAT GDP binding in lean rats was reduced by 40% after propranolol treatment, while no effect on BAT GDP binding was found with obese fa/fa rats. The increase in BAT GDP binding which was found in adrenalectomized obese fa/fa rats, could be blocked by propranolol *(York et al., 1985)*, (which suggested that sympathetic activity was increased in adrenalectomized obese fa/fa rats). However, an increase in GDP binding in fa/fa rats after ACTH treatment, has been established. Therefore, it would be of interest to see whether this increase could be blocked (prevented) by propranolol treatment. Obese fa/fa rats were treated with 50 μg ACTH/rat and propranolol, as described in Section 2.6.3, 24 hr before killing. Results in Table 15, show that the ACTH-induced increase in BAT GDP

TABLE 15

EFFECT OF THE β-ANTAGONIST PROPRANOLOL ON BROWN ADIPOSE TISSUE OF OBESE fa/fa RATS INJECTED WITH ACTH

	Obese fa/fa		
	Control	+ ACTH	+ACTH +Propranolol
Brown adipose tissue:			
Wet weight (g)	0.40 ± 0.03	0.39 ± 0.04	0.43 ± 0.04
Protein (mg)	11.7 ± 1.51	12.6 ± 1.90	10.6 ± 0.50
Succinate cytochrome C oxidoreductase (μmol/min/tissue)	1.1 ± 0.10	1.6 ± 0.21	0.83 ± 0.10
GDP binding (pmole/mg protein)	144 ± 12	254 ± 13***	146 ± 8†††

4-5 week old obese fa/fa rats were injected with 50 μg ACTH/rat, intramuscularly, 24 hrs before killing. Rats were given (during the 24 hrs) propranalol (2 mg/100 g body weight) or vehicle subcutaneously, as described in Section 2.6.3, three times over a 15 hr period (beginning 6 hrs after ACTH injection) at 16.00, 24.00 and 08.00 h. Rats were killed 1 hr after the final injection. Other details were as described in Table 14. Values represent means ± S.E.M. for 8 rats in each group. ***$p < 0.001$ compared with control group; †††$p < 0.001$ compared with ACTH group.

binding in obese rats was blocked completely by propranolol treatment. Table 16 shows the effect of propranolol on serum corticosterone and insulin concentrations. Propranolol had no effect on either serum corticosterone or serum insulin levels.

3ℓ. The acute effect of Metopirone injection on brown adipose tissue in obese fa/fa rats

It has been shown from the present results that ACTH restored GDP binding in obese fa/fa rats to the same level observed in lean animals. As ACTH administration continued, a significant rise in serum corticosterone levels was evident, and this was accompanied by a decrease in mitochondrial GDP binding to the low level observed in untreated obese fa/fa rats. Increasing serum corticosterone concentrations are normally associated with a reduction in BAT thermogenic function. Therefore, it was of interest to see whether the inhibition of corticosterone secretion could cause an increase in BAT mitochondrial GDP binding. So, Metopirone (Metyropane), a corticosterone biosynthesis inhibitor, was used in the following experiment. Four week old obese fa/fa rats were housed individually, and they were injected with metopirone two times, 16 hr and 8 hr before killing them. The results of this experiment are shown in Table 17. A redution in food intake during the 16 hr period was observed in the metopirone treated obese rats, although serum corticosterone was not altered at the time of sacrifice. This observation agrees with a previous report by *Schefzig et al.* (1978) in human subjects, who observed a marked fall in serum corticosterone (30, 90) mins after metopirone injection, which then started to rise after 8 hr until it reached control values. However, no effect was observed on BAT wet weight or tissue protein, but there was a significant increase in succinate cytochrome C oxidoreductase, which suggests that the mitochondria content of brown fat was increased by metopirone treatment. BAT mitochondrial GDP binding was increased significantly after metopirone injection, and reached the value previously observed in control lean rats. Total tissue mitochondrial GDP binding was increased by over 250% in the metopirone treated obese rats.

TABLE 16

EFFECT OF THE β-ANTAGONIST PROPRANOLOL ON SERUM CORTICOSTERONE AND INSULIN IN OBESE fa/fa RATS INJECTED WITH ACTH

	Control	+ ACTH	+ ACTH + propranolol
Serum corticosterone (µg/dl)	20.75 ± 2.3	35.1 ± 2.8***	36.1 ± 3.4***
Serum insulin (ng/ml)	11.5 ± 2.1	16.1 ± 5.8	16.4 ± 3.2

Obese fa/fa rats were injected with ACTH, propranolol as described in previous Table 15. Serum corticosterone and insulin were measured as described in Section 2.12, 2.14 respectively. Values represent means ± S.E.M. for 5 rats in each group. ***$p < 0.001$ compared with control group.

TABLE 17

THE ACUTE EFFECT OF METOPIRONE INJECTION ON BROWN ADIPOSE TISSUE IN OBESE fa/fa RATS

	Obese fa/fa	
	Control	+ Metopirone
Body wt. (g)	91.8 ± 3.2	92.5 ± 2.5
Food intake (KJ)	109 ± 5.4	80 ± 8.1**
Serum corticosterone (µg/dl)	19.2 ± 1.97	18.4 ± 1.04
Brown adipose tissue		
wet weight (g)	0.49 ± 0.1	0.51± 0.1
protein (mg)	16.1 ± 0.9	19.0 ± 2.3
Succinate Cytochrome C Oxidoreductase activity (µmol/min/Tissue)	1.6 ± 0.1	2.7 ± 0.4**
GDP Binding (pmol/mg protein)	118 ± 11	243 ± 31***

Obese fa/fa rats were housed individually. Food intake was measured. Rats were injected with metopirone (25 mg/100g body weight), subcutaneously, as described in Section 2.6.4 16 hrs before killing. Control rats received vehicle. They were injected two times during the 16 hrs, starting at 16.00, 24.00, then they were killed at 09.00. BAT, protein, succinate cytochrome C oxidoreductase and mitochondrial GDP binding were measured as described in Section 2.9, 2.10, 2.8 respectively.

Values represent means ± S.E.M. for 8 rats in each group.

** $p < 0.01$; *** $p < 0.001$ compared with control group.

3m. The *in vitro* effect of corticosterone on brown adipose tissue GDP binding

Present results suggest that corticosterone has an inhibitory effect on BAT mitochondrial GDP binding, which agrees with previous reports by *Holt et al. (1983); York et al. (1984), Galpin et al. 1983.* This inhibitory effect of corticosterone has been associated with an inhibition of sympathetic activity of BAT *(York et al., 1985).* However, in order to see whether corticosterone has a direct inhibitory effect on BAT mitochondrial GDP binding, the following test was carried out: BAT was minced, incubated in Krebs ringer bicarbonate buffer (pH 7.2) in the presence and absence of corticosterone (0.1 mg/mls), for the times indicated in Figure 10. At various times, the tissue was harvested, homogenised and mitochondria prepared for assay of GDP binding. Results in Figure 10 show the time course of the *in vitro* effect of corticosterone on BAT mitochondrial GDP binding. An increase in BAT mitochondrial GDP binding was observed after 60 min of incubation after which there was a subsequent fall in binding, back to the pre-incubation levels. A similar rise was observed in tissue incubated with corticosterone. However, a small, but statistically significant, increase in BAT GDP binding was observed in samples incubated with corticosterone for 120 and 360 minutes.

FIGURE 10 EFFECT OF CORTICOSTERONE ON BROWN ADIPOSE TISSUE GDP BINDING IN VITRO

Brown adipose tissue from control lean rats was dissected, chopped and incubated in Krebs Ringer bicarbonate buffer (pH 7.2) + (o----o) and - (●——●) corticosterone (0.1 mg/ml), for the time indicated. BAT mitochondria were prepared as described in Section 2.7. BAT, mitochondrial GDP binding was measured as described in Section 7.8. Values represent means ± S.E.M. for 4 samples in each group.

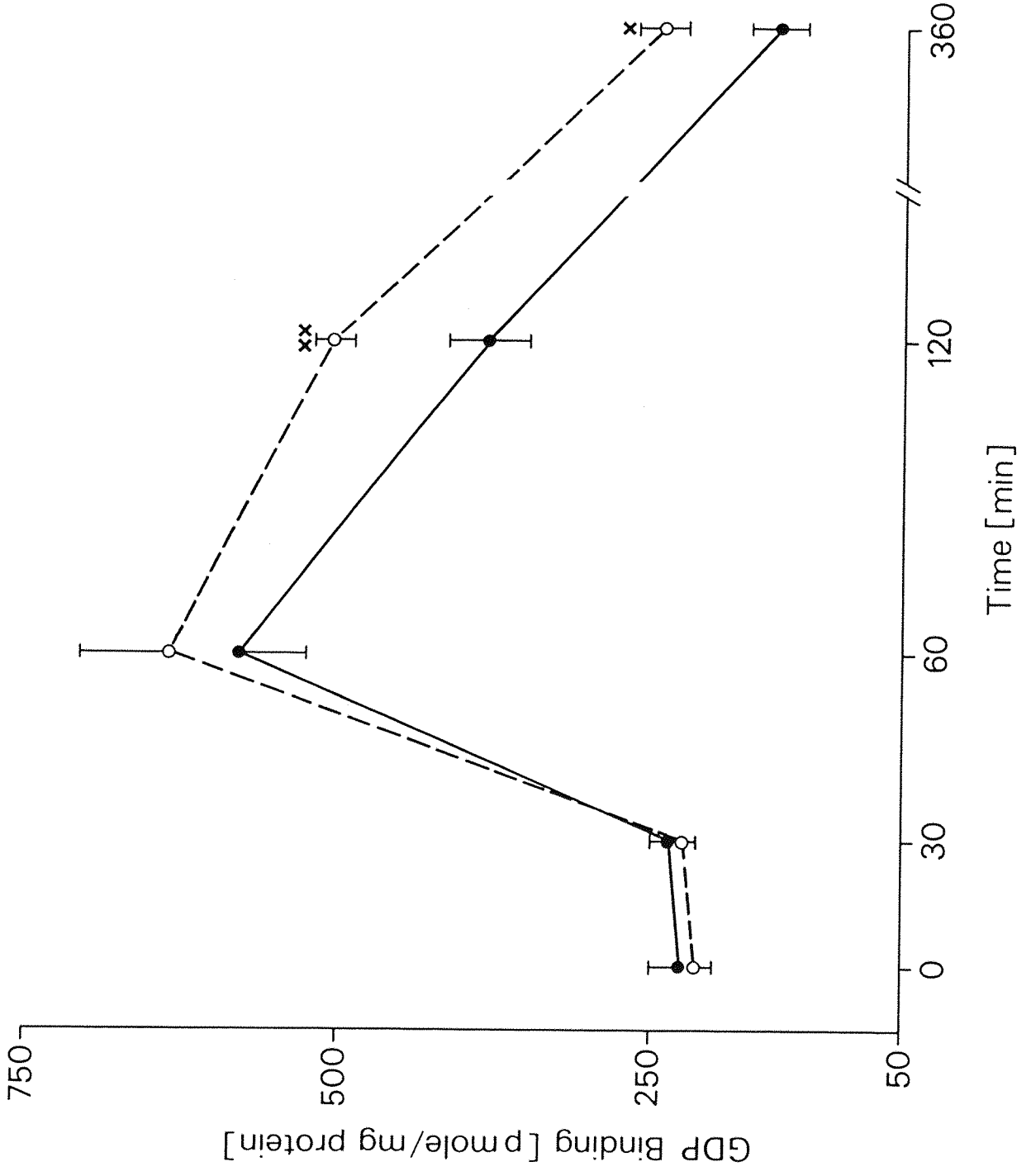

GDP Binding [p mole/mg protein]
750
500
250
50
0
30
60
120
360
Time [min]
xx
x

TABLE 18

EFFECT OF CORTICOSTERONE ON BROWN ADIPOSE TISSUE GDP BINDING IN VITRO

Incubation Time (mins)	% Stimulation of GDP Binding (above Control Tissue)
30	-
60	8.5 ± 2.5
120	18.5 ± 3.5
360	31.8 ± 5.5

Brown adipose tissue from control lean rats was dissected, chopped and incubated in Krebs Ringer bicarbonate buffer (pH 7.2), with absence and presence of corticosterone (0.1 mg/ml), for the time indicated. BAT mitochondria were prepared as described in Section 2.7. BAT mitochondrial GDP binding was measured as described in Section 7.8.

Values represent means ± S.E.M. for 4 samples in each group.

CHAPTER 4

DISCUSSION

The obesity in obese fa/fa rats is thought to result from a decrease in energy expenditure, since an increase in fat deposition occurs even in the absence of hyperphagia *(Radcliffe and Webster, 1976; Bray et al., 1973)*. The increase in energetic efficiency which this suggests has been clearly demonstrated by *Marchington et al. (1983)*. Evidence has shown that this increase in energetic efficiency is associated with a decrease in the maintenance energy requirement *(Pullar and Webster, 1977)*, and the loss of the normal thermogenic response to feeding, whereas the thermogenic response to environmental cold is normal *(Holt et al., 1983; Triandafillou and Himms-Hagen, 1983)*. BAT has been shown to be the major site of both cold (non-shivering) and dietary induced thermogenesis *(Rothwell and Stock, 1979)*. The specialised thermogenic function of BAT resides in the unique mitochondrial proton conductance pathway, the activity of which can be monitored by the binding of GDP to the 32000 mol. wt. protein *(Nicholls, 1979; Brooks et al., 1980)*.

A reduction in BAT mitochondrial GDP binding in fa/fa rats has been widely reported *(Holt et al., 1983; Triandafillou and Himms-Hagen, 1983; York et al., 1984)*, and this defect has been confirmed in the studies reported in this thesis. This defect is thought to result from the failure to increase BAT thermogenesis in response to dietary signals, since they show a normal increase in BAT thermogenesis when exposed to cold ($4^{o}C$) *(Holt et al., 1983; Rothwell et al., 1981; Triandafillou and Himms-Hagen, 1983)*. This defect, reflected in a reduction in BAT mitochondrial GDP binding, is present shortly after birth *(Bazin et al., 1984; York et al., 1984)*, and appears to be the earliest identifiable change in the fa/fa genotype. The reduction in BAT mitochondrial GDP binding which was observed in fa/fa rats at 14 days of age, and the defect in oxygen consumption following a meal, both show a close relationship to fa gene-dosage *(York et al., 1984)*. For instance, the increase in oxygen consumption following a meal was 21% in (Fa/Fa) homozygous lean, compared with 12.7% in hetrozygote lean (Fa/fa) and 7.2% in obese fa/fa rats *(York et al., 1984)*.

Adrenalectomy of fa/fa rats has been shown to normalize energetic efficiency, restore BAT mitochondrial GDP binding and prevent the

development of obesity *(Marchington et al., 1983; Holt et al., 1983; Yukimura et al., 1978)*. However, the increase in BAT thermogenesis which occurred after adrenalectomy could result from either the removal of circulating corticosterone or from the parallel increase in serum ACTH which would follow adrenalectomy. Previous results from this laboratory have shown that the beneficial effect of adrenalectomy on BAT function in the obese fa/fa rat were prevented by corticosterone replacement therapy *(Holt et al., 1983)*. In addition, corticosterone has been shown to suppress diet-induced, but not cold-induced, BAT thermogenesis in both mice *(Galpin et al., 1983)* and rats *(York et al., 1985)*.

ACTH and the Restoration of BAT Mitochondrial GDP Binding

Present results have shown that ACTH increased BAT mitochondrial GDP binding in the obese fa/fa rats to the same level as the lean control, and suggest that ACTH may be the major effector of the increase in BAT thermogenesis after adrenalectomy. In contrast, ACTH had no acute effect on BAT mitochondrial GDP binding of lean rats. However, it appeared that as the administration of ACTH continued, there was a significant decrease in mitochondrial GDP binding of lean rats, associated with a rise in serum corticosterone. Similarly, mitochondrial GDP binding of obese rats declined with chronic ACTH treatment. These results suggest that ACTH may stimulate, while corticosterone inhibits, BAT mitochondrial GDP binding. An inhibitory response to corticosterone has been shown previously by *Galpin et al. (1983)* and *York et al. (1985)*. The absence of any acute ACTH stimulation of GDP binding in the lean rats may have resulted from the parallel increase in serum corticosterone. Indeed, this seems likely, since an acute stimulation of BAT mitochondrial GDP binding, in response to ACTH, was observed in adrenalectomized lean rats, in which no endogenous secretion of corticosterone was possible. However, a stimulation in BAT mitochondrial GDP binding can be observed also acutely in lean rats after high doses of ACTH (100 µg) in which BAT GDP binding was increased by 25%. The ACTH stimulation of GDP binding in obese rats was attenuated at high doses of ACTH, possibly as a reflection of an enhanced corticosterone secretion.

Metopirone is an adrenostatic agent; it has a specific effect on 11-beta hydroxylation of steroids, inhibiting adrenal 11 hydroxylase

enzyme, the enzyme responsible for the hydroxylation of II deoxycorticosterone to corticosterone *(Caballeira et al., 1974)*. It has been shown that serum corticosterone conconcentration falls rapidly 30-90 mins after metopirone administration *(Schefzig et al., 1978)*. This fall in serum corticosterone was followed by a compensatory increase of ACTH secretion from the pituitary and an increase in secretion of II-deoxycorticosterone from the adrenal cortex *(Schefzig et al., 1978; L'age and Schöneshöfer, 1965)*.

Werner and Wunnenberg (1980) reported the effect of metopirone injection on thermoregulatory heat production in the European Hedgehog. They observed an increase in metabolic rate and an increase in BAT heat production after metopirone injection (150-450 mg/kg i.p.). The present results, which showed an increase in BAT mitochondrial GDP binding in fa/fa rats after acute metopirone injection, confirm this effect of metopirone in a different species of animal and are consistent with the inhibitory and stimulatory effect of corticosterone and ACTH, respectively, which have been proposed. However, in the present experiments, serum corticosterone levels were normal in metopirone treated rats. It has been shown previously that while serum corticosterone started to fall 30 mins after metopirone administration, it had returned to normal after 8 hrs *(Schefzig et al., 1978)*. However, glucocorticoid normally requires several hours for expression, so the increase in BAT GDP binding would probably reflect the serum corticosterone/ACTH profiles of several hours previously. Thus, the demonstration of a metopirone stimulation of BAT GDP binding is again consistent with an inhibitory effect of corticosterone on BAT function, confirming previous results in this thesis and other reports *(Galpin et al., 1983; Holt et al., 1983; York et al., 1985)*. Another possible explanation of the increase in GDP binding could be the increase in II-deoxycorticosterone secretion which follows metopirone injection. It has been reported that deoxycorticosterone injection (3 mg/kg; im) caused an increase in NST in the hedgehog, similar to that which was observed after metopirone injection *(Werner and Wunnenberg, 1980)*. Other explanations for the increase in BAT GDP binding might be the increase in endogenous ACTH which would follow metopirone injection, since it has been found that an increase in ACTH concentration occurred 4 hr after metopirone injection, and was maintained for a further 4 hr. Both ACTH and metopirone reduced food

intake in obese rats and increased BAT mitochondrial GDP binding. Similarly, adrenalectomy of fa/fa rats leads to a reduction in food intake and an increase in BAT mitochondrial GDP binding *(Holt et al., 1983; Yukimura et al., 1978)*. Thus, although it is not possible from the present data to attribute the metopirone effect to either the fall in serum corticosterone or the rise in serum ACTH, the experiment again demonstrated the role of the pituitary-adrenal hormones in regulating BAT thermogenic function.

Present results and previous reports by *Galpin et al. (1983) and York et al. (1985)*, showed that corticosterone has an inhibitory effect on BAT GDP binding. However, the in vitro study of the effect of corticosterone on BAT mitochondrial GDP binding shows an increase in GDP binding. This might reflect the lipolytic effect of corticosterone on BAT, since an increase in free fatty acids was observed from the in vitro and in vivo study of the effect of corticosterone on BAT *(Fain, 1965; Hahn et al., 1969)*. Corticosterone like T_3 is required permissively for BAT thermogenesis, especially in animals being exposed to cold temperature *(Fellenz et al., 1982)*. However, these data suggest that corticosterone may inhibit BAT GDP binding indirectly, presumably through the sympathetic nervous system. This suggestion is in agreement with previous reports by *Hahn et al. (1969) and York et al. (1985)*, who observed a decrease in BAT noradrenaline content after corticosterone treatment.

It has been difficult to explain the glucocorticoid dependence of the obesity of Zucker fa/fa rats, since serum corticosterone concentrations have been shown to be normal in the young fa/fa rats during the period of excessive fat deposition. Data presented in this thesis confirms these observations of normal serum corticosterone *(Shargill et al., 1983; Yukimura et al., 1978; York et al., 1984)*. The diurnal rhythm of plasma ACTH and corticosterone level was examined in lean and obese fa/fa rats and shown to be similar in both groups, by both *Shargill et al. (1983) and Yukimura et al. (1978)*, although *Martin et al. (1978)* found enhanced serum corticosterone in older fa/fa rats at certain times of the day. Thus the (fa/fa) obese rat is unlike the obese (ob/ob) mouse, where both an increase in plasma ACTH level and serum corticosterone have been observed *(Edwardson and Hough, 1975; Dubuc, 1976, Naeser, 1974; Herberg and Kley, 1975)*.

Circulating corticosterone can exist either free in the serum or bound to protein, either the specific corticoid binding globulin (CBG), which is the major binder of corticosteroid, or to albumin. Only the free corticosterone is biologically active. Present results have shown that serum free corticosterone concentration was the same in both lean and obese fa/fa rats. However, the capacity of corticosterone binding globulin was higher in obese fa/fa rats than in lean. Animals used for these experiments were at the same age (10 weeks) and the same sex (♂), since it has been shown that corticosterone binding capacity was increased in mature female rats, compared with their male littermates *(Gala and Westphal, 1965)*. The observation of normal levels of free and bound corticosterone in the obese rat despite increased binding capacity of CBG for corticosterone is unexpected. It suggests that the affinity of CBG for corticosterone is reduced in the fa/fa rat. The differences in CBG concentration between lean and obese rats may contribute to the differing acute response of BAT GDP binding to ACTH. Normally, any increase in total corticosterone is followed by an increase in CBG-bound steroid, while the unbound corticosterone remains at the same percentage of the total level. At a higher level of corticosterone, CBG binding sites become saturated due to the limited capacity, so the additional (excess) corticosterone is distributed only in the unbound corticosterone and albumin-bound fraction *(Ballard, 1979; Gala and Westphal, 1965)*. So as the obese fa/fa rats have higher CBG capacity and thus possibly have a lower affinity for corticosterone, it seems likely that the increase in corticosterone concentration which followed ACTH administration would be accompanied by differing profile of free corticosterone concentration. If a greater percentage of the initia- excess corticosterone secreted were bound in the fa/fa rat this might explain why the ACTH stimulation was observed in fa/fa but not lean rats. Further experiments would be required to verify this. Alternatively, the ACTH response of BAT in fa/fa rats could reflect an increased sensitivity to ACTH stimulation. The demonstration that at a high dose of ACTH, an ACTH-stimulation was apparent in lean BAT, would be consistent with this suggestion. However, the change in sensitivity would have to be tissue specific since *Yukimura et al. (1978)* have shown a normal response of adrenals to ACTH stimulation in the obese fa/fa rats.

Mechanism of ACTH Stimulation of BAT GDP Binding

The mechanism through which ACTH stimulates BAT function is not clear. Previous reports have shown an increased metabolic rate (by 25%) during infusion of ACTH *(Jansky, 1973)*. *Laury and Portet (1977)* also observed an increase in metabolic rats after chronic treatment with ACTH. Indeed, in studying the effect of corticotropin (ACTH) and glucagon infusion on BAT of newborn rabbits, *Heim and Hull (1966)* found that ACTH caused a large increase in the rate of oxygen consumption, and also stimulated heat production in BAT. It seemed probable that the calorigenic effect of ACTH was due to the direct action of this hormone on BAT *(Heim and Hull, 1966)*. The effect of ACTH on BAT thermogenesis in the rat has also been reported by *Rothwell and Stock (1982c)*. A significant increase in BAT GDP binding was observed after chronic ACTH injection (1 i.u./kg/day for 14 days). Another study was performed by *Lafontan and Agid (1979)*, on the lipolytic effect of ACTH on white adipose tissue in obese rabbit, in which they showed that ACTH is one of the most potent lipolytic hormones. They proposed two pathways of physiological regulation of lipolysis, one of them is pituitary dependent and the other one is mediated by catecholamines from the adrenal medulla.

Many studies have been reported on the in vitro and in vivo lipolytic effect of ACTH on white adipose tissue *(Levovitz et al., 1965; Hollenberg et al., 1961; Zinder et al., 1971)*; they all observed a rapid increase in free fatty acids mobilisation . *Laury and Portet (1980)* reported that chronic treatment with ACTH heightened the sensitivity of BAT to the lipolytic action of noradrenaline, and that ACTH led to a pseudo-acclimation of BAT in non-acclimated animals. *Harri (1981)* studied the effect of chronic ACTH and alprenolol (a β-antagonist) on muscle and brown fat oxidative enzymes. No changes in the enzyme activities were observed after chronic ACTH treatment, but an increase in BAT wet weight was observed. This effect was abolished when alprenolol was given together with ACTH. Finally, in order to test the role of pituitary of hormones in BAT thermogenesis (NST), experiments were performed on hypophysectomized rats by *Laury*

et al. (1984). They suggested that pituitary hormones are necessary to optimize the cold stimulation of BAT thermogenesis. However, the mechanism through which ACTH caused an increase in BAT thermogenesis is unclear. It could be either a direct or indirect action. BAT thermogenesis is mediated mainly through the sympathetic nervous system *(Landsberg and Young, 1982)*. Recent reports have shown that sympathetic stimulation of BAT, as measured by noradrenaline turnover, is impaired in obese fa/fa rats *(Levin et al., 1982; York et al., 1985)*, and restored to normal after adrenalectomy *(York et al., 1985)*. It seems possible that the ACTH stimulation of BAT mitochondrial GDP binding in fa/fa rats might be through regulation of the sympathetic response to dietary signals. This is likely since the stimulation effect of ACTH on BAT thermogenesis (after 24 hrs) in obese fa/fa rats was blocked by propranolol (present results). This confirmed the previous reports by *Harri (1981)*, that propranolol inhibits the ACTH effect. In contrast, chronic ACTH injection (50 µg/day for 3 days) did not change noradrenaline concentrations or turnover in BAT of obese fa/fa rats *(York and Marchington, unpublished observation)*. This may reflect the opposing effects of ACTH and corticosterone on noradrenaline turnover, since the ACTH stimulation of BAT GDP binding in obese fa/fa rats was decreased as the administration of ACTH continued and serum corticosterone level increased.

A small increase in BAT mitochondrial population appears after ACTH treatment, as indicated by the increase in total activity of the mitochondria marker enzyme, succinate cytochrome C oxidoreductase (Table 8). These changes would be consistent with a stimulation of the sympathetic tone of BAT mitochondrial proliferation, a characteristic of the changes induced by sympathetic stimulation, e. g. noradrenaline infusion or cold adaptation. This might support the previous suggestion, that ACTH acts on BAT indirectly through the sympathetic innervation.

Serum T_3 levels were shown to be reduced in fa/fa rats, in confirmation of a previous report by *York et al. (1984)*. T_3 levels of obese fa/fa rats were raised to the same value as those observed in control lean rats after ACTH injection. Most of the metabolic

effects of thyroxin (T_4) are thought to be due to triiodothyronine (T_3), which is produced from T_4 by a process of 5'-monodeiodination. 5'-deiodinase, the enzyme responsible for this process, has been found in BAT *(Leonard et al., 1983)*. *Silva and Larsen (1983)* have shown that this enzyme is under adrenergic control. This suggestion was consistent with previous reports by *York et al. (1984)*, of increased serum T_3 levels in lean and obese fa/fa rats in all situations when sympathetic stimulation of BAT was enhanced, e.g., after housing at 4°C for 7 days or after adrenalectomy of obese rats. The increase in T_3 which followed ACTH injection might provide further support for the suggestion that ACTH might stimulate BAT thermogenesis through the sympathetic nervous system.

Serum insulin levels were increased after ACTH treatment, although a reduction in food intake was observed. However, this is most likely to reflect the ACTH induced increase in corticosterone secretion, since glucocorticoids are known to enhance insulin secretion. The consequences of the hyperinsulinemia are not clear. However, insulin is thought to be essential for the normal response of BAT to dietary stimulation *(Rothwell and Stock, 1981a)*. It is possible that an insulin resistance rapidly develops in response to the hyperinsulinemia and this might in turn be responsible in part, at least, for the gradual reduction in BAT GDP binding with prolonged ACTH treatment. Indeed, *Mercer and Trayhurn (1983)* have suggested that insulin resistance develops much more rapidly in lipogenic pathways of BAT of obese ob/ob mice than in lipogenesis in other tissue.

The other possibility, a direct effect of ACTH on BAT, would be suggested from the work of *Lafontan and Agid (1979)*, showing that ACTH has a highly lipolytic effect, which causes an increase in free fatty acids. Free fatty acids are supposed to be the main substrate for BAT thermogenesis. The thermogenesis capacity of BAT is shown by the unique ability of BAT mitochondria to translocate protons across the mitochondrial inner membrane without coupling to ATP synthesis, which is called the proton conductance pathway *(Nicholls, 1979)*. The proton conductance pathway is associated with a specific protein of 32,000 molecular weight. This protein has an affinity for purine nucleotide such as GDP, which binds to the 32,000 mol. wt. protein and closes the proton leak. However, the increase in free fatty acids would not only provide extra substrates for oxidation by the BAT

mitochondria, but might also lead to an increase in fatty acyl CoA concentration which in turn would lead to unmasking of the proton conductance pathway. Fatty acyl CoA, rather than fatty acids, are thought to be the physiological regulators of the proton conductance channel *(Nicholls, 1979)*.

It has been found that chronic ACTH treatment increased blood flow to BAT *(Smith and Horwitz, 1969; Laury and Portet, 1980)*. So it is possible that ACTH which caused an increase in blood flow to BAT, would increase both oxygen supply and fatty acid production. An increased concentration of fatty acyl CoA would open the proton short circuit and an increase in thermogenesis would follow. It was previously reported that noradrenaline infusion and cold exposure, which both cause an increase in BAT thermogenesis, also increased blood flow to BAT by 3 and 1.5 fold respectively, more than control *(Laury and Portet, 1980; Foster and Frydman, 1978)*.

ACTH effect on mitochondria 32K Protein

It has been reported that the reduction in BAT mitochondrial GDP binding in obese fa/fa rats is due to the reduced number of low affinity binding sites, since the number of the high affinity binding sites is normal compared with lean control rats *(Holt, 1984; French et al., 1985)*. In the work reported in this thesis, Scatchard analysis of mitochondrial GDP binding in obese rats again indicated the presence of 2 binding sites, with high and low affinities for GDP. The K_d and B_{max} values for the high affinity site of obese rats were similar to those observed by *French et al. (1985)*, (0.18 µM and 51 pmole/mg protein and 0.20 µM and 59 pmole/mg protein, for K_d and B_{max} values in the two studies, respectively). For the low affinity site, both K_d (3.07 µM) and B_{max} (137 pmole/mg protein) were lower than those observed by *French et al. (1985)*, (10.2 µM and 320 pmole/mg protein for K_d and B_{max}, respectively). The reason for these differences is not clear. It may represent either a seasonal effect or change in animal housing temperature. After ACTH injection, there was an increase in B_{max} of both the high affinity and low affinity sites without any major change in the K_d of the sites. Since the response of ACTH treatment was measured after 24 hrs, it is possible that the increase in GDP binding in the obese fa/fa rats is largely due to

unmasking of pre-existing binding sites rather than synthesis of new uncoupling protein.

The increase in GDP binding in cold acclimated rats has been ascribed to the increase in the number of low affinity sites with no change in the affinity of binding *(Bryant et al., 1983)*. In those animals the change in GDP binding was accompanied by increased mitochondria concentration of 32K protein *(Ashwell et al., 1983; Desautels and Himms-Hagen, 1979)*, presumably reflecting an increased synthesis of the uncoupling protein. The acute increase in GDP binding which occurs within 24 hrs of cold exposure was not accompanied by any marked change in the concentration of 32,000 protein in the mitochondria, so it is thought that the initial increase in GDP binding results from unmasking of pre-existing binding sites *(Desautels and Himms-Hagen, 1979)*. Similarly, after adrenalectomy of obese fa/fa rats BAT GDP binding increases rapidly in the initial 24 hrs. This might also be due to the unmasking of binding sites. The increase in GDP binding which was observed 7 days after adrenalectomy, represents an increase in B_{max} for the low affinity site, only with no change in their affinities of either site (K_d). This might reflect an increase in synthesis of new binding sites *(French, Holt and York, unpublished observation)*. However, Scatchard analysis of GDP binding within 24 hours of adrenalectomy of obese rats has not been performed. Thus, the apparent dissimilarity lies in the responses to adrenalectomy and to ACTH and may reflect the different times post-treatment at which the experiments were performed. Indeed, *Bryant et al. (1983)* have shown that changes in B_{max} and K_d of the high affinity site are normally associated with acute responses, while changes in the low affinity site are shown in both acute and chronic responses. Indeed the maximal response of BAT to exogenous noradrenaline may be attained with 30-60 minutes. This is associated with an increase in B_{max} for the high affinity site as well as the low affinity site, similar to that observed in our experiments after ACTH treatment. In contrast, after cold adaptation or cafeteria feeding for 10 days, the increase in BAT GDP binding resulted from increases in low affinity binding sites without any changes in the maximum binding of high affinity sites. The similarity in the response of BAT to noradrenaline and to ACTH would possibly further support the suggestion that ACTH may be acting through or stimulated by the sympathetic innervation in BAT.

Body composition/weight in response to ACTH

ACTH reduced food intake and lowered the rate of weight gain in fa/fa rats to the value observed in lean rats. A similar effect of adrenalectomy on food intake and weight gain of fa/fa rats has been previously reported *(Holt et al., 1983; Yukimura et al., 1978)*. Since ACTH injection restored BAT mitochondria GDP binding despite a marked reduction in food intake, it is possible that ACTH might restore the BAT response to dietary stimulation in fa/fa rats. Chronic treatment of obese fa/fa rats with ACTH (21 days), reduced body weight gain and food intake. These effects were accompanied by a reduction in inguinal adipose tissue weight, and a reduction in weight/length ratio (Table 10). Such data would suggest that ACTH treatment, like adrenalectomy, may prevent the development of obesity in obese fa/fa rats. However, the Lee Index, which is normally regarded as a good indicator of obesity *(Simson and Gold, 1982)*, was not reduced after ACTH treatment. This might reflect the effect of prolonged excess corticosterone secretion in the ACTH treated rats which resulted in a stunting of linear growth, rather than the maintenance of obesity. However, it seems difficult to explain the chronic effect of ACTH on the obesity in obese fa/fa rats, since a reduction in body weight gain, and body fat was observed although BAT thermogenesis, as indicated by GDP binding, was reduced. However, in both short and long term (chronic) ACTH treatment serum corticosterone was increased (51; 55 µg/dl, respectively). Therefore, it might be that the inhibition of BAT GDP binding which occurred after 21 days of ACTH treatment was due to the effect of corticosterone, as it has been suggested from present results and reports by *Galpin et al. (1983) and York et al. (1985)*, that corticosterone has an inhibitory effect on BAT GDP binding. But since the reduction in BAT thermogenesis was accompanied by a reduction in body weight gain, it seems possible that a general corticosteroid-dependent increase in the catabolic pathway might be responsible for the weight loss. A similar response has been observed after treatment with thyroid hormones which may inhibit BAT GDP binding even under conditions where extra BAT thermogenesis is required, e.g. cold acclimation *(Sundin, 1981)*. Like thyroid hormones, gludocorticoids do also appear to be required in permissive amount for normal BAT thermogenesis *(Fellenz et al., 1982)*.

The reduction in BAT GDP binding of obese fa/fa rats has been associated with the lack of sympathetic stimulation of BAT as indicated by measurement of noradrenaline turnover *(Levin et al., 1983; York et al., 1985)*. Adrenalectomy restored BAT GDP binding and in parallel with this is the restoration of normal levels of sympathetic activity in the tissue *(York et al., 1985)*. These changes are also associated with the restoration of the adaptive increase in GDP binding in response to sucrose intake. However, this sucrose stimulation appears to be independent of sympathetic activity, since it was not prevented by propranolol. Thus, it has been suggested that a certain sympathetic tone is required before diet-induced response are apparent in BAT *(York et al., 1985)*. Diet induced changes in BAT activity, independent of extra sympathetic activity, have been reported by *Levin and Co-Worker* and also *Levin et al. (1983)*. Thus it was of interest to see if a sucrose induced increase in BAT GDP binding would be observed in ACTH treated obese fa/fa rats, since the initial ACTH induced BAT function appears to be sympathetically mediated. However, experiments performed (Section 3j) did not provide any evidence to support this hypothesis, since no net increase in food intake was attained in the ACTH treated fa/fa rats given extra sucrose; the reduction in chow intake induced by ACTH was only matched by the level of sucrose intake. In contrast, in the lean rats, the total energy intake in sucrose fed/ACTH treated rats was increased, compared with control and ACTH treated lean rats. This increase was not associated with any increase in the rate of weight gain, but was associated with a large increase in BAT mitochondrial GDP binding, suggesting a compensatory increase in BAT thermogenesis. In the obese rats the intake of sucrose by ACTH treated animal was associated with a small increase in body weight gain, but no increase in BAT mitochondrial GDP binding, compared to the chow fed/ACTH treated rats, which suggested that there was no increase in BAT thermogenesis. However, this may not have been anticipated since the total energy intake was not increased in the ACTH sucrose fed obese rat. So the increase in weight gain (compared to ACTH/chow rats) may well just reflect the change in the composition of diet. Further experiments are required on obese fa/fa rats to see if ACTH treatment, like adrenalectomy, is associated with the restoration of BAT response to sucrose intake.

SUMMARY AND CONCLUSION

ACTH treatment restored BAT mitochondrial GDP binding in obese fa/fa rats to the same level observed in lean rats. The stimulatory response to ACTH was absent from lean rats unless endogenous secretion of corticosterone was prevented. There were a number of similarities between the effects of ACTH and of adrenalectomy on the development of obesity in obese fa/fa rats. Both treatments reduced body weight gain, reduced food intake, restored BAT mitochondrial GDP binding to the level seen in lean rats, and increased serum T_3 levels towards normal *(Holt et al., 1983; Yukimura et al., 1978)*. The only difference between the acute response to these two treatments observed in the present studies was the responses of serum insulin level, which was reduced after adrenalectomy, but increased after ACTH treatment, presumably as a result of the increase in endogenous corticosterone secretion. Another difference was that adrenalectomy permanently prevents the development of obesity, while the response to ACTH was biphasic; the initial stimulation of GDP binding after ACTH treatment was followed by a secondary decline, presumably as a result of the increase in corticosterone secretion.

The pituitary-adrenal system is important in BAT function, hypophysectomized rats cannot survive cold exposure unless they are treated with corticosterone and thyroid hormones *(Fellenz et al., 1982; Laury et al., 1984)*. However, the reasons for the dependency of obesity in obese fa/fa rats on the pituitary-adrenal system is unclear. Serum corticosterone and the percentage of unbound hormone is similar in both lean and obese fa/fa rats, as shown in this thesis and by other previous reports *(Yukimura et al., 1978; York et al., 1984)*. Serum ACTH has been reported to be the same in both lean and obese fa/fa rats at the age of 10 and 30 weeks *(Yukimura et al., 1978)*. However, *Margules (1978)* assumed that obese fa/fa rats had a higher serum level of ACTH, since β- endorphin level in plasma and pituitary was higher in obese fa/fa rats than in lean rats. β-endorphin and ACTH are produced from the cleavage of a single large precursor protein (pro-opiocortin) *(Mains et al., 1977)*. ACTH and β-endorphin are released concomitantly from the pituitary of rats *(Guillemin et al., 1977)*,

therefore it is possible that the increase in β-endorphin level which was found in obese fa/fa rats might inhibit BAT function. Indeed, recent work in our laboratory *(Allars and York - unpublished observation)* has shown that naloxone - an inhibitor of β-endorphin, increases BAT mitochondrial GDP binding of obese rats in a similar manner to the response to ACTH. Therefore it is possible that the stimulation of BAT function by ACTH represents an ACTH inhibition of β-endorphin secretion. The other possibility is that serum ACTH level is reduced in obese fa/fa rats; this hypothesis is supported by recent measurements of serum ACTH in fa/fa rats in our laboratory *(Holt et al. - unpublished observation)*.

It is possible that pituitary ACTH content is similar in both lean and obese fa/fa rats, but there are disturbances in the secretion of the ACTH from pituitary in obese fa/fa rats. Two factors effect the release of ACTH from the pituitary gland, corticotropin releasing factor (CRF), produced in the hypothalamus, stimulates ACTH secretion whereas there is a negative feedback by adrenal glucocorticoid hormones which inhibit the secretion of ACTH and CRF. CRF is released from the medial basal hypothalamic region. It is possible that there might be a defect in this area which is associated with fa/fa genotype, which effects the release of pituitary ACTH. Since it has been reported that lesion or widespread lesions in this area (medial hypothalamus) decrease or block ACTH release in response to some stressful function *(Makara, 1979; Brodish, 1963)*, while electrical stimulation in this area results in release of ACTH *(Dunn and Critchlow, 1973)*. However, *Albert et al. (1971)* have reported that lesions placed in this area between the lateral and ventromedial hypothalamus produced obesity and hyperphagia.

After adrenelectomy, serum ACTH rises to the same level in lean and obese fa/fa rats *(Yukimura et al., 1978)*, although the level was lower in the intact fa/fa rats than in the intact lean rats. This suggests that the adrenal feedback may be responsible for the impaired serum ACTH level in obese fa/fa rats. Two kinds of negative feedback affect the pituitary-adrenal system - a fast feedback response to serum corticosterone levels and a delayed feedback, independent of circulatory corticosterone levels, which may be neurally-mediated from the adrenal to the hypothalamus. Since circulating corticosterone level is similar in both lean and obese fa/fa rats, it is possible that the

reduction in serum ACTH reflects the effect of this delayed feedback in obese fa/fa rats.

However, further experiments are required to clarify the precise mechanism of brain-pituitary adrenocortical system regulation of BAT function.

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