STUDIES IN HORMONAL AND METABOLIC ASPECTS OF
INHERITED OBESITY AND INDUCED HYPERLIPAEemia

by

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SUMMARY

For the present thesis, the Zucker fatty rat was studied as a model of inherited obesity alongside the CoCl₂-treated rat which represented a model of induced hyperlipaemia. Carbohydrate and lipid metabolism in these two rats were investigated. To elucidate the role of insulin and glucagon receptors in obesity and hyperlipaemia, liver and adipose tissue insulin and glucagon receptor-interactions were examined in freshly prepared liver and adipose tissue plasma membranes from these two rats and compared to their controls.

In the Zucker fatty rat obesity was found to be associated with gross hypertriglyceridaemia and hyperinsulinaemia. The insulin resistance associated with this rat could be accounted for by a decrease in the number of insulin receptors at its target tissues examined in the present study. There was no alterations in the number or avidity of the glucagon receptors in the liver or adipose tissue of the Zucker fatty rat. Hypertriglyceridaemia of this rat was found to be associated with high activity of lipoprotein lipase. A mechanism for insulin resistance and another for hypertriglyceridaemia in this rat will be described in some detail.

The polycythemia of the CoCl₂-treated rat was found to be accompanied by moderate hypertriglyceridaemia compared to that of the Zucker fatty rat. The postulated glucagon resistance of the CoCl₂-treated rat as well as for the Zucker rat was investigated in some detail. Glucagon basal level as well as after arginine stimulation was found to be within the normal range in the CoCl₂-treated rats as set by their untreated controls. Furthermore, there was no abnormality detected in glucagon or insulin binding to liver or adipose tissue plasma membranes in these rats. The hypertriglyceridaemia of the CoCl₂-treated rat was found to be due to a decrease in the plasma
triglycerides removal rate. The role of cobalt in the aetiology of this induced hypertriglyceridaemia will be discussed in detail and a mechanism will be presented. Longitudinal studies in the effect of cobalt on rats was also investigated and results reported.
ACKNOWLEDGEMENTS

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My special thanks to Dr. Linda Morgan for reading and correcting the manuscript and Mrs. M. Whatley for typing it.
DEDICATION

To my wife for her love, patience and most of all her sacrifice.
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMP.PNP</td>
<td>Adenylyl imidophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Adenosine-3',5'-phosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanine-3',5'-phosphate</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Cpm</td>
<td>Count per minute</td>
</tr>
<tr>
<td>DG</td>
<td>Diglyceride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetracetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis-(β aminoethylether) N, N'-tetracetic acid.</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KRP</td>
<td>Krebs-Ringer phosphate buffer</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>MG</td>
<td>Monoglyceride</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>POPPOP</td>
<td>1,4-bis-[2-(4-methyl-5-phenyloxazoly)]benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-Diphenyloxazole</td>
</tr>
<tr>
<td>SA</td>
<td>Specific activity</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<tr>
<td>W/A</td>
<td>Wistar Albino</td>
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CHAPTER ONE

GENERAL INTRODUCTION
1.1 Obesity and hyperlipaemia

Obesity is the most prevalent metabolic disorder in affluent societies. Despite wide recognition of the adverse health consequences of obesity including cardiovascular dysfunction, digestive disturbances, diabetes mellitus, skin ailments and degenerative arthritis, its prevalence remains high (1).

There are many definitions of obesity. It is perhaps best defined as that bodily state in which there is excessive accumulation of fat in both the relative and absolute sense (2). This greatly expanded adipose tissue mass is caused by an increase in the number as well as the size of fat cells (hyperplasia) in obesity of early onset. In obesity of later onset there is predominantly increased "packing" of the fat cells with triglycerides (hypertrophy) (3). Experiments by Hirsch and Han (4) indicate that the adipose tissue in young rats grows by an increase in both cell number and size. By the 15th week of life cell number in the rat becomes fixed and further change in the size of the tissue occurs by an increase in the cell size. Extreme changes in depot size (induced by experimental obesity or starvation) were ineffective in changing the final cell number. Hirsch and his colleagues suggest that (a) There are a fixed number of mature adipocytes, unaffected by changes in the depot size; (b) the adipocytes (or their precursors) are formed early in life and that during a finite postnatal period, certain influences (eg. nutritional) may determine adipose cell number.

Hyperlipaemia is a major consequence of obesity and the incidence of hypertriglyceridaemia is high (5). The reported frequency being dependent on the definitions of normal values for body weight and serum triglyceride levels. Because it is a relative term, hyperlipaemia
is rather difficult to define. Statistical analysis of population samples of apparently "healthy" individuals give so-called normal values. Individuals with values greater than these have an increased risk of developing coronary heart disease.

There is therefore a growing body of workers who prefer to draw the limits of normality for plasma lipid levels at about those concentrations which correspond to an excessive increase in mortality or morbidity from heart disease (5).

In a recent survey of a healthy population, the mean body weights of men and women with an equivocal Type 4 hyperlipoproteinaemia were six to eight Kg higher than in the respective normolipaemic subjects (7). In the plasma the excess lipid is transported predominantly in the very low density lipoprotein (VLDL) fraction, which also contains increased amounts of cholesterol, even when the cholesterol concentration in whole plasma is normal (8, 9, 10). Indeed, the cholesterol content in other lipoproteins, notably the high density class (HDL) may be reduced (10). The combination of obesity and hypertriglyceridaemia is a common finding among younger men presenting with coronary heart disease (11). In a recent study of men under the age of 50 in whom coronary artery disease was established by angiography, obesity and hypertriglyceridaemia were frequent findings (12). The respective incidence of these two risk factors (71% and 49%) were greater than that for any other risk factor e.g. hypercholesterolaemia, smoking cigarettes, abnormal glucose tolerance or hypertension.

Earlier epidemiological studies failed to demonstrate a significant relationship between fatness and serum cholesterol levels (13). However, two more recent studies of larger populations have shown marginally significant correlations, at least in some age groups (14, 15). Despite
this evidence for some association between weight change and the serum cholesterol level, there is no question that this is not nearly as impressive as that between obesity and serum triglyceride. It is highly likely that obesity accentuates those metabolic disorders that give rise to hypertriglyceridaemia. Obese diabetics are more likely to have raised serum triglyceride levels than lean diabetic patients (though both obesity and hypertriglyceridaemia are independently related to atherosclerosis in diabetic patients) (16).

Other metabolic aberrations are common in obese humans and animals. These include impaired glucose tolerance, inadequate mobilization of fatty acids, a sluggish free fatty acid response to exogenous insulin, resistance to ketosis and a diminished growth hormone response to hyperglycaemia, exercise and starvation (17).

Attempts have been made to mimic the hyperlipaemia of obesity in normal subjects by overfeeding. Sims and his colleagues have studied the metabolic departures from normal observed during induction of obesity in a group of volunteers in the Vermont State Prison (18). These subjects ingested two or three times their normal daily calorie diet and gained an average of 26% above their initial lean weight. As the subjects became obese, the following metabolic alterations were noted compared to observations made when body weight of the experimental subjects was normal:

1. Serum cholesterol and triglycerides increased and plasma free fatty acids decreased.

2. Fasting plasma insulin levels increased.

3. Despite the increase in fasting and postglucose insulin levels, there was a significant reduction in oral and intravenous glucose tolerance.
4. With weight gain there was a progressive impairment in the rise in plasma human growth hormone levels.

5. Cortisol levels increased.

6. Cell number of adipose tissue cells remained constant but cell size increased with adiposity.

Three outstanding differences between the findings in experimental and spontaneous obesity were observed in the above study. First, in contrast to the findings in spontaneous obesity, there was no increase in adipose tissue cell number (hyperplasia). Second, there was a fall in the plasma free fatty acid levels in the experimental obesity compared to spontaneous obesity. Third, in general the volunteers who gained weight by eating a mixed diet consumed more excess calories than could be accounted for by the gain in weight and once they had gained the weight, it required approximately 50% more calories per square metre of body surface area for them to maintain the added weight. Thus their nutritional efficiency appeared to be reduced and this is certainly not the case with spontaneous obese humans and animals. An obese patient usually maintains his weight while eating 1500 calories per square metre per day as compared with 2700 calories per square metre per day required to maintain peak weight in the induced obesity cases. The above experiment summarizes the major and important findings in obesity.

1.2 The role of insulin and glucagon in obesity and hyperlipaemia

Central to the discussion of the relationship of obesity to hypertriglyceridaemia is the role of insulin. The association of obesity, hypertriglyceridaemia, diminished glucose disposal and raised plasma insulin levels has been reported in several studies (19, 20, 21). Perhaps the most striking endocrine alteration in obesity is the combination of
hyperinsulinaemia and the presence of peripheral insulin resistance, with increased responsiveness of the pancreatic B cells to a variety of stimuli including amino acids, insulin secretagogues and pharmacological agents (2). It has proved difficult to show that the above metabolic abnormalities stem directly from obesity. It is nevertheless, tempting to assume that obesity reduces responsiveness to the action of insulin in key tissues such as liver, muscle and adipose tissue, which in turn lower glucose utilisation and raises plasma triglyceride levels. In recent studies (21) in overweight subjects, the production of triglyceride was correlated to a highly significant degree with insulin resistance, estimated from the plasma glucose response to insulin infusion. After weight reduction the i) resistance to insulin, ii) post-glucose insulin concentration, iii) plasma triglyceride concentration and iv) triglyceride turnover rate fell significantly, emphasising the modulating effect of fatness on the metabolic abnormalities.

Despite the strong association between hyperinsulinism and hypertriglyceridaemia it has been difficult to demonstrate directly that insulin stimulates triglyceride formation. Studies with rat liver have provided only suggestive evidence (22). By contrast subjects with pancreatic islet cell tumours are not necessarily hypertriglyceridaemic (23), whereas insulin deficient diabetics may (24). This is not surprising since insulin affects triglyceride metabolism in many ways: its antilipolytic effect reduces the flux of free fatty acids a substrate for triglyceride production, while its stimulation of lipoprotein lipase activity enhances triglyceride removal. If the hyperinsulinaemia of obesity is related to hypertriglyceridaemia then this may be an association rather than a casual relationship (25). Hyperinsulinaemia may reflect resistance in liver, muscle and possibly adipose tissue to the action of insulin, leading to increased secretion from the pancreas. The diminished
responsiveness to insulin may on one hand result in an increased flux of free fatty acids and precursors of glucose to the liver leading to overproduction of triglyceride, while on the other hand, the uptake of triglyceride in adipose tissue and muscle may be reduced.

It is well established that the first step in the action of hormone is binding to a specific recognition site on the target cell known as a receptor. This hormone-receptor complex then activates additional cellular processes, which in turn are followed by the cellular response characteristic of that particular hormone (26, 27, 28). Thus, it seemed appropriate to begin assessing the cellular basis for insulin resistance in obesity and hyperlipaemia by focusing on this aspect of insulin action. In this regard Kahn et al. (29) were the first to study the insulin receptor in obesity. These workers found a striking decrease in insulin binding to liver plasma membranes from the genetically obese (ob/ob) mouse. This decrease in binding was entirely accounted for by a decrease in the number of insulin receptor sites. Subsequent work from the same group (30) demonstrated that although fewer in number, the insulin receptor of the obese mouse were functionally normal. Decreased numbers of insulin receptors have also been found in fat (31), cardiac muscle (32) and lymphoid tissue (33) from the ob/ob mouse. Therefore it seemed reasonable to conclude that as a generalized phenomenon, cells from these animals had fewer insulin receptors than cells from lean controls.

Decreased numbers of insulin receptors have been found in a variety of other obese states both in animals and humans. Thus, decreased numbers of insulin receptors have been found in liver membranes prepared from mice made obese by hypothalamic injection of gold thioglucose (34); adipocytes (35); adipocyte membranes (36); liver membranes (37) and skeletal muscle (38) from old spontaneously obese rats; and of circulating monocytes (39, 40) and isolated adipocytes (41, 42)
from obese humans. Thus, decreased insulin binding is widely distributed in a variety of obese states.

In any insulin-resistant state the insulin resistance can theoretically be due to one or more of four major causes:-

1) abnormal B-cell secretory products. Since resistance to exogenous insulin is seen in obesity and hyperlipaemia, it seems highly unlikely that the insulin resistance of obesity and hyperlipaemia is related to secretion of abnormal endogenous insulin molecules or altered insulin-to-proinsulin ratios. Indeed considerable evidence against these latter possibilities exists(43).

2) insulin degradative processes: The possibility that in insulin-resistance states, insulin may be degraded faster has been ruled out. On the contrary experiments carried out on lean and obese mice indicate that membranes from various tissues of lean mice degrade insulin faster than those from their obese counterparts (29,30,31).

3) circulating insulin antagonists: The case for circulating insulin antagonists in obesity has recently been reviewed (1) and although slight differences in cortisol (44) and growth hormone (45,46) secretion have been described in obesity, the evidence indicates that this is not responsible for the insulin resistance of this state.

Antireceptor antibodies have been implicated in the pathogenesis of three diseases i.e. Grave's disease, with antibodies to the thyroid stimulating hormone receptor, myasthenia gravis, with antibodies to the acetylcholine receptor and a new syndrome associated with extreme insulin resistance and acanthosis nigricans, in which antibodies to the insulin receptor have been found. In each of these conditions, antibodies compete with the hormone or transmitter for receptor binding on the cell membrane (49).

Like patients with obesity, patients with insulin resistance due to acanthosis nigricans have decreased binding of insulin to its membrane receptors. However, these patients differ from obese patients in that
1) their metabolic defect is more severe than that seen in obesity, 
2) analysis of the binding data shows that the defect is primarily one 
of reduced receptor affinity rather than of a reduced concentration of 
normal receptors (47), and 3) fasting does not restore binding toward 
normal as it does in obese patients. These findings, together with the 
immunologic features seen in this syndrome, suggest that it is due to 
the presence of an antibody that competes with insulin for binding to 
the insulin receptor (48). Such antibodies are absent in obese 
patients.

4) The fourth possible cause of insulin resistance is tissue insulin 
insensitivity at the target cell level, implicating either a receptor 
or post-receptor defect. At the receptor level, the formation of 
insulin-receptor complexes could be altered by changes in receptor 
affinity or number. Post-receptor defects, such as alterations in 
intracellular pathways that mediate the cell response to insulin, could 
also result in insulin resistance. However, evidence from studies 
mentioned above, show that the defect causing insulin resistance in 
obesity is at the target cell receptor level and specifically involves 
the insulin receptor. First, obese humans and animals bind less insulin 
to target cells because of a decrease in insulin receptor concentration 
on the target cell. Secondly, chronic diet ameliorates the clinical 
insulin resistance and results in increased concentration of receptors 
that are able to now bind insulin in normal amounts. Thirdly, in 
obesity the insulin receptor can change its affinity for insulin without 
any change in receptor number (49).

Glucagon is another pancreatic hormone which has a role in fat and 
adipose tissue metabolism. However, the role of glucagon in obesity is 
not as clearly defined as that of insulin. To date, the correlation
between obesity and circulating glucagon levels is unresolved. Despite early reports that glucagon concentrations may be increased in obesity (50), the bulk of evidence now points to normal fasting glucagon concentrations, normal suppression following glucose administration and variable responses to stimulation by amino acid infusions. There are conflicting reports on the A-cell response to aminogenic stimuli and starvation in obesity. Some investigators have recorded an excessive response to intravenous arginine which was normalised following reduction to a lean body weight (51). Others (52) have shown that A-cell response to intravenous arginine is subnormal in obesity. However, in none of these studies was the composition of the diet strictly controlled, and it is well known that the amount of dietary carbohydrate affects both the basal plasma glucagon and the A-cell response to amino acid stimulation (53). Likewise, obese subjects have been reported to exhibit either subnormal (54) or normal (55) increases in plasma glucagon levels during starvation. The status of A-cell function is further confused by the finding that dexamethasone elevates the basal plasma glucagon and enhances the A-cell response to intravenous alanine to a greater extent in obese than in lean individuals (56). Thus, on the basis of information presently available, glucagon does not appear to play a clear role compared to insulin in obesity.

Glucagon belongs to the lipolytic hormone group. In several species glucagon enhances the release, in vitro, of glycerol and free fatty acids from pieces of adipose tissue or isolated adipose cells but not always to the same degree. Its lipolytic activity has been demonstrated in man, dog, fowl and rat (57, 58, 59, 60, 61).
In man a single dose of glucagon given to patients with primary hyperlipoproteinaemia lowered plasma triglycerides over a period of four hours, but did not produce any significant change in plasma cholesterol. When glucagon was given daily over a period of ten days plasma cholesterol was lowered as were plasma triglycerides \((62,63)\). The fall in plasma triglyceride levels has been ascribed to reduce hepatic triglyceride output \((64)\) or reduced apoprotein synthesis \((65)\) caused by the action of glucagon.

Endogenous hypertriglyceridaemia may be caused by various factors. It has been suggested that one of these factors might be resistance to the action of glucagon \((66,67)\), especially since many patients with hypertriglyceridaemia are hyper glucagonaemic. However, Elkeles and Hambley tested the above hypothesis, by studying the effects of exogenous glucagon in patients with hypertriglyceridaemia, and found no evidence of glucagon resistance from such study \((63)\).

1.3 Proposed models of action of insulin and glucagon

As previously mentioned, the primary event in the action of polypeptide hormones and catecholamines is their binding to a specific site on the plasma membrane of the cell known as "receptor site". Indirect evidence that polypeptide hormones are able to act while on the surface of cells, is of several types and has been reviewed by Kahn et al. \((68)\). Although this evidence has recently been questioned \((69,70)\) it was taken to mean that the hormone need not enter the cells in order to act. Based on these observations, and the discovery of cyclic AMP, Sutherland and co-workers proposed the "second messenger" hypothesis of hormonal action \((71)\) as a model for peptide hormonal action. This is illustrated in Fig.1.1.
According to this model, peptide hormones travel in the circulation to their target cells and attach to surface receptor sites. This binding reaction activates an effector system, which then generates an intracellular "second messenger" of hormonal action. In the case of glucagon, the effector system is the enzyme adenylate cyclase and the second messenger is cyclic AMP.
The mechanism by which glucagon is thought to stimulate lipolysis has been extensively studied by Rodbell and his associates (72). Based on the "second messenger" hypothesis Steinberg and Huttunen (73) put forward a mechanism by which glucagon is believed to exert its action on the adipocyte to stimulate its lipolytic activity (Fig. 1.2).

Fig 1.2 The glucagon-induced lypolytic cascade
As a first step the hormone binds to a specific protein receptor situated on the outer surface of the adipose cell plasma membrane. As, a consequence of the glucagon-receptor interaction, a cascade of subsequent events ultimately result in the stimulation of lipolysis (Fig. 1.2). These events involve successively adenylate cyclase, protein kinase, cyclic AMP, hormone-sensitive triglyceride lipase and finally triglyceride breakdown. The intimate mechanisms linking these events are only partially known.

The mechanism by which glucagon exerts its glycogenolytic activity also involves an effector-receptor complex (Fig. 1.1). The second messenger is also cyclic AMP.

Despite over fifty years of research concerning the mechanism of the action of insulin, it is still not known how the hormone regulates its diverse effects on its target cells. Our ignorance of the action of insulin constrasts sharply with our knowledge of the action of glucagon (74). Glucagon was discovered ten years later than insulin, but its mechanism of action, as presented above has been appreciated for over a decade.

For many years investigators have searched for a single mechanism that would explain all of the known effects of insulin. At present there is a widely held belief that all of insulin's actions are the result of its binding to a specific receptor on the surface of target cells (69). With the discovery that cyclic AMP was the second messenger for glucagon, an intensive search ensued for an analogous second messenger mechanism for insulin. It had been known that insulin can antagonize the effects of hormones that act via cyclic AMP, and it was later found that under certain circumstances insulin can decrease the
hormoned-induced elevations of this nucleotide (75). Further, it has been demonstrated that insulin can increase the activity of cyclic AMP phosphodiesterase (76), the enzyme that degrades cyclic AMP, and, under certain circumstances, insulin can inhibit adenylate cyclase (77).

Several other substances have been proposed as second messengers for insulin. Cyclic GMP has been implicated (78). Because insulin is known to alter the ion content of target cells it has also been postulated that ions may be second messengers for insulin (79). Calcium has been suggested as an alternative candidate to the cyclic nucleotides in the action of insulin. In this regard the stimulation by insulin of the calcium/magnesium dependent ATPase and the binding of calcium by the isolated plasma membranes of fat cells may provide important clues to determining the nature of the second messenger for insulin (80).

To complicate the issue further, there has been some evidence indicating that insulin not only induces its effects via a second messenger but also enters its target cells. Using radioiodinated insulin, it has been shown that insulin is associated with nuclear, mitochondrial and microsomal fractions (81, 82) both in hepatocyte and lymphocyte subcellular fractions.

It is certainly possible that insulin regulates all of its actions via a second messenger generated at the plasma membrane (74). However, since intracellular structures have specific binding sites for insulin, and since it is possible that insulin does enter the intact cell an alternative theory provides a simpler explanation for the diverse effects of this hormone. According to this formulation insulin in the extracellular fluid binds to receptors on the cell surface and, as a direct result of this interaction, there are changes in plasma membrane function, such
as transport and enzyme activity. In addition to this, insulin enters the interior of the cell, where it binds to the nucleus, endoplasmic reticulum, Golgi apparatus and other structures. As a direct result of its binding to these intracellular structures, insulin itself then mediates various intracellular functions leading to the synthesis of DNA, RNA and protein. This is shown diagrammatically in Fig.1.3.

![Diagram of Insulin Action](image)

**Fig.1.3** Proposed Model of Insulin Action. Receptor-Transducer-Internalization Model. Modified after Steiner (80)
Fig. 1.3 shows a proposed model of insulin action. On the left of the figure is the conventional model based on reversible external binding of insulin and its receptor. In this scheme insulin must dissociate from its receptor in order to be degraded by enzyme system in the extracellular space. As a consequence of insulin binding at the receptor, a second messenger designated X' is generated to produce some of its biological activities (e.g. permeability and transport), via modulation of protein kinase and/or phosphatase activities within the cell and perhaps within its plasma membrane. On the right of the figure insulin monomer passes into the cell through a channel in the membrane or via pinocytosis. It is believed that only 2% of the mass of insulin-receptor complex behaves in this way. Once inside the cell the insulin is degraded rapidly and further reduced to smaller fragments by proteolytic systems. Some of the insulin, or perhaps, more likely, specific fragments of the hormone might act directly on cellular components either as second messengers or by reinforcing the action of a receptor-generated second messenger X. The action is possibly carried on the intracellular components by altered protein phosphorylation to activate RNA, DNA synthesis to produce enzymes for protein, lipid and glycogen synthesis (80).

1.4 Animal models of obesity and hyperlipaemia

Obesity can manifest itself in a wide number of ways. Investigators interested in the field of obesity have a number of models from which to choose. For ease of classification these models can be divided into five major groups (83):

1) Obesity following ventromedial hypothalamic injury.

2) Endocrine imbalance.

3) Physical inactivity.
4) Nutritional obesity.

5) Genetic obesity.

Detailed studies on many of these experimental forms of obesity have provided insight into some of the mechanisms by which human obesity develops (84). Genetic obesity will be one of the subjects studied in detail in this thesis. Animals exhibiting obesity have recently been reviewed by Herberg and Coleman (85). There are many types of mice which exhibit obesity with hyperglycaemia and hyperinsulinaemia. These include the yellow obese, obese, diabetes, KK, New Zealand obese and Wellesley hybrid mice.

1.4.1. The Zucker Rat

Of special interest to us is the genetically obese Zucker rat (Rattus norvegicus). The type of obesity exhibited by this strain of rat is associated with marked peripheral insulin resistance yet with complete absence of hyperglycaemia, a combination which is always associated with obesity (85).

The Zucker (fa/fa) rat is one of a group of animals that inherit obesity as an autosomal Mendelian recessive gene (86). Many features of the fatty Zucker rat are similar to those found in other obese animals; they include increased food intake, hyperinsulinaemia, enlarged fat cells (hypertrophy) and altered function of the endocrine system. The fatty condition can be first detected by weight and appearance as early as three weeks of age and is very apparent by five weeks (86).

While the male fatty rats have sex organs of generally normal appearance and have occasionally been fertile, the females show a small, underdeveloped uterus and are uniformly sterile. Therefore, phenotypically normal heterozygotes of both sexes must be depended upon for continuing the stock (86).
Second to the obese appearance, the most striking sign of the fatty rat is lactescence (milky appearance) of the blood serum. This sets in shortly after weaning, and soon becomes very intense. Total fatty acid content of the serum reaches ten times the level observed in non-obese rats, and cholesterol and phosphatides are raised by about a factor of four. Blood lipid levels remain very high even after an 18-hour fast. On the other hand, blood sugar does not rise much above the normal range in fed or fasted fatty rats.

The increase in serum lipoproteins was one of the earliest abnormalities described in the fatty rat (87) and is associated with a substantial increase in triglycerides (88). The level of lipoproteins increases with age. The major increase is in the very low density lipoprotein fraction which has a higher percent of triglyceride (78%) as compared to their lean counterparts (60%). Low density lipoprotein (LDL) and high density lipoproteins (HDL) are also increased in serum from fatty rats (89).

Blood sugar, basally and after glucose load is normal in the Zucker fatty rats as compared to their lean controls (90). The rate of disappearance of blood glucose following intraperitoneal injection of glucose, is similar in fatty and control rats aged 5-6 weeks and again at 50 weeks. At the intermediate age of 20 weeks the glucose disappears from the blood of fatty rats more slowly than from the blood of their lean controls.

Differences in the activity of several glycolytic and gluconeogenic enzymes in the liver and adipose tissue have also been reported in the Zucker rats. Measurement of glycerokinase showed an elevation in the activity of this enzyme in the adipose tissue of the fatty rat (91). Increases in activity of glucose-6-phosphate dehydrogenase, malic enzyme and citrate cleavage
enzymes and which declined with age have also been reported in the Zucker fatty rats (92). The increased rate of lipogenesis shown by these animals is associated in an increased rate of the fatty acid desaturase (93).

Endocrine changes in the Zucker fatty rat have been the subject of considerable study. Hyperinsulinaemia appears as early as 3 to 4 weeks of age and is influenced by the quantity of carbohydrate in the diet and by the size of the litter (90). Insulin concentration in the pancreas is also increased (94) and the release of insulin from isolated islets in response to glucose is enhanced (95). Restriction of food intake by pair-feeding lowers insulin though not to normal (94). Only with restriction of dietary intake to three-fourths of that eaten by lean animals, or by total starvation, can insulin be restored to normal (90). Insulin resistance of the Zucker fatty rat diaphragm to glucose uptake in vivo both basally and after injected insulin has been demonstrated (90), as well as insulin resistance of adipose tissue and skeletal muscle (96).

In contrast to insulin, glucagon levels in the pancreas of these rats are normal but the circulating concentration of glucagon is said to be decreased (97). Insulin, proinsulin and glucagon extracted from Zucker obese rats were said to be indistinguishable by radioimmunoassay, radioreceptor assay and bioassay from the insulin, proinsulin and glucagon of their lean littermates (98).

Other endocrine changes have also been demonstrated in the Zucker fatty rat and summarised by Bray (99). These include an increase in follicle-stimulating hormone and luteinizing hormone, diminished uptake of radioactive iodine and reproductive function.
1.4.2 - Cobalt-Chloride Induced Hyperlipaemia:

In contrast to the hereditary hyperlipaemia associated with obesity as exhibited by the Zucker fatty rat, another animal model of hyperlipaemia not associated with obesity was chosen for the present study. This model of induced hyperlipaemia is the cobalt chloride-treated Wistar albino rats.

It has been known for over 40 years that many animals including man, develop polycythemia in response to small doses of cobalt (100). Thus, cobalt has been used for the treatment of some types of anaemia. Commonly associated with the polycythaemia produced by cobalt treatment is an induced hyperlipaemia. Hyperlipaemia after cobaltous chloride treatment has been shown to occur in a variety of animal species, e.g. rabbits (101), chicken (102), and rats (103).

In animals, different responses were observed depending upon the dose given. Caren and Corbo (104) demonstrated that a single intravenous injection of cobalt chloride into rabbits caused marked elevation of serum cholesterol levels within 24 hours, peaking at 2-3 days and returning to normal by 6-7 days. They did not perform quantitative studies on other lipid fractions, but did notice that at 24 hours, the serum from these animals was extremely lipaemic (milky). Their findings in the rabbit were confirmed by others (101), who also reported depletion of liver glycogen, focal necrosis and fatty changes in liver and myocardium with less severe fatty changes in the kidneys.

Caplan and Block (105) found that cobalt chloride administration to rabbits resulted in large increases in total serum lipids and of these, triglycerides increased most consistently and to the largest extent. Tennent et al (102) found that subcutaneous injections of cobalt chloride
into chickens were associated with high levels of serum cholesterol and increased atheroma formation.

Caplan et al (106) reported the case of a patient in which cobalt toxicity was responsible for secondary hyperlipaemic xanthomas, goitre and marked hypothyroidism. In this report they reviewed all previous cases of cobalt-induced goitre, together with experimental studies in animals treated with cobalt chloride, in an attempt to establish whether the elevated serum lipids associated with cobalt chloride were secondary to hypothyroidism. They concluded that the elevation of serum lipid levels following cobalt chloride treatment, in different animal species and in man, was not secondary to hypothyroidism, but due to the effect of cobalt on lipid metabolism through unknown mechanisms.

The effect of chronic cobalt administration on the concentration of glucagon and its physiological activity in the rat was examined by Eaton (103). Basal plasma levels of the hormone were elevated and rose markedly in response to an arginine infusion. However, the rise in serum glucose in response to this exaggerated elevation in serum glucagon concentration, was subnormal. Similarly, the hyperglycaemic response to exogenous glucagon was subnormal. Insulin levels were similar in the cobalt treated rats to those in the controls. It was therefore concluded that the hyperglucagonaemia observed in the cobalt chloride-treated rat was associated with the induced hepatic resistance to the glucose-mobilizing action of glucagon. It was further suggested by Eaton and his colleagues (66,67) that loss of the normal hypolipaemic action of glucagon activity might be responsible for the development and/or maintenance of endogenous hyperlipaemia observed in the cobalt chloride-treated rats and other genetic and acquired types of hyperlipaemia.
Even if this explanation is correct the mechanism of cobalt chloride-induced hyperlipaemia in experimental animals is poorly understood (107,108, 109). The hypothesis that prevailed as a result of the work of Van Compenout and Cornelius (110) was that cobalt selectively destroyed the A cells (the glucagon producing cell) of the pancreatic islets. This led to acute glucagon release followed by chronic glucagon deficiency which gave rise to elevated plasma glucose and lipid levels.

1.5 Investigation on obesity and hyperlipaemia for the present studies:

As already mentioned, two animal models of hyperlipaemia were chosen for study. The Zucker fatty rat, a model of gross hereditary hyperlipaemia associated with obesity; and the cobalt chloride-treated rat, a model of moderate induced hyperlipaemia. The following investigations were carried out on these two models:

1. The effect of obesity and hyperlipaemia on carbohydrate metabolism and the role of insulin and glucagon.

2. The role of lipases in particular hormone sensitive lipase and lipoprotein lipase in obesity and hyperlipaemia and the effect of cobalt on these two enzymes.

3. The role of insulin and glucagon liver and adipose tissue receptors in the genesis of the insulin resistance of the Zucker fatty rat and the proposed glucagon resistance of the cobalt chloride-treated rat.

4. The effect of induced chronic hyperglucagonaemia on the Zucker fatty rat.

5. Cellular and subcellular distribution of cobalt in the cobalt chloride-treated rat and the correlation between cobalt levels and the duration of hypertriglyceridaemia.
Conclusions drawn from these studies and possible mechanisms for insulin resistance of the Zucker fatty rat and its hypertriglyceridaemia as well as that of the cobalt chloride-treated rat will be presented.
CHAPTER TWO

MATERIALS AND METHODOLOGICAL DEVELOPMENT
1. Animals
   i. Wistar Albino (W/A) Rats: Male Wistar albino rats aged 4-6 weeks were used throughout this study. These animals, bred at the University of Surrey Animal Unit, served as a model for induced hyperlipaemia following cobalt chloride treatment.

   Male rats (100-120 gm) were treated with cobalt chloride. 4 mg CoCl₂ per g body weight per day was given subcutaneously for 10 days with 9 days rest after the first five injections. Normally, 20-24 animals were treated per batch.

   Unless otherwise stated all CoCl₂-treated rats used throughout this study were experimented on after 10-15 days of last CoCl₂ injection.

   ii. Zucker Rats: Rats, homozygous for the fatty gene (fa) and bred from heterozygous thin strain in which the (fa) gene has been incorporated, were used together with their non-fatty litter mates. These animals were bred at the University of Surrey Animal Unit; an example is shown in Fig.2.1.

   All rats were fed a commercial pellet diet (Spratt Lab. Diet No. 1) ad libitum. The diet contained 4.5% fat, 21.5% protein and 42% carbohydrate.

2. Chemicals

   Adenylyl-imidophosphate tetra lithium salt (Boehringer Mannheim, Germany)

   Adenosine-5'-triphosphate (Boehringer Mannheim, Germany)

   Creatine Kinase (E.C.2.7.3.2) (Boehringer Mannheim, Germany)

   Creatine phosphate (Boehringer Mannheim, Germany)

   Lactoperoxidasesuspension (E.C.1.11.1.7, 160U/mg) (Boehringer Mannheim, Germany)

   Adenosine-5'-monophosphate (BDH Chemicals, Poole, England)
Polyethylene glycol 6000
Chloramine T
Theophylline
3-Isobutyl-1-methylxanthine
Crystalline bovine insulin
Crystalline porcine glucagon
Long-acting glucagon
Dextran T-500
QAE-Sephadex A-25
DEAE-Sephadex A-25
Sephadex G-25
PPO and POPOP
Instagel
Hyamine hydroxide
Synperonic Lissapol-NXP
Bovine Serum albumin
Carrier free Na\textsuperscript{125}\textsubscript{I} Solution (S.A. \textsuperscript{11-17mCi}/\textsubscript{ug})
D- U\textsubscript{14}C glucose (S.A. 230mCi/m mol.)
D- U\textsubscript{14}C Fructose (S.A. 250mCi/m mol.)
cyclic 8-\textsuperscript{3}H cAMP (S.A. 26 Ci/m mol.)
UDP-[U-\textsuperscript{14}C]glucose (S.A. 200 mCi/mmol)
Intralipid
Triolein (99% pure)
Triton X-100
Trasylol
Routine Chemicals of Analar Grade

(BDH Chemicals, Poole, England)
(BDH Chemicals, Poole, England)
(BDH Chemicals, Poole, England)
(Aldrich Chem., U.S.A.)
(Eli Lilly Co., U.S.A.)
(Eli Lilly Co., U.S.A.)
(Novo, Copenhagen, Denmark)
(Pharmacia Fine Chemicals, Sweden)
(Pharmacia Fine Chemicals, Sweden)
(Pharmacia Fine Chemicals, Sweden)
(Packard, Switzerland)
(Packard, Switzerland)
(Packard, Switzerland)
(ICI, Billingham, England)
(Sigma, U.S.A.)
(Radiochemical Centre, Amersham)
(Radiochemical Centre, Amersham)
(Radiochemical Centre, Amersham)
(Radiochemical Centre, Amersham)
(Kabi Vitrum, Sweden)
(Sigma, U.S.A.)
(BDH Chemicals, Poole, England)
(Bayer, W. Germany)
(BDH Chemicals, Poole, England)
Fig. 2.1 Fifteen week-old female Zucker fatty rat and its lean litter-mate.
B: Methodological Development.

2.1. Isolation and Purification of Rat Liver Plasma Membrane

The plasma membrane plays a very important role in cellular metabolism and function. The method of isolation originally developed by Neville (111) and later modified by Enmelot et al. (112) made possible the study of its biochemical properties. However, these methods give a low yield of membranes. Other workers, Tackenchi et al. (113) for example, developed methods for plasma membrane isolation using isotonic medium. In some of these methods the membrane yield was increased but activity of the functional enzymes were comparatively low.

The above methods, now termed the "classical" procedures, relied on sucrose density gradient techniques and have been shown to cause structural damage as a result of sedimentation through solutions having a high interfacial tension (114).

Later methods have used a two-phase polymer (dextran-polyethylene glycol) system. This has a more stabilizing effect on the plasma membrane and other cell organelles than the sucrose gradient technique (115).

Crude plasma membranes can also be isolated using a standard differential centrifugation technique. In these techniques different plasma membrane fractions which sediment with different cellular organelles, can be obtained e.g. nuclear, mitochondrial and microsomal membranes. These fractions, although not fit to use for hormone-receptor interaction studies, were found to be very useful for comparative studies on plasma membrane marker enzymes and adenylate cyclase estimations.
Methods:

Three methods representing each of the above three principles were chosen for experimentation in order to choose the appropriate procedure for subsequent work.

1. Two-phase polymer (polyethyleneglycol-dextran): This was described in detail by Lesko et al. (115) and is summarized below.

   W/A rats were killed by cervical dislocation. Their livers were quickly removed and washed in cold homogenizing medium (0.5mM calcium chloride + 1mM sodium bicarbonate pH 7.5). The livers were homogenized in a Potter-Elvehjem glass homogenizer with a teflon pestle. The homogenate was diluted 100 times the wet weight of the liver in the homogenizing buffer. It was then filtered and poured into plastic centrifuge cups and centrifuged at 2,600 rpm for 30 min. at 4°C.

   The supernatant was discarded and the pellet suspended in the same buffer, mixed and gently homogenized. The suspension was diluted to half the previous volume and centrifuged at 2,400 rpm for 15 min. The supernatant was discarded and the inside of the cups dried with gauze.

   The low speed pellet was then suspended in the upper phase of the two polymers and aliquoted into 4 x 50 ml MSE oak ridge polycarbonate centrifuge tubes. After shaking the tubes were centrifuged for 15 min. at 3,000 rpm. The plasma membranes, which formed a thin white sheet at the interface of the two polymers, were removed and suspended in fresh top phase. This procedure was repeated a total of three times. The purified plasma membranes were washed three times in 20mM Tris-HCl buffer, pH 7.5, and stored in small aliquots in liquid nitrogen. This method will be referred to as Method I.
2. **Sucrose-density gradient technique**: The method is described in detail by Ray (116) and is summarized below:

The low speed pellet was prepared exactly as for the previous two-phase polymer. The inside of the cups were thoroughly dried and 48% (w/w) sucrose solution was added. The resulting suspension was divided equally between 4 x 50ml oak ridge MSE polycarbonate centrifuge tubes. Solutions containing 45%, 41%, 37% (w/w) sucrose were added in that order to each tube. The tubes were then spun at 4°C for 2 hours at 25,000 rpm. The plasma membrane layer suspended between 41% and 37% sucrose layers was removed and washed three times as for the previous method. It was then aliquoted in small quantities and stored in liquid nitrogen until used. This method will be referred to as **Method 2**.

3. **Sucrose-density gradient flotation technique**:

The low speed pellet was prepared exactly as described above. The inside of the cups were thoroughly dried and the resulting suspension was divided equally between 4 x 50ml oak ridge tubes. After mixing with 48% (w/w) sucrose solution, 42% (w/w) sucrose solution was layered on it followed by 0.25M sucrose in 50mM Tris-HCl buffer pH 7.5 as a protection against dehydration. The spinning procedure was repeated as for method 2 and the membranes were washed and stored as above. This method will be referred to as **Method 3**.

4. **Standard differential centrifugation technique**:

Four grammes of minced liver tissue were homogenized for 90 sec. (set at 3) in 9 volumes of 0.25M sucrose-50mM Tris-HCl buffer pH 7.5 in a Polytron homogenizer. The unbroken tissue fragments were separated by straining through 4 layers of medical gauze. Standard differential centrifugation was used to obtain nuclear (600 x g, 10 min), mitochondrial
(12,000 x g, 15 min), and microsomal (45,000 x g, 30 min in the presence of 0.1M NaCl and 0.5mM MgSO₄ fractions. Each pellet was washed twice. The pellet obtained from the 12,000 x g centrifugation was collected into two different fractions. The bottom layer was very tightly packed and the reddish (mitochondrial) pellet was separated from the loosely packed upper layer of the pellet by very gentle stirring with the buffer. The lighter upper fraction "heavy membrane" was separated. This method will be referred to as Method 4.

A pictorial summary of the above methods is shown in Fig. 2.2.

Protein Measurements:

The method of Goodwin and Choi (117) was used. In this method trinitrobenzene sulfonic acid is used with sodium sulphite which forms a coloured complex of trinitrophenyl protein-sulphite in borate buffer. The coloured complex is yellow and stable with maximal spectral absorbance at 420nm. The coloured complex gave a linear calibration curve up to 400μg protein/ml.

All plasma membrane and other fractions were diluted with sodium tetraborate solution and measured as above.

Plasma Membrane Marker Enzymes:

Two plasma membrane marker enzymes were carried out on all plasma membrane preparations as a routine procedure.

1) Mg++-stimulated ATPase (E.C.3.6.1.3)

2) 5-Mononucleotidase (E.C.3.1.3.5)

Mg++-stimulated ATPase was assayed at pH 7.5 using ATP as substrate. The assay system contained in a final volume of 1ml the following:
Liver Homogenate

Spin thrice at 2,500 r.p.m.

Nuclear Pellet

Two-Phase Polymer
Spin at 3000 r.p.m. x 3

Sucrose-Gradient
Spin at 25,000 r.p.m. for 2 hrs

Floatation
Spin at 25,000 r.p.m. for 2 hrs

Supernatant

Differential Centrifugation
Spin at 12,000 r.p.m.

Mitochondrial Fraction + Heavy Membrane Fraction

Spin at 45,000 r.p.m.

Microsomal Membrane

Fig.2.2 Diagrammatic representation of plasma membrane preparation as summarized in text
50mM Tris buffer pH 7.5
10mM MgCl₂
5mM Disodium salt of ATP
80-150μg membrane protein suspension

After incubating the mixture at 37°C for 30 min. 1.5ml of 6% trichloroacetic acid solution was added to the tubes and centrifuged using the bench centrifuge. Blanks were set up exactly as for the tests except that no incubation was carried out. All determinations were carried out in duplicate.

5'-mononucleotidase was assayed exactly as for Mg++-stimulated ATPase but at a different pH (7.8) and using 5'-AMP as substrate.

Released phosphate was measured using the Technicon Autoanalyzer, adopting the technique described by Robinson (118). Briefly, the sample was diluted with and dialyzed into 10% sulphuric acid. The inorganic phosphate in the dialysate was coupled with a molybdivanadate reagent. The resulting complex was yellow; the absorbance of which was measured at 405nm. A calibration curve was set up covering the range 0.02-0.2mM P.

Results:

The yield and purity of rat liver plasma membranes using the first three of the above methods is summarized in Table 2.1. The results are compared with other published work in Table 2.2.

2.2. Insulin and Glucagon-Receptor Interactions:

As previously mentioned, the first step in the action of a hormone is its binding to a specific recognition site on the target cell known as a receptor. This hormone-receptor complex then activates additional
Table 2.1  Yield and purity of rat liver plasma membrane separated by methods 1 - 3.  
The yield is expressed as mg of membrane protein/gm wet weight of liver.  
The enzyme activities are expressed as umoles phosphate released/mg protein/30 min.  
Results mean ± S.E.M. Number of determinations in parenthesis

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total Protein (10 gm liver used)</th>
<th>Mg$^{2+}$-Stimulated ATPase</th>
<th>5'-Mononucleotidase</th>
<th>Yield</th>
<th>Fold Purification (5'-M.Nase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>9100 ± 110 (7)</td>
<td>1.98 ± 0.21 (7)</td>
<td>2.2 ± 0.4 (7)</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>1st spin pellet</td>
<td>4080 ± 76 (7)</td>
<td>3.21 ± 0.31 (7)</td>
<td>3.98 ± 0.52 (7)</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td>2nd spin pellet</td>
<td>2800 ± 62 (7)</td>
<td>3.01 ± 0.26 (7)</td>
<td>4.7 ± 0.36 (7)</td>
<td>2.15</td>
<td>7.40</td>
</tr>
<tr>
<td>Purified plasma membrane twofold polymer method</td>
<td>18.9 ± 0.28 (14)</td>
<td>13.72 ± 4.1 (14)</td>
<td>15.4 ± 3.1 (14)</td>
<td>1.89 ± 0.21 (14)</td>
<td>7.40</td>
</tr>
<tr>
<td>membrane sucrose gradient method (Method 2)</td>
<td>7.9 ± 0.14 (6)</td>
<td>13.76 ± 2.1 (6)</td>
<td>15.25 ± 2.0 (6)</td>
<td>0.79 ± 0.08 (6)</td>
<td>6.9</td>
</tr>
<tr>
<td>Purified plasma membrane flotation method (Method 3)</td>
<td>20.0 ± 0.11 (8)</td>
<td>8.9 ± 1.6 (8)</td>
<td>22.6 ± 2.0 (8)</td>
<td>2.0 ± 0.12 (8)</td>
<td>10.3</td>
</tr>
</tbody>
</table>
### Table 2.2

Comparison of yield and purity of rat liver plasma membrane between the methods employed in the text and other published work. Mean ± S.E.M. Units as for Table 2.1

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>1.89 ± 0.21</td>
<td>0.79 ± 0.08</td>
<td>2.0 ± 0.12</td>
<td>1.3</td>
<td>2.69 ± 0.28</td>
<td>1.3</td>
<td>0.41</td>
</tr>
<tr>
<td>Mg^{++}-stimulated ATPase</td>
<td>13.7 ± 4.1</td>
<td>13.7 ± 2.1</td>
<td>8.9 ± 1.6</td>
<td>100.00</td>
<td>21.0 ± 2.6</td>
<td>12.1</td>
<td>22.1</td>
</tr>
<tr>
<td>5'-Mononucleotidase</td>
<td>15.4 ± 3.1</td>
<td>15.25 ± 2.0</td>
<td>22.6 ± 2.0</td>
<td>41.0</td>
<td>28.38 ± 3.9</td>
<td>27.1</td>
<td>16.1</td>
</tr>
</tbody>
</table>

Other results relating to the binding of labelled glucagon and its displacement by glucagon and adenylate cyclase will be given and discussed later under the appropriate sections of this chapter.
cellular processes which, in turn, are followed by a cellular response characteristic of that particular hormone.

Most contemporary workers use the term "Receptor" to describe the natural molecular components of the cell that serve to recognize a biologically active chemical messenger or hormone. This definition will be used in this study.

Hormone-receptor studies have been applied successfully for the study of endocrine physiology (49). At least four different areas have been investigated.

1. Mechanism of hormone-action and structure activity relationships for peptide hormones.
3. The role of the receptor in altered states of hormone sensitivity.
4. Factors regulating hormone-receptor interaction.

In the present study the last two areas will be investigated in relation to obesity and hyperlipaemia in Chapters to follow. This Chapter will concentrate on the technical aspects of these interactions. The basic methods and techniques applied to the direct study of the hormone-receptor interaction are similar to those used in other competitive binding techniques e.g. radioimmunoassays. They require an isotopically labelled hormone, a suitable receptor preparation and an appropriate means for separating the hormone-receptor complex from free hormone.

Methods:

1. Hormone Iodination:

a) Chloramine T Method -[Method 1]

i) Glucagon: The method of Jørgensen and Larsen (120) was used and summarized as follows:
1 mg of porcine glucagon was dissolved in 2.5 ml of 0.01 N NaOH. 25 μl of this solution was pipetted into a small tube and mixed with 25 μl of 0.4 M phosphate buffer pH 7.2 and 0.5 mCi of Na\textsuperscript{125}I. The following reagents (all in 0.04 M phosphate buffer pH 7.4) were then added, with continuous stirring (using a small magnetic stirrer): 5 μl of 0.4% Chloramine T, ten seconds later 20 μl of 0.24% Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}, 50 μl of 1% KI. 5 μl were then taken for electrophoresis and the rest was applied to a QAE Sephadex-A25 and eluted at a rate of 10 ml/hr, using 20 mM Tris-HCl pH 8.5 + 4% BSA. Two ml fractions were collected and counted using an LKB Sample Counter.

ii) Insulin: The same method was employed for insulin using three times more insulin than glucagon in the reaction mixture and DEAE Sephadex-A25 instead of QAE Sephadex-A25. The same rate of elution was applied with the same eluting buffer as above.

b) Chloramine T Method -[Method 2]

i) Glucagon: The method of Goldfine et al. (121) was employed. In this method the concentrations of all reactants were kept relatively high and the amount of chloramine T was reduced markedly below that used in Method 1. This, as claimed by the authors reduces the risk of di and triiodination of the hormone which renders it biologically inactive (unbindable to membranes). The purification was carried out on 1 ml column packed with cellulose powder after taking samples for electrophoresis 1 ml fractions were collected.

ii) Insulin: The method of Freychet et al. (122) was employed. In this method as for glucagon, very small amounts of chloramine T were used. The proportion of hormone:iodine:chloramine T was 1:1:0.5 to 1 in molar equivalents. Purification was carried out as above for glucagon, using cellulose powder.
c) Insulin Iodination by Lactoperoxidase Method – [Method 3]

Using high concentrations of Lactoperoxidase and H₂O₂

The method of Thorell and Johansson (123) was employed, and summarized as follows:

5μg insulin (10μl), 10μl of 0.4M acetate buffer pH 5.6 were added followed by 1mCi of Na⁺¹²⁵. 10μl of 0.003% H₂O₂ and 10μl (1mg) lactoperoxidase were then added. After 10 sec. 500μl of 0.05M phosphate buffer pH 7.6 was added. After taking a sample for electrophoresis, the mixture was purified in a cellulose powder column as above.

d) Insulin Iodination by Lactoperoxidase Method – [Method 4]

Using low concentrations of lactoperoxidase and H₂O₂

The method of Suzuki et al. (124) was employed.

This method is similar in principle to Method 3 except that low concentrations of lactoperoxidase (200μg) and H₂O₂ (0.001%) were employed and the reaction was allowed to proceed for 20 min. at 30°C. Purification of the labelled hormone was first carried out on Sephadex G-25 column followed by cellulose column.

All preparations were stored in small aliquots at -20°C until used.

Chromatography-electrophoresis of all different preparations was carried out as follows:

5μl of the reaction mixture was spotted on Whatman 3M chromatography paper (2 x 26cm) 4cm from the cathode end of the paper. Chromatography was first carried out until the solvent front reached half way up the paper. The solvent employed for both chromatography and electrophoresis was 0.05M barbitone buffer. Electrophoresis followed using constant
voltage at 180 v for 60 min. The papers were dried and scanned using Dünnschicht Scanner II (Camlab, Cambridge) (Unpublished method).

Specific activities of all iodinated hormones were calculated by trichloracetic acid precipitation as described in detail by Cuatrecasas and Hollenberg (125).

Results:

The yield of TCA-precipitable radioactivity for Method 1 was 90% for glucagon corresponding to a specific radioactivity of 450µCi/µg of glucagon and a degree of iodination 0.7 atoms of $^{125}$I per molecule of glucagon.

For insulin TCA-precipitable radioactivity for Method 1 was about 80% and a specific activity of 380µCi/µg of insulin.

In Method 2 for glucagon about 50% of the radioactivity was incorporated into the hormone as measured by TCA precipitation. This low incorporation was due to the low concentrations of oxidizing agent used as compared to Method 1. About 10% of the applied radioactivity, which was equivalent to about 25% of the intact $^{125}$I-glucagon that had been applied to the column, was recovered in the albumin effluent. Calculated specific activity was 150µCi/µg of glucagon.

In Method 2 for insulin 90% was precipitated by TCA with a specific activity of 92µCi/µg of insulin.

In Method 3 for insulin only 35% of the total radioactivity was precipitated by 5% TCA with very low specific activity of 10µCi/µg of insulin.
In Method 4 for insulin high specific activities were obtained in different iodinations ranging between 250-350µCi/µg of insulin.

The biological activity of all the above preparations as judged by their ability to bind and be displaced by unlabelled hormones (hormone-receptor interaction) will be shown later in this section.

2. The Development of Hormone-Receptor Interaction Methods:

1) Glucagon

Rat liver plasma membranes were prepared either by the two-phase polymer (Method 1) or the flotation technique (Method 3) as above. 80-100µg of membrane protein in 100µl buffer (20mM Tris pH 7.5) were incubated in LP₃ tubes with 100µl of Krebs-Ringer phosphate buffer pH 7.5 (KRP) containing either 1% BSA or 1% gelatine. Different concentrations of glucagon standards (1-250ng/ml reaction mixture) in 100µl KRP buffer were added to the appropriate tubes. This was followed by the addition of 10,000 cpm of labelled glucagon (120pg) in 100µl KRP buffer. The total volume was 400µl per tube. The tubes were then mixed and incubated for 30 min. at 30°C. They were then spun at 3,000 rpm at 4°C using MSE Minstral 4 L centrifuge for 15 min. The supernatant from each tube was removed by means of a Pasteur pipette using a suction pump without disturbing the pellet. 1ml of the KRP buffer was added to each tube and spun again. The washing was repeated a total of three times. The radioactivity was then counted using a LKB-Gamma Sample Counter.

Non-specific binding (NSB) tubes representing the percentage of ¹²⁵I-radioactivity bound to membrane pellets in the presence of high concentrations of unlabelled glucagon (10µg) were included for each batch of tests. The NSB values obtained were subtracted from each of the test results.
All tests, total and NSB were carried out in duplicate and when possible in triplicate.

The following factors for glucagon-receptor interactions were investigated:

1) Effect of membrane concentration:
W/A rat liver plasma membranes were prepared by the two-phase polymer method. Various concentrations of membrane protein (8-500μg) were incubated with $^{125}$I-glucagon (10,000 cpm) prepared by Method 1, for 30 min. at 30°C.

2) Effect of incubation time and temperature on binding:
W/A rat liver plasma membranes were prepared by the two-phase polymer method. A series of tests containing 100μg membrane protein and $^{125}$I-glucagon (10,000 cpm) prepared by Method 1 were counted at 1, 1, 2, 3, 4, 5 and 24 hours at 4°C and 30°C.

ii) Insulin:

Insulin-receptor interactions were carried out exactly as for glucagon with one modification - 4% glucose was added to the KRPO buffer and calcium was omitted. This was shown to increase insulin binding to the membranes (126).
Insulin-receptor interaction was also investigated as for glucagon, for the effect of membrane concentration, time and temperature.

After the establishment of the optimal conditions for the glucagon and insulin-receptor interactions as will be discussed later, $^{125}$I-labelled hormones prepared by the different methods described above were tested for their ability to bind to hormone receptors and to be displaced by excess "cold" hormone.

Glucagon-receptor studies were carried out to check the suitability of liver plasma membranes prepared by the above methods.

Results:

Fig. 2.3 shows the effect of the liver plasma membrane concentrations on glucagon and insulin binding. Half maximal binding (50%) is calculated to be at $100\mu g/ml$ approximately for insulin and glucagon concentration. Fig. 2.4 shows the effect of time and temperature on the course of hormone binding. Results of glucagon and insulin binding to rat liver plasma membranes using the above iodination methods are represented in Fig. 2.5 and 2.6 for glucagon and insulin respectively.

Fig. 2.7 shows $^{125}$I-glucagon binding and its displacement by native glucagon using the three methods of plasma membrane preparation discussed in Section 1 of this chapter.

2.3 Adenylate Cyclase

Adenylate cyclase is the primary enzyme system through which a number of peptide hormones and biogenic amines alter the metabolism structure
Fig. 2.3 Effect of membrane concentration on hormone binding of (○) glucagon and (■) insulin under conditions described in text.
Fig. 2.4 Effect of incubation time and temperature on the binding of (○) glucagon and (□) insulin under conditions described in text.
Fig. 2.5  Glucagon binding to rat liver membranes for different methods of glucagon iodination employed (○) Method 1, (□) Method 2.
Fig. 2.6  Insulin binding to rat liver membranes for different methods of iodination employed (○) Method 1, (O) Method 2, (■) Method 3, (□) Method 4.
Fig. 2.7 Glucagon binding to rat liver plasma membranes as prepared by (▲) Method 1, (■) Method 2, and (○) Method 3.
and function of their target cell. This system selectively receives, translates and amplifies the "message" contained within the structure of the hormone to give a new message through a second messenger adenosine 3',5' cyclic phosphate (cAMP) formed by the catalytic hydrolysis of ATP.

The aim of this section is to find a fairly sensitive, simple and reproducible assay for adenylate cyclase. The conversion of ATP to cAMP by the enzyme adenylate cyclase was first described in detail by Sutherland et al. (127). Their assay (for this conversion) though sensitive and fairly accurate is a bioassay and depends upon a multicomponent system whose activity is influenced by a number of adenine nucleotides and certain hexoses. The method requires extensive purification of cAMP before assay. When appropriately radiolabelled ATP became available, bioassays were replaced by radiometric procedures for the determination of cAMP formed from radioactive ATP in various broken cell fractions. This type of assay is hard to apply to preparations of mammalian tissue homogenates which contain numerous enzymes using ATP as substrate. All methods using radioactive ATP require extreme care in isolating the product cAMP from other radioactive metabolic products of ATP which are often produced in greater quantities than cAMP itself (128). Much valuable data has been obtained with the method of Krishna et al. (129) using labelled ATP as substrate. However, this method is limited by sensitivity and is laborious as each determination requires separation on Dowex-50 columns.

For the present work two methods were assessed:

a) Using adenylyl imidophosphate (AMP.PNP) as developed by Maguire et al. (130). AMP.PNP is an analogue of ATP. The replacement by nitrogen of the oxygen between the $\beta$ and $\gamma$ phosphates renders the molecule
essentially immune to enzymatic cleavage at the β-γ position by ATPase and other phosphohydrolases (131). This eliminates the use of an expensive ATP regenerating system.

b) Using ATP as substrate with an ATP regenerating system.

The cAMP produced was estimated by competitive protein binding method as developed by Brown et al. (132).

Methods:

1) AMP-PNP as substrate:  

The standard adenylate cyclase mixture contained in a total volume of 250μl the following concentrations of materials:

- 20mM Tris HCl buffer pH 8.5
- 1mM Theophylline or 0.2mM 1-methyl-3-isobutyl xanthine
- 4mM MgSO$_4$·7H$_2$O
- 0.5mM AMP-PNP substrate
- 0.1% BSA

The reaction was started by the addition of 20-50μg enzyme protein prepared by the two-phase polymer method. Incubation time was 10-15 min. at 30°C. NaF (10mM) or glucagon (10μg/ml) were added as needed. The reaction was stopped by immersing the tubes in a boiling water bath for 2 min.

Samples were frozen at -20°C until cAMP was determined.

Cyclic AMP Estimation:

Cyclic AMP as mentioned above was determined by competitive protein binding. A binding protein with a high affinity for cAMP was extracted
from bovine adrenal cortex and prepared as follows:

Bovine adrenals were collected fresh after slaughter, the cortex were separated, chopped and homogenized using a Polytron homogenizer (2g tissue in 3 volumes medium). The homogenizing medium contained the following:

0.25M sucrose
0.025M KCl
0.005M MgCl₂
0.05M Tris buffer pH 7.4

The resultant suspension was spun at 2,000 x g for 5 min. The supernatant was separated and spun at 5,000 x g for 15 min. at 4°C, and stored in small quantities at -20°C. Negligible loss of binding activity has been found after 9 months of storage.

A binding protein dilution curve was set up for each preparation of binding protein to determine the appropriate dilution of the binding protein to be used in subsequent work.

Serial dilutions of the protein were set up in LP₃ incubation tubes each containing:

100µl of the buffer (0.05M Tris-HCl 6mM 2-Mercaptoethanol and 8mM Theophylline)
50µl Tracer (9.1mCi/mg tritiated cAMP)
≈ 5000 cpm/tube
100µl diluted binding protein

After mixing, the tubes were incubated at 4°C for 90 min. The
free and bound fractions were separated as follows:

1g. of charcoal was mixed in a magnetic stirrer with 50ml distilled H₂O for 15 min. After spinning at 2,200 rpm for 15 min. the supernatant was discarded and the charcoal was mixed with 50ml buffer that contained 0.2g BSA. With continuous mixing on the magnetic stirrer 500μl was added to each of the above incubation tubes. The tubes were mixed using a Whirlmixer and spun for 15 min. at 2,500 rpm at 4°C. 500μl of supernatant which contained the bound cAMP were mixed with 4.5ml of Scintillation liquid which contained:

- 0.4% P.P.O. 10g
- 0.01% P.O.P.O.P. 0.25g
- Lissapal-LXP 830ml
- Toluene 1670ml

Total of 2,500ml.

After whirlmixing the tubes were counted using an LKB ultra-beta counter at a counting efficiency of 55%. The dilution of the binding protein which was used subsequently was that which gave 20-30% of total activity bound.

For each batch of cAMP determination the following tubes were set up:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total</th>
<th>NSB</th>
<th>Zero</th>
<th>Standard or test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>250μl</td>
<td>250μl</td>
<td>150μl</td>
<td>100μl</td>
</tr>
<tr>
<td>Standard or sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50μl</td>
</tr>
<tr>
<td>Tracer</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>Binding Protein</td>
<td>-</td>
<td>-</td>
<td>100μl</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Standard range 1-50ng/ml cAMP.
After mixing and incubating for 90 min. at 4°C 500μl of buffer was added to the "total" and 500μl of charcoal prepared as above was added to all other tubes including the "zero" and "NSB" tubes. Separation and counting was carried out as above. A typical standard curve is shown in Fig. 2.8

2) ATP as substrate

The standard adenylate cyclase mixture contained in a total volume of 250μl the following concentration of materials:

- 30 mM Tris-HCl buffer pH 7.5
- 5 mM MgSO₄·7H₂O
- 0.25 mM Theophylline
- 1.0 mM ATP
- 0.5% (w/v) BSA
- 1.0 mM EDTA
- 1 mg/ml Creatine Kinase
- 20 mM Phosphocreatine

To start the reaction of 20-50 g membrane protein was added and incubated for 10-15 min. at 30°C. Sodium fluoride or glucagon was added as required. The reaction was terminated as for Method 1. cAMP was estimated as described above.

Results:

Method 1 using AMP-PNP as substrate was first investigated with W/A rat plasma membranes isolated by the two-phase method.

Table 2.3 Results of adenylate cyclase basal and stimulated levels expressed in pmoles cAMP/mg protein/10 min. Mean ± SEM. Fluoride concentration was 10 mM and glucagon 10μg/ml. Number of determinations on different plasma membrane determinations are shown in parenthesis.

<table>
<thead>
<tr>
<th>Basal Level</th>
<th>+ Fluoride</th>
<th>+ Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.2 ± 0.8 (5)</td>
<td>14.1 ± 0.7 (5)</td>
<td>16.4 ± 1.1 (5)</td>
</tr>
</tbody>
</table>
Fig. 2.8  Cyclic-AMP Standard Calibration Curve
The results shown in Table 2.3 are very low compared to other workers findings. Using the same substrate Rodbell et al. (133) reported a basal level of 125 pmoles cAMP/mg protein/10 min., 890 with fluoride and 950 with glucagon stimulation. Using ATP as substrate values ranging between 100-500 pmoles cAMP/mg protein/10 min. for basal levels while fluoride stimulation gave 1 - 2 nmoles cAMP and glucagon stimulation values as high as 5 nmoles cAMP were reported (134, 135).

Investigations were made into the purity of the substrate which was bought from Boehringer and was claimed to be 96% pure. A gross solution of the substrate was spotted on silica gel plates with 1-propanol-conc. ammonia-water (6:3:1, v/v) as the solvent (131). The substrate was found to be only 75% pure with the major contaminant being 5'-adenylyl phosphoamidate (ADPNH₂). This contaminant was reported to lower the enzymatic activity (130). When the pure substrate was scraped off the plates, dried and resuspended in distilled H₂O then used, there was a slight improvement in the basal levels but the fluoride and glucagon stimulated levels remained unaltered (results not shown).

It has been reported that lithium inhibits the TSH-induced stimulation of adenyl cyclase activity in beef thyroid membranes without affecting the basal activity (136). As the AMP-PNP substrate was supplied by Boehringer as tetra-lithium salt, it was thought that the presence of lithium might have affected the adenylate cyclase assay. The substrate was put through a Dowex 50 H⁺ (50-100 mesh) column and the resultant lithium free substrate was collected and checked for the presence of lithium before and after purification by means of an atomic absorption
method for lithium. However, results obtained after using lithium free substrate were not much better than previously obtained before purification (results not shown).

Following this failure, ATP was used as substrate as in Method 2. The rat liver plasma membranes were again prepared by the two-phase polymer and adenylate cyclase was estimated using Method 2. Once again the results were not very encouraging.

The two-phase polymer method was abandoned and the sucrose density gradient methods were tried using ATP and AMP-PNP as substrates. Results are shown in Table 2.4.

Table 2.4

Adult rat liver plasma membranes were prepared by Method 2 (sucrose gradient) and Method 3 (flotation) as in Section 1 of this chapter. Comparative results of the two methods for adenylate cyclase using ATP and AMP-PNP as substrates are shown below and expressed as pmoles cAMP/10 min/mg protein. Each experiment contained triplicate enzyme analysis and the results are given as the mean ± S.E.M.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Basal level + 10 mM Fluoride + 10µg/ml Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Method 2</td>
</tr>
<tr>
<td>ATP</td>
<td>141±3.1</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>92±2.7</td>
</tr>
</tbody>
</table>

Different fractions which correspond to different cellular particles collected at different sucrose density gradients using Method 2 and 3 were checked for adenylate cyclase and 5'-Mononucleotidase activities and results are shown in Figs. 2.9 and 2.10 for the respective methods.
Fig. 2.9 Adenylate cyclase and 5'-mononucleotidase activities of different fractions collected at different sucrose density gradients of Method 2.

Fig. 2.10 Adenylate cyclase and 5'-mononucleotidase activities of the two fractions collected at the two sucrose density gradients of Method 3.
Adenylate cyclase activity was further investigated to test the heterogeneous nature of the rat liver plasma membrane. A standard differential centrifugation method was used (Method 4). Different fractions were collected and tested for basal and glucagon stimulated activities of adenylate cyclase using ATP as substrate. Results are shown in Fig. 2.11.

2.4 Isolation of Rat Adipose Tissue Plasma Membranes:

The method of Cutrecasas (137) was modified and summarized as follows:

After the rats were sacrificed by cervical dislocation the epididymal fat pads were collected, cut into small pieces and washed with cold saline. They were then incubated in KRP buffer pH 7.5 containing 4% BSA, 0.5 mg/ml glucose and 0.3 gm/ml collagenase for one hour at 37°C mixing gently at intervals.

After centrifugation at very low speed the floating adipocyte layer was separated from the stromal-vascular and other cells by aspiration, and washed twice in KRP buffer. They were then homogenized using a Polytron (setting 2.2) for 1 min in a lysing medium containing 2.5 mM MgCl2, 2.5 mM CuCl2, 1mM KHCO3 in 10 mM Tris-HCl buffer, pH 7.5.

The suspension was then spun at 23,000 x g for 30 min. The supernatant and the creamy layer were discarded. The pellet, which contained most of the plasma membranes, was washed in the lysing medium, suspended in 0.03M Tris-HCl buffer, pH 7.5, and stored in small aliquots at -40°C until used.

2.5 Carbohydrate Measurements:

a) Liver glycogen

Liver glycogen was measured by the method of Chang et al (138) and
Fig. 2.11 Adenylate cyclase activity of the different fractions collected by differential centrifugation.

- Basal level
- Glucagon-stimulated level (10 µg/ml)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Basal level</th>
<th>Glucagon-stimulated level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Fraction</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Heavy Membrane</td>
<td>600</td>
<td>800</td>
</tr>
<tr>
<td>Microsomal Fraction</td>
<td>300</td>
<td>500</td>
</tr>
</tbody>
</table>
summarized as follows:

In this method the liver was homogenized in 0.03N HCl. After heating for 5 min. in a boiling water bath glucose was released by incubation in 0.2M acetate buffer pH 4.8, with amylα-1,4-1,6 glucosidase for 90 min. at 37°C. Glucose produced was measured.

b) Glucose

Blood samples were collected in fluoride tubes and kept at 4°C until measurement (within the same day). Glucose was measured using GOD-Perid method with a kit supplied by Boehringer Mannheim (Cat. No. 15155). This method uses the chromogen ABTS, ammonium salt of (2,2' azino-di,3-ethyl-benzothiazoline-6-sulphonic acid) as redox indicator with glucose oxidase and peroxidase. The method has a coefficient of variation (cv) of 3.3% at 5 mmoles/1.

2.6 Lipid Measurements:

Lipids in plasma and liver homogenates were measured.

a) Cholesterol

Cholesterol was measured colorimetrically using a kit method supplied by Boehringer Mannheim (Cat.No. 15949). This method utilises the Lieberman-Burchard reaction of acetic anhydride and concentrated sulphuric acid with a reproducibility value of + 6.0%.

b) Free Fatty Acids

FFA levels were also measured colorimetrically using a kit method supplied by Boehringer Mannheim (Cat. No. 15997). In this method the FFA are converted to chloroform-soluble copper salts. The copper in the organic layer is subsequently measured colorimetrically. The concentration of FFA is proportioned to the absorbance of the copper-containing chloroform. The method has a reproducibility level of + 6.0%.
c) Triglycerides:

Triglycerides were measured spectrophotometrically using a kit method supplied by Boehringer Mannheim (Cat. No. 15989). The principle of the method is as follows:

\[
\begin{align*}
\text{Triglycerides} & \xrightarrow{\text{KOH}} \text{glycerol} + \text{FFA} \\
\text{Glycerol} + \text{ATP} & \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP} \\
\text{ADP} + \text{PEP} & \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} + \text{H}^+ & \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+
\end{align*}
\]

The disappearance rate of NADH+H⁺ is measured at 340 nm which is proportional to the levels of triglycerides present in the sample. The method has a CV of 4.2% at 1.7 mmole/l triglyceride concentration.

Quality control sera, Liponome^R (BDH, Poole) and Seronorm^R (Nygard, Norway) were used with all batches.

2.7 Determination of Cobalt Levels in Tissues of CoCl₂-treated Rats:

A weighed portion (1 g.) of each tissue or 1 ml of plasma was digested in concentrated nitric acid and perchloric acid (5:1). The digest was then heated on a hot plate at a temperature of 300°C until the solution was evaporated to dryness. The cobalt in the residue was re-dissolved in 1 ml of 4.5N nitric acid solution and heated again at 150°C for 2 min. After cooling, 1 ml aliquots were then buffered by the addition of 0.3 ml ammonium citrate buffer (5% w/v ammonium citrate in 6N ammonia). The pH was adjusted to 3.0 with ammonia solution. 0.1 ml of ammonium pyrrolidinedithiocarbamate (APDC) was added and the cobalt was extracted in 0.5 ml of methyl isobutyl ketone. A series of cobalt standard solutions (5-500 μg/100 ml) were extracted in the same manner.

The organic layer was then separated from the aqueous layer following centrifugation and aspirated into 1L 353 atomic absorption spectrophotometer (Instrumentation Laboratory UK Ltd.,) and the absorption at 240.7 nm
was determined. The detection limit of the method was 1 μg Co/100 ml, 10 ng/gm tissue.

2.8. Plasma Insulin and Glucagon Determinations:

Plasma insulin and glucagon were determined by radioimmunoassay using human insulin and porcine glucagon as standards. The glucagon antiserum used had a low cross-reactivity with gut extracts (GLI). Glucagon radioimmunoassay had a sensitivity of 25-40 ng/l with a coefficient of variation of 5.7%. Glucagon assays were kindly carried out by Dr. Y. Al-Tamer and Dr. D. Tsiolakis at the University of Surrey, Biochemistry Department. Insulin assays were carried out by Miss Wendy Green, Clinical Biochemistry Department, St. Luke's Hospital, Guildford, Surrey.

2.9 Discussion

As this thesis is partly concerned with insulin and glucagon liver receptors in normal and pathological states as exhibited by the rat models investigated, much effort was concentrated on the preparation of these receptors in purified plasma membranes. Not only the yield and purity of these membranes was important, but also the ability to bind radioactive labelled hormones which can be displaced by unlabelled hormones. Furthermore, the ability to measure in these preparations the basal and stimulated levels of adenylate cyclase, an enzyme system by which glucagon is believed to exert its metabolic action, is of utmost importance for this particular type of study. In order to achieve some meaningful results a fairly pure and viable liver plasma membrane preparation should be obtained.
With that in mind, three different techniques of liver plasma membrane preparation were tried, investigated and summarized in Fig. 2.2. The first method was that of the two-phase polymer system (dextran-polyethylene glycol). This method, as shown in Table 2.1 gave a good yield of liver plasma membranes, with a fairly pure preparation as judged by the plasma membrane marker enzymes, 5'-mononucleotidase and Mg\(^{++}\)-stimulated ATPase. The advantage of this method is that it utilizes low speed centrifugation and thus eliminates the need for expensive high speed centrifugation. It is also comparatively simple and highly reproducible. Hormone-receptor interaction results for both insulin and glucagon using this method of plasma membrane preparation, as shown in Fig. 2.6 and 2.7 for insulin and glucagon respectively, were excellent. However, adenylate cyclase basal and stimulated levels were both low as shown in Table 2.3. This led to a time-consuming investigation into the method employed for the determination of this enzyme system utilizing AMP-PNP as substrate part of which is reported under section 2.3 of this chapter. Eventually, after different methods of liver plasma membrane preparations were tried, it was discovered that the fault lay within the plasma membrane preparation using the two-phase polymer and not the method employed for adenylate cyclase determination. It appears probable that either one or both the polymers used in the membrane preparation might be blocking or interfering in some way with the action of adenylate cyclase through some stage of its cascade system. Up to the present no published work has been cited in any literature using the two-phase polymer method for adenylate cyclase estimation.
This discovery necessitated the return to the more conventional methods of plasma membrane preparation i.e. the sucrose gradient techniques. The method developed by Ray (116) was tried using four sucrose gradients 48, 45, 41 and 37% (w/w), (Method 2) and its modification using only two sucrose gradients 48 and 42%, (Method 3). In Method 2 fairly good results for membrane purity, as shown by the plasma marker enzymes as well as adenylate cyclase levels, were obtained. However, the yield was very poor when compared to Method 1 and Method 3 as shown in Table 2.1, but the hormone-receptor interaction results for insulin and glucagon was just as good as the other two methods.

Pohl et al. (139) have reported an increase in adenylate cyclase activity in partially purified rat liver plasma membranes as compared to the fully purified preparations. This led to the development of Method 3, in which only two sucrose gradients were used, 48 and 42%. Using this method for the rat liver plasma membrane preparation, it was found that there was an increase in adenylate cyclase levels both in the basal and stimulated states as shown in Figures 2.9 and 2.10. Not only the adenylate cyclase levels were elevated but also the 5'-mononucleotidase levels, compared to the fully purified procedure, Method 2.
Table 2.2 shows a comparison of the yield and purity of the plasma membrane preparations though the present work and other methods using similar techniques. The Table shows an appreciable drop in the plasma membrane marker enzyme of the preparations for the present work as compared to the preparations of other workers. If these enzymes are taken to be solely located at the plasma membranes then the above preparations seem to be contaminated with other subcellular fractions. However, Chang et al (140) have shown that this is not the case and only in exceptional cases have such enzymes been shown to be located uniquely in plasma membrane fractions. Different patterns of localization may occur between the same tissue in different species or between different tissues in the same species. They have also shown that, for instance, 5'-mononucleotidase activity in fat cell homogenates was found to change during fractionation and very substantial activity was present in non-plasma membrane fractions.

All the above methods utilize the material present in the low speed nuclear fraction, assuming that the intact large sheets of membranes will sediment with this fraction, while all of the other fractions being discarded. Chang et al (140) in their studies have labelled the specific cell surface membrane receptors with low concentrations of highly radio-active $^{125}\text{I}$-ligand and counted the tracer after conventional plasma membrane preparation procedures. They have reported that substantial quantities of plasma membranes (> 50%) were not recovered in the interface of the two-phase polymer system. Conventional liver fractionation procedures, as employed here, which retain for further purification only the readily sedimented pellet (2000 x g) discarded a very large (at least 60%) fraction of the total plasma membranes.
In the iodination procedures the Method of Jørgensen and Larsen (120) for glucagon iodination proved to be the method of choice since as much as 60% of labelled glucagon was bound by rat liver plasma membrane and was readily displaceable by unlabelled glucagon. Method 2 of the glucagon iodination using very low concentrations of chloramine T gave much lower binding results as shown in Fig. 2.5.

Insulin proved to be more difficult to iodinate with retention of its biological activity as judged by its ability to bind to liver plasma membranes. This is due to the fact that the insulin molecule contains four tyrosine molecules which are readily iodinated. Di- and tri-iodinated insulin have been shown to be non-binding to membranes in insulin-receptor interaction studies (141). Only mono-iodinated insulin is easily displaceable for such studies. Here, once again, the modified method of Jørgerson and Larsen proved to be the best, followed by Method 4 of Suzuki et al, as shown in Fig. 2.6. The other two methods gave unsuitable insulin label for the present studies. For reasons of simplicity and reproducibility, Method 4 was used for all subsequent work.

Fig. 2.3 shows the effect of membrane concentration on hormone binding. Half maximal binding (50% of total binding) was chosen for all subsequent work which was calculated to be approximately 100μg/ml incubation medium.

Fig. 2.4 shows the effect of incubation time and temperature on the course of binding. Both the amount and the rate of binding are temperature-dependent. After 30 min. of incubation at 4°C the percentage of \(^{125}\)I-insulin bound was about one-third of that observed at 30°C and only one-fifth of that for glucagon. The reaction reached a maximal binding state at 4°C after 24 hours incubation, compared to 30-60 min. at 30°C. For all
subsequent work incubation at 30°C was carried out.

Fig. 2.7 shows the suitability of plasma membrane preparations by different methods for glucagon-receptor interaction studies which were used as a model. As can be seen from the graphs, there was no significant difference between the three methods employed.

In the adenylate cyclase results shown in Table 2.4 ATP proved to be a better substrate for this enzyme than AMP.PNP for basal and stimulated levels. Although Method 2 utilizing ATP as the substrate is more expensive due to the presence of the ATP-regenerating system, it was chosen for all subsequent work.

Adenylate cyclase estimated on different fractions prepared by differential centrifugation, as represented in Fig. 2.11, shows a considerable variation of distribution of this enzyme system between different fractions.

The "heavy membrane" fraction which layers loosely on top of the mitochondrial pellet, contains the highest activity of this enzyme. This is followed by the nuclear fraction from which intact membranes, used for this study are prepared in the above three methods. The microsomal fraction contains a considerable activity of adenylate cyclase, while the mitochondrial contains very little. The distribution of adenylate cyclase activity in differential centrifugation shows the heterogeneous nature of the so-called liver plasma membrane. If adenylate cyclase is taken as a marker enzyme for liver plasma membranes, then this confirms the findings of Chang et al (140) that only a small fraction of the total plasma membrane is recovered in the low speed nuclear fraction utilized in the available conventional procedures for plasma membrane preparations, and are represented in the three methods employed here.
2.10 Summary of methods chosen for work to follow:

1) All rat liver plasma membranes will be prepared by two-sucrose gradient flotation technique (Method 3).

2) Hormone-receptor interaction studies will be assayed at 30°C using Jørgenson and Larsen's method (Method 1) label for glucagon, and Suzuki et al (Method 4) label for insulin.

3) ATP will be used as the substrate for adenylate cyclase determination in the presence of an ATP-regenerating system.

4) Other methods will be used as described in this chapter and under other appropriate chapters in this thesis.
CHAPTER THREE

CARBOHYDRATE METABOLISM IN THE ZUCKER AND CoCl₂-TREATED RATS
3.1. **Introduction:**

Much work on intermediary metabolism has been carried out on Zucker and CoCl₂-treated rats. Preliminary work in this Chapter was directed towards confirmation of previous studies by other workers on these two animal models. However, in order to elucidate further the role of carbohydrate metabolism in the production of obesity in the Zucker fatty rat and the hyperlipaemia of the CoCl₂-treated rat, other lines of investigations were pursued.

1. Plasma glucose, insulin and glucagon levels were measured in fasted and fed animals.
2. Glucose tolerance tests were performed.
3. Arginine tolerance tests were performed.
4. Glucose and fructose tracer studies were carried out on CoCl₂-treated rats.
5. Liver glycogen was measured in the two types of animal models. Glycogen and glycogen synthase levels in response to a glucose loading test was carried out on the CoCl₂-treated rats.

3.2. **Methodology**

a) **Glucose Tolerance Test:**

After an 18 hr. fast, fatty and lean 8 weeks old male Zucker rats were anaesthetised with Nembutal (6 mg/100 g body weight). Fasting blood samples were withdrawn from the tail (50µl). 3g/kg of 50% (w/v) sterilized glucose solution were given i.p. Tail blood was collected after 15, 30, 60, 90, 120 and 150 min of glucose administration. All blood samples were collected in 0.33N perchloric acid (to deproteinise), centrifuged at room temperature and kept at 4°C until glucose was assayed.

The same procedure was carried out on CoCl₂-treated rats and their controls.
b) Arginine Tolerance Test:

Following anaesthesia with Nembutal, blood samples were collected by cardiac puncture from 12 weeks old, fasted, fatty and lean male Zucker rats. Arginine was given i.p. at a dosage of 2g/kg body weight. Blood samples were collected via cardiac puncture after 15, 30 and 60 min. Plasma was assayed for insulin, glucagon and glucose. Glucose samples were collected in small fluoride-oxalate tubes, whilst glucagon and insulin samples were collected into heparinised tubes containing 2,000KIU Trasylol per ml of blood. 1ml blood samples were collected each time. All samples were centrifuged immediately and the plasma was separated and stored at -20°C until assayed.

The same procedure was repeated on CoCl₂-treated rats and their controls.

c) Glucose and Fructose Tracer Studies:

Following an overnight fast CoCl₂-treated rats and their controls were given 1g glucose containing 25μCi D-[U-¹⁴C] glucose orally per rat. After 3 hr the animals were anaesthetized with Nembutal and blood, liver epididymal fat pads, muscle and kidneys were collected for analysis. The livers were quickly washed with saline and immediately dropped in liquid nitrogen to preserve their glycogen content.

The tissues were processed as follows:

i) Liver

A liver homogenate (10%) was made using a Polytron homogeniser (setting 3) in ethanol/water mixture (7:3 v/v). An aliquot of 0.4ml was placed in a scintillation vial and 3ml hyamine hydroxide added as protein digestant. After heating in a water bath for 3 hours the digest was cooled, decolourized by the addition of 0.4ml of a 30% H₂O₂ solution
and left overnight. The mixture was made acidic next morning by the addition of 0.25ml concentrated HCl. 9 ml Insta-gel scintillant was subsequently added and the mixture counted, after a further 12 hours, in a LKB β-Counter.

Liver glycogen was isolated using a standard potassium hydroxide-ethanol extraction procedure (142). 1g of liver + 8ml of 30% KOH solution were heated in a boiling water bath until digestion was completed giving a clear solution. After cooling in an ice bath 10ml of 95% ethanol were added. After 30 min the white precipitate of glycogen was separated by centrifugation and the ethanol extraction procedure was repeated. The resultant glycogen was dissolved in 4ml of boiling water; 9ml Insta-gel were added and the samples counted in an LKB-β-counter.

Liver total lipids were isolated using a standard chloroform-methanol procedure. A portion of liver (2g) was homogenized in 38ml chloroform-methanol mixture (2:1 V/v), in a Polytron homogenizer. The resulting suspension was filtered through glass wool and 20ml transferred to a 50ml glass stoppered conical flask. 6.4ml methanol was added followed by 10.6ml of water. After mixing the suspension was separated into two phases by centrifugation. 3 ml aliquot of the chloroform phase was evaporated to dryness. The dried residue was mixed with 9 ml Insta-gel and counted in an LKB-β-Counter.

ii) Muscle, kidneys and adipose tissue

Homogenates of these tissues were made (10%) digested and counted as described above for total liver.

iii) Plasma

One ml of plasma was added to 9ml Insta-gel mixed and counted.

Fructose tracer studies on the cobalt chloride-treated rats and
their controls were carried out exactly as for glucose except that 1g of "cold" fructose was given orally with 25µCi of D[U-^{14}C] fructose.

^{14}C-n-hexadecane (1 x 10^6 dpm/g) was used as an external standard. Counting efficiency for each tissue was calculated and all reported results were corrected accordingly.

d) Glucose loading Test, Glycogen and Glycogen Synthase:

A glucose loading test was carried out on the CoCl_2-treated rats and their controls. After an overnight fast each rat was given 1 g of glucose orally plus 1 g glucose I.P.. Three hours later the rats were anaesthetised with Nembutal, their livers were removed and immediately placed in liquid nitrogen for glycogen-estimation as described above.

Liver glycogen was measured in Zucker lean and fatty rats as well as in CoCl_2-treated rats and their controls.

Glycogen synthase [EC 2.4.1.11] was determined by measuring the incorporation of ^14C-glucose from UDP-[U-^{14}C] glucose into glycogen. The ^14C-labelled glycogen was measured after adsorption on filter paper. Active synthase (I) and total synthase (I + D) activity were estimated at pH 7.8 as described in detail by Watts and Gain (142).

Statistical analyses were carried out using the Mann-Whitney U-test (169).
3.3. Results

   a) Zucker Rats

      i) Plasma glucose and hormone concentrations:

      As shown in Table 3.1, lean Zucker rats had similar plasma glucose levels to their fasted and fed fatty counterparts. Plasma glucagon levels were slightly higher in the fasting fatty rats than in their lean litter mates, but not significantly so. However, in the fed fatty animals plasma glucagon levels though lower than those in fasting fat animals, were nonetheless significantly lower than in their lean controls (p<0.02). Plasma insulin showed a 14-fold increase in the fatty rats compared to the lean ones in the fasted state; and a 9-fold increase in the fed state.

Table 3.1  Average weights, plasma glucose and hormone concentrations of 12 weeks old male Zucker lean and fatty rats in the fasted and fed states. Fasted assays were obtained after 18 hrs of food deprivation. Values are the mean + SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Fasted</th>
<th>Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>350±6</td>
<td>490±5</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.5±0.7</td>
<td>6.3±0.5</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>22±5</td>
<td>315±30</td>
</tr>
<tr>
<td>Glucagon (ng/l)</td>
<td>196±25</td>
<td>243±40</td>
</tr>
<tr>
<td></td>
<td>7.5±0.8</td>
<td>8.2±0.9</td>
</tr>
<tr>
<td>Zucker Fatty</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucagon (ng/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>&lt; 0.001 &lt; 0.02</td>
</tr>
</tbody>
</table>

   ii) Glucose tolerance test:

      As shown in Fig. 3.1, fasting blood glucose levels were similar
Fig. 3.1  Glucose Tolerance Curves for Fatty and Lean Zucker Rats.

Glucose (3g/kg body weight) was given i.p. in 50% solution. Results are expressed as the Mean ± SEM. (●) Lean rats, (□) Fatty rats. n = 5 for each group of animals.
in the fatty and lean Zucker rats as in the previous experiment. Fig. 3.1.
also shows that the rate of disappearance of blood glucose following i.p.
glucose administration was similar in both groups of rats.

iii) Arginine tolerance test:

To evaluate the secretory status of the alpha cell in these animals,
the amino acid arginine was administered in very large doses (143,144).
As shown in Fig. 3.2, both lean and fatty rats demonstrated a steady rise
in plasma glucose concentration which continued up to the termination of
the experiment at 60 min. The fatty rats had significantly higher blood
levels of glucose at 60 min than their lean litter-mates.

Basal plasma glucagon levels were higher in the fatty rats (220±50 ng/l)
than in the lean animals (200±20 ng/l) but not significantly so. Arginine
stimulation caused higher rise in plasma glucagon levels in the fatty rats;
mean maximal levels were 1075±90 ng/l at 30 min in the fatty rats compared
to only 440±50 ng/l after 60 min in the lean ones.

Plasma insulin levels rose 13-fold after 30 min as a result of
arginine stimulation in the lean rats. This was followed by a drop,
after 60 min, to about a 9-fold increase above basal levels. The fatty
rats exhibited a less dramatic rise amounting to only a 1.5-fold increase
after 30 min compared to the basal state. The difference in the magnitude
of insulin response is probably due to the pre-existing elevated levels
of insulin in the fatty rats.
Fig. 3.2  Arginine Tolerance Test for Zucker Lean and Fatty Rats.

Effect of high-dose arginine administration (2g/kg body weight) on plasma glucose, insulin and glucagon concentration in (○) lean rats, (■) fatty rats. Each point represents mean ± SEM of 4 animals.

(*) p < 0.05, (**) p < 0.001
iv) Liver Glycogen:

Table 3.2 compares the liver glycogen content of lean and fatty Zucker rats. Liver glycogen was roughly 9-fold increased in the fatty rats as compared to their lean litter mates in the fasted states. After feeding the ratio was reduced to about two fold only. The difference in the liver glycogen content might explain the greater rise in blood glucose levels in the fatty rats compared with their lean litter mates after arginine stimulation (Fig. 3.2).

<table>
<thead>
<tr>
<th></th>
<th>Fasted</th>
<th>Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td>31 ± 7</td>
<td>239 ± 42</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>272 ± 55</td>
<td>416 ± 33</td>
</tr>
</tbody>
</table>

b) Cobalt-chloride Treated rats:

i) Plasma glucose and hormone concentrations

Table 3.3 shows that CoCl$_2$-treated animals had similar plasma glucose levels to their fasted and fed controls. Very similar results were also obtained for plasma insulin and glucagon levels in the two groups of animals: both in the fasted and fed states.

ii) Glucose tolerance test

Glucose tolerance curves are shown, in Fig. 3.3, for CoCl$_2$-treated animals and their controls. The CoCl$_2$-treated rats demonstrated mild but
Fig. 3.3 Glucose Tolerance Curves for Cobalt Chloride-Treated and Control Rats.

Glucose was given i.p. in 50% solution at 3g/kg body weight. Results are expressed as the Mean ± SEM. (○) Control rats, (■) CoCl$_2$-treated rats. n = 5 for each group of animals. p = ns for all points.
statistically insignificant intolerance to a glucose load compared to their controls.

iii) Arginine tolerance test

To examine pancreatic glucagon secretory potential, a high dose arginine stimulation test was performed on the CoCl$_2$-treated rats as for the Zucker rats above, (Fig. 3.4.).

Both groups of animals showed similar responses to arginine. Plasma glucose demonstrated a steady and similar rise in both groups of animals up to the termination of the experiment at 60 min. Mean plasma glucagon levels reached a peak of 170±19 ng/l in the control animals and 180±35 ng/l in the CoCl$_2$-treated ones (p=ns), 15-30 min after arginine administration. The slightly higher rise in mean plasma insulin levels observed in the CoCl$_2$-treated animals compared to their controls was not statistically significant.

Table 3.3 Average weights, plasma glucose and hormone concentrations in CoCl$_2$-treated and control rats in the fasted and fed states. Fasted assays were obtained after 18hr of food deprivation. Values are the mean ± SEM of 6 animals in each group.
Fig. 3.4  Arginine Tolerance Test for CoCl$_2$-treated and Control Rats.

Effect of high-dose arginine administration (2g/kg body weight) on plasma glucose, insulin and glucagon concentration in (●) Control rats, (■) CoCl$_2$-treated rats. Each point represents mean ± SEM of four animals. p = ns for all points.
iv) Glucose and fructose tracer studies

As shown from the above studies no differences in carbohydrate metabolism had been demonstrated between CoCl$_2$-treated rats and their controls. In order to further elucidate a possible role of carbohydrate metabolism in the hyperlipaemia of the CoCl$_2$-treated animals the labelled glucose and fructose tracer studies were performed. Results of the glucose tracer studies are shown in Fig. 3.5. Liver, adipose tissue, kidney and muscle showed a higher incorporation of the label in the CoCl$_2$-treated rats than in their controls. However, only in the case of the liver were the results obtained significantly different between the two groups of animals (p<0.05). In the CoCl$_2$-treated animals 2.25% of the total label administered was incorporated into the liver per g m$^{-1}$ wet weight as compared to only 1.65% in the controls. The bulk of the label taken up by the liver in both groups of animals was incorporated into glycogen. Of the label taken up by this organ 76% was incorporated into glycogen in the CoCl$_2$-treated animals and 54.5% in the controls (p<0.001). The control animals incorporated more of the label into liver lipids; the values being 5.5% and <2% respectively for the control and CoCl$_2$-treated animals (p<0.05). The remainder of the label taken up by the liver, i.e. 40% for the controls and 22% for the CoCl$_2$-treated rats, was used up in other routes.

The clearance from the plasma of the labelled glucose was similar in both groups of animals as shown in Fig. 3.5.

Fig. 3.6 shows the fate of labelled fructose in the CoCl$_2$-treated rats and their controls. The incorporation of this label was approximately
Fig. 3.5  Labelled Glucose Tracer Studies on Cobalt Chloride-Treated and Control Rats.

Rats were given 1g glucose + 25 μCi D-[U-^{14}C] glucose orally. Three hours later rats were sacrificed and tissues isolated. Results are expressed as the Mean ± SEM of 5 animals. (□) Control rats, (□□) CoCl₂-treated rats.

(*) p < 0.05
half that of the labelled glucose in the various tissues examined in both groups of rats. However, the overall picture remained similar as that for labelled glucose, with CoCl$_2$-treated rats incorporating more of the label than their controls except for the muscle tissue. The liver once again showed the higher incorporation of the fructose label where 0.97% of the total label was utilized per gram of liver in the CoCl$_2$-treated animals compared to 0.72% in the controls (p<0.05). Out of the total fructose label taken up by the liver 56% was incorporated into glycogen by the CoCl$_2$-treated rats and 47% by their controls (p<0.05), 1% was present in liver lipids in the CoCl$_2$-treated rats and 11% in their controls. The remainder, which was approximately the same in both groups of rats, i.e. 43% for the CoCl$_2$-treated animals and 42% for their controls, was presumably used up by other routes. Unlike the labelled glucose the clearance rate of labelled fructose from the blood was slower in the CoCl$_2$-treated rats than in their controls, judging by the plasma levels of labelled fructose after 3 hr (Fig. 3.6).

Table 3.4  Liver glycogen content after labelled glucose and fructose tracer studies and glucose loading test. Results are expressed as μmoles glucose/gm wet liver and are the mean + SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>After $^{13}$C-glucose</th>
<th>After $^{14}$C-fructose</th>
<th>After glucose loading test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ig glucose + label orally</td>
<td>Ig fructose + label orally</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>125 ± 12.0</td>
<td>134 ± 13</td>
<td>230 ± 28</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>179 ± 25</td>
<td>166 ± 13</td>
<td>292 ± 14</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.005</td>
</tr>
</tbody>
</table>
Fig. 3.6  Labelled Fructose Tracer Studies on Cobalt Chloride-Treated and Control Rats.

Rats were given 1 gm fructose + 25μCi D-[U-14C] fructose orally. Three hours later, rats were sacrificed and tissues isolated. Results are expressed as the Mean ± SEM of 5 animals. (□) Control rats, (■■) CoCl₂ -treated rats

(*) p < 0.05
Following the finding from the tracer studies that the $\text{CoCl}_2$-treated livers incorporate more of the tracer into glycogen than their normal controls, glycogen was estimated in the livers which had been kept at -20°C after the tracer studies. A glucose loading test (see Methods) was carried out. The results are shown in Table 3.4, from which it can be seen that in livers from all three tests $\text{CoCl}_2$-treated rats contained significantly more glycogen than their controls.

As a result of these findings it was decided to measure glycogen synthase activity in the two groups of rat livers after glucose and fructose tracer studies. Results are shown in Table 3.5.

From these results it can be seen that although there was a slight increase in the total enzyme activity in the $\text{CoCl}_2$-treated rats. This was not statistically significant. However, the active form of the enzyme did show a significant increase in $\text{CoCl}_2$-treated rats compared to their controls particularly in the glucose tracer studies.

Table 3.5 Glycogen synthase activity in the $\text{CoCl}_2$-treated and control rats

The enzyme activity is expressed as units per mg protein. A unit of enzyme activity catalysed the incorporation of 1 μmol of glucose from UDP-glucose into glycogen in 1 min. under the conditions of the assay. Values are the mean ± SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Livers from animals used in glucose tracer studies</th>
<th>Livers from animals used in fructose tracer studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total enzyme I &amp; D</td>
<td>Active enzyme I</td>
</tr>
<tr>
<td>Controls</td>
<td>10.1±1.9</td>
<td>1.1±0.23</td>
</tr>
<tr>
<td>$\text{CoCl}_2$-treated</td>
<td>11.3±1.7</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>$P$</td>
<td>ns</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
3.4 Discussion

The present studies confirm and extend preliminary observations which indicated that obesity in the Zucker fatty rat is associated with hyperinsulinaemia (90, 145). The high concentration of plasma insulin in fed fatty rats, and the persistence of relatively high concentrations of plasma insulin during fasting might result either from slow clearance of the hormone or from its excessive production. From previous study the latter possibility was thought more likely by the finding of a higher rate of release of insulin from isolated pancreatic islets (145), from Zucker fatty rats and a normal clearance of insulin in this strain of rats (90).

This study confirms previous reports (90) that obesity and hyperinsulinaemia present in the Zucker fatty rat are associated with normal glucose metabolism in both the fasted and fed states. A glucose tolerance test is also normal.

Plasma glucagon levels in the fatty rats were found to be significantly elevated compared to their lean littermates in the fed state as well as in response to arginine. These results are contradictory to previous reports in similar studies carried out by Eaton et al. (144). These workers reported that plasma glucagon levels in this strain of fatty rats were lower in fed animals than in their lean controls. Following fasting the reduction in plasma glucagon levels in the fatty rats was even greater compared to the lean controls. In response to arginine the same group have also reported a subnormal rise in glucagon levels in the fatty rats compared to their lean counterparts. My results, however, are more in agreement with plasma glucagon values reported by Laburthe et al. (98) who found increased glucagon values in the fatty animals compared to the lean.
Obese non-diabetic subjects have elevated basal and stimulated insulin levels. Obese diabetic subjects on the other hand have elevated basal insulin levels, but their stimulated insulin levels are less than these of weight-matched non-diabetics (146). According to this classification and from the results presented above, the Zucker fatty rats fall into the first category.

CoCl$_2$-treated rats as demonstrated by the present studies show no significant difference when compared to their controls in plasma glucose, insulin and glucagon levels either in the fasted or fed states. CoCl$_2$-treated rats also demonstrated a normal response to a glucose load compared to their controls. This finding is in agreement with previous report by Eaton (147).

An arginine tolerance test showed a normal response in glucagon secretion in the CoCl$_2$-treated rats compared to their controls. Eaton (103) has shown that glucagon secretion was markedly elevated after arginine infusion. He also showed a reduced plasma insulin response in the CoCl$_2$-treated rats. This was not demonstrated in the present studies (Fig. 3.4).

From the tracer studies and the glucose loading test it was shown that CoCl$_2$-treated rats incorporated more glucose and fructose into hepatic glycogen compared to their controls. Elevated hepatic glycogen in CoCl$_2$-treated rats on a normal daily diet have been reported by Eaton (147) and Alexander (148). The tracer studies showed decreased incorporation of the glucose and fructose label into liver lipid in the CoCl$_2$-treated rats compared to their controls. This is probably due to the higher incorporation of glucose and fructose by the CoCl$_2$-treated rats into glycogen than their controls.
Eaton (147) has reported low plasma insulin levels in the CoCl₂-treated rats following an oral glucose tolerance test. He attributed the normal glucose tolerance in spite of much lowered insulin levels (five-fold decrease) in the CoCl₂-treated rats to a higher insulin efficiency in terms of glucose utilization in these animals. This was supported by observations by Dixit and Lazarow (149), that cobalt potentiated and stimulated insulin action on adipose tissue. The present study indicates that the probable reason for the higher glycogen levels shown by CoCl₂-treated rats is the significant increase in the activity of hepatic glycogen synthase. The other possibility which can contribute to this finding is decreased glycogenolysis. However, Dingle et al., (150) who examined the effect of cobalt on glycogenolysis in the rat, reported that cobalt caused no inhibition of glycogenolysis in CoCl₂-treated rats.
CHAPTER FOUR

LIPID METABOLISM IN THE ZUCKER AND CoCl₂-TREATED RATS
Chapter 4

4.1 Introduction

The progressive development of obesity in the Zucker fatty rat must result from the deposition as body fat of calories consumed in excess of expenditure. The question thus arises as to whether the Zucker fatty rat as compared to its lean mate, has an increased capacity to synthesize fat in the form of endogenous glycerides from fatty acids, monosaccharides and amino acids and to secrete those glycerides produced in the liver into the plasma as lipoproteins. To achieve this, activities of plasma triglyceride clearing factors as well as adipose tissue triglyceride depositing processes will have to be greatly increased. If the Zucker fatty rat could be shown to have such increased capacities, this finding would then point to one inherent mechanism which might result in a greater proportion of the dietary intake being deposited in the adipose tissue.

The CoCl$_2$-treated rat on the other hand, as has been discussed in Chapter 1, exhibits a hypertriglyceridaemic syndrome without the development of obesity. The concentration of plasma triglycerides in this animal as well as other cases of hypertriglyceridaemia, might theoretically be governed by changes in the rate of triglyceride production, by the rate of triglyceride removal, or both. The kinetic behaviour of plasma triglycerides in the Zucker fatty and CoCl$_2$-treated rats has not, so far, been investigated.

In adipose tissue the processes of uptake of circulating triglycerides and its intracellular release requires the hydrolysis of the triglyceride molecule. These hydrolytic reactions are mediated by two distinct lipase systems (151, 152). The mobilization of adipose tissue triglyceride as glycerol and fatty acids requires the action of a hormone-sensitive lipase.
that is activated by glucagon, catecholamines and a variety of other hormones. Glucagon is believed to exert its lypolytic action via the activation of this enzyme (153, 154, 155), the mechanism of which has been discussed in Chapter 1. The second adipose tissue lipase, lipoprotein lipase, regulates the rate of uptake of plasma triglycerides by adipose tissue because of its action in catalysing the hydrolysis of circulating chylomicrons and lipoprotein triglyceride that must occur prior to the uptake of fatty acid components (156, 157, 158).

Lipoprotein lipase is released into the plasma by heparin. The lipolytic activity of post-heparin plasma against different triglyceride substrates was widely used as a measure of tissue lipoprotein lipase activity until it became evident that heparin is able to liberate several lipases from tissues. A triglyceride lipase released from the liver contributes significantly to the post-heparin plasma lipoprotein lipase activity (PHPLA) (159, 160, 161). The hepatic lipase is resistant against high concentrations of sodium chloride and protamine sulphate (155, 159, 160). Lipoprotein lipase activity is inhibited by catecholamines and enhanced by insulin (155, 156).

Studies in this Chapter were designed to investigate the activities of the above two enzymes in the Zucker and CoCl₂-treated rats and their possible role in the pathogenesis of the hyperlipidaemia in these two animals.

4.2. Methodology

a) Plasma, liver and total body lipids in the Zucker and CoCl₂-treated animals

i) Plasma lipid: Fourteen week-old male Zucker fatty and lean animals were fasted for 18hr. Nembutal was given (6 mg/100 g body weight),
blood was collected via cardiac puncture and pipetted into heparinised tubes. After centrifugation, plasma cholesterol, free fatty acids (FFA) and triglycerides were estimated as explained in detail in Chapter 1.

ii) Liver lipid: Livers were immediately removed after cervical dislocation, washed in cold saline and stored at -20°C. Lipids were extracted and estimated within 6 hrs. Lipids were extracted in methanol-chloroform as in Chapter 3.

iii) Total body lipids: Total body lipids were estimated as follows:- Fourteen week-old male Zucker fatty and lean rats were used. After weighing the animals were sacrificed by cervical dislocation. The intestinal contents were removed. The animals were dried in a vacuum oven at 70°C for 48 hr. They were then weighed and minced 3 times through a meat and bone grinder. The material was bottled and stored at -20°C pending analysis. A portion (3g) of each animal was analysed for lipid content by extraction with methanol-chloroform method as described above. An aliquot of the chloroform phase was evaporated to dryness and weighed (total body fat). Total body triglycerides, FFA and cholesterol were also determined on the chloroform phase.

The same procedure was repeated in CoCl₂-treated and control rats.

b) Hormone-Sensitive Lipase

Enzyme extract from fat tissue was prepared according to the method of Huttenen and Steinberg (164) and summarized as follows:

Rats epididymal fat pads were incubated in KRP buffer pH 7.4 containing 4% BSA (1.5g of fat/4 ml buffer) in small flasks for 1 hr. The fat pads were then homogenised in 10 vol. of ice-cold 0.25M sucrose containing 1 mM EGTA. The homogenate was then centrifuged for 60 min. at 30,000 r.p.m. The clear fat free supernatant was used as a source of hormone-sensitive lipase. The protein content was determined.
Twelve week-old male Zucker fatty and lean rats were examined for triglyceride lipase activity. The livers of these animals were also homogenized and subcellular fractions prepared using a standard differential centrifugation technique as described under Methods (Chapter 2).

Hormone-sensitive lipase activity in epididymal fat pads was also investigated for the CoCl_2-treated and control rats.

The enzymatic assay was performed according to the method of Huttenen and Steinberg (164):

The assays were performed in 13 ml glass-stoppered centrifuge tubes in a volume of 0.2 ml containing 0.8 μmoles triolein (Sigma, 99% pure), 4 mg BSA, 15 μmoles NaCl and 10 μmoles sodium phosphate buffer pH 7.0. After sonication using a Biosonik microprobe sonicator for 2 min, the reaction was started by the addition of 1-2 mg enzyme protein extract. Incubation was at 30°C for 1 hr. The reaction was stopped by the addition of buffered cupric solution (from the Boehringer kit method) followed by chloroform. Reagent blanks were added containing all of the above reagents but with no incubation. FFA was then estimated. Blank values were subtracted from test results.

c) Lipoprotein Lipase (EC3.1.1.3)

Lipoprotein lipase was determined on plasma before and after heparin administration (blood was collected in EDTA tubes) as well as on epididymal adipose tissue extracts. Adipose tissue extracts were prepared by the method of Schonfeld et al (1965) which can be summarized as follows: 5g epididymal fat was removed and homogenized in a Polytron homogenizer (set at 2) in 10 vol. of n-butanol. The homogenate was filtered under suction and the residue washed with 20-30 vol. of petroleum ether. Powders were dried in a vacuum desiccator and stored at room temperature. The tissue powders were extracted twice with 5mM Tris-HCl buffer pH 7.4
at 4°C for 15 min. and the extracts were combined. The solution was then centrifuged at 4°C for 10 min at 2000 x g. The supernatant was used for lipoprotein lipase determination immediately after protein estimation.

Lipoprotein lipase was determined by the modified method of Scholtz et al. (166). A mixture of 0.15g triolein (Sigma grade, 99% pure), 0.9ml of 1% BSA, 0.9 ml of 1% Triton X-100, 7.2 ml of 0.2M Tris-HCl buffer pH 8.0 and 3 ml of overnight fasted rabbit serum, was made and sonicated with a Biosonik microprobe sonicator for 4 min. The incubation medium contained:

0.4 ml of the above mixture
0.1 ml of plasma or adipose tissue extract (3-5 mg protein).

The mixture was incubated at 37°C for 20-30 min and the reaction was stopped as for hormone-sensitive lipase. FFA was determined at the beginning and end of incubation. Blanks were also set up and were subtracted from test values.

Blood for lipoprotein lipase activity measurement was collected in EDTA. Statistical analysis was carried out using the Mann-Whitney U-test (169).

4.3 Results
a) Zucker Rats:
(i) Plasma, liver and total body lipids

Table 4.1 shows fasting lipid levels of the Zucker lean and fatty rats. It is clearly demonstrated that the fatty rats are hypercholesterolaemic, with an increase of slightly under two-fold in their plasma cholesterol levels. This hypercholesterolaemia is accompanied by gross hypertriglyceridemia where values for plasma triglycerides show about 4-fold increase in the fatty compared to the lean rats. There was no significant difference in the plasma FFA levels between the two groups.
### Table 4.1 Plasma lipid levels of 14 week-old male Zucker fatty and lean rats. Animals were fasted for 18hr, anaesthetized and blood was collected in heparinized tubes via heart puncture. Results are the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>FFA mmol/l</th>
<th>Cholesterol mmol/l</th>
<th>Triglycerides mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker Lean</td>
<td>0.4 ± 0.05</td>
<td>1.7 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Zucker Fatty</td>
<td>0.5 ± 0.04</td>
<td>3.7 ± 0.4</td>
<td>4.8 ± 1.1</td>
</tr>
</tbody>
</table>

### Table 4.2 Liver weight and lipid content of 14 week-old male Zucker fatty and lean rats. Total lipid was estimated on the chloroform phase of methanol-chloroform extract of homogenized livers. Results are the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>g liver/100 g body weight</th>
<th>% Liver lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker Lean</td>
<td>220 ± 5</td>
<td>12.4 ± 0.5</td>
<td>5.6 ± 0.4</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>Zucker Fatty</td>
<td>410 ± 4</td>
<td>22 ± 1.9</td>
<td>5.1 ± 0.4</td>
<td>9.0 ± 0.7</td>
</tr>
</tbody>
</table>

### Table 4.3 Total body lipid of 14 week-old male Zucker lean and fatty rats. Lipid was estimated on the chloroform phase of the methanol-chloroform extract of dried and minced rats. Results are the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Whole body lipid (g)</th>
<th>% Body lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker Lean</td>
<td>230 ± 7</td>
<td>33.4 ± 5</td>
<td>14.5%</td>
</tr>
<tr>
<td>Zucker Fatty</td>
<td>410 ± 6</td>
<td>173 ± 1.5</td>
<td>42.2%</td>
</tr>
</tbody>
</table>
Table 4.2 shows liver weight and its lipid content of the Zucker fatty and lean rats. Although the liver weight was greater in the fatty than the lean rats when expressed on a weight to weight basis the situation is reversed when results are expressed in terms of liver weight/100g body weight. The liver lipid content showed over a two-fold increase in the fatty rats compared to their lean controls.

Table 4.3 shows total body lipid results of the Zucker lean and fatty animals. Body lipids were approximately three times as great in the fatty as in the lean rats.

ii) Hormone-Sensitive Lipase

Fig. 4.1 shows basal and stimulated levels of hormone-sensitive lipase in the epididymal tissue of Zucker lean and fatty rats. Basal activity of this enzyme is nearly halved in the fatty rats compared to the lean ones. To check for the presence of any inhibitor of this enzyme in the fatty rat or an activator in the lean rat adipose tissue, an equal amount of enzyme protein from lean and fatty rats adipose tissue extracts were mixed and the activity was determined. As can be seen from the results no such inhibitor or activator was found. A value of 3.4 mmol/min/mg protein was obtained for the mixture. This represents the mean of the activities in the adipose tissue of the two independent groups.

Upon stimulation with glucagon the activity of the hormone-sensitive lipase in the adipose tissue of the lean group increased by 51%, while a higher increase of 100% of basal activity was demonstrated in the fatty rats. Insulin demonstrated its antilipolytic activity by lowering the levels of hormone-sensitive lipase activity in the lean rats by 24% of basal activity. However, it had no such influence in the fatty group. When these two hormones were combined and added in equal amounts to the
Fig. 4.1 Basal and stimulated levels of hormone-sensitive lipase in epididymal adipose tissue of 12 week old male Zucker lean and fatty rats.

For basal activity determination, fat pads were incubated for 1 hr in KRP buffer pH 7.5 containing 4% BSA, homogenized and centrifuged. The enzyme activity was then determined on the fat-free supernatant. In the hormone-activated experiments, incubation for 1 hr in KRP buffer pH 7.5 containing 4% BSA was followed by further 15 min incubation after the hormone was added (5μg/ml reaction mixture). Fat pads were then treated as above. Results are expressed as the mean ± SEM of 4 animals in each group. (□) lean rats, (△) fatty rats and (×) lean + fatty rats.
reaction mixture the lipolytic activity was increased by 50% in the lean group and by 80% in the fatty group.

Triglyceride lipase distribution and activity in the Zucker lean and fatty rats are shown in Fig. 4.2. The total activity in the crude liver homogenates was lower in the fatty rats than the lean ones. The lean rats had an activity of 22 ± 3, while the fatty rats had only 13 ± 4 nmol FFA/min/mg protein. This decrease was also reflected in all subcellular fractions of hepatocytes in the fatty compared to the lean rats, particularly in the microsomal and soluble fractions. The lysosomal fraction had the highest activity in both groups of rats.

The term hormone-sensitive lipase was not used for the liver enzyme because it did not show any stimulation by glucagon or adrenaline (results not shown). A more general term "triglyceride lipase" was used instead.

iii) Lipoprotein lipase.

Plasma post-heparin lipoprotein lipase activity in the Zucker lean and fatty rats are shown in Table 4.4. Zucker fatty rats showed more than a 4-fold increase in total activity compared to the lean. 45% of this activity remained after the addition of protamine sulphate in both fatty and lean rats. This shows that approximately half of the enzyme activity present in the plasma after heparin is of hepatic origin. Sodium chloride inhibited 50% of the basal activity in the lean animals and 66% in the fatty rats. This indicates that PHLA of extrahepatic origin is less resistant to high concentrations of sodium chloride than to protamine sulphate.

Table 4.5 shows adipose tissue lipoprotein lipase activity in the Zucker lean and fatty rats. Fatty rats showed about two-fold increase
Fig. 4.2  Triglyceride lipase distribution and activity in the liver of 12 week old Zucker lean and fatty rats. Livers were homogenized in 10 vol. 0.25M sucrose + 1 mM EGTA. Subcellular fractions were collected using a standard differential centrifugation technique. The enzyme activity was then determined on each fraction. Results are the mean ± SEM of 3 rats in each group.
(☐) lean rats, and (☑) fatty rats.
Table 4.4  Plasma-post-heparin lipoprotein lipase activity in 14 weeks male Zucker lean and fatty rats. Animals were anaesthetized with Nembutal after 18hr fast. Heparin was given through the heart in a dose of 200 IU/kg body weight. Ten minutes later blood was collected in EDTA tubes. Lipoprotein lipase activity was determined. Activity is reported as nmol. FFA released/ml plasma/min. Results are the mean ± SEM of 6 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>+ 2 mg/ml Protamine Sulphate</th>
<th>+ 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td>178 ± 14</td>
<td>79 ± 12</td>
<td>89 ± 14</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>757 ± 50</td>
<td>344 ± 23</td>
<td>502 ± 45</td>
</tr>
</tbody>
</table>

p for all results <0.001

Table 4.5  Adipose tissue lipoprotein lipase activity in 14 weeks old male Zucker lean and fatty rats. N-Butanol extracts were prepared from epididymal fat pads. After extraction in 5 mM Tris-HCl buffer lipoprotein lipase activity was determined. Activity is reported as nmol FFA released/min/mg protein. Results are the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>+ 2 mg/ml Protamine Sulphate</th>
<th>+ 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td>1.16 ± 0.006</td>
<td>0.27 ± 0.06</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>2.1 ± 0.07</td>
<td>0.33 ± 0.04</td>
<td>0.5 ± 0.03</td>
</tr>
</tbody>
</table>

p for all results <0.05
in lipoprotein lipase activity. 23% of this activity remained after the addition of protamine sulphate in the lean rats and 15% in the fatty rats. Upon addition of 0.5M NaCl 36% in the lean and 23% in the fatty rats of adipose tissue lipoprotein lipase activity remained.

b) CoCl₂-Treated Rats

Table 4.6 Plasma lipid levels of CoCl₂-treated and control rats. Animals were fasted for 18 hr, anaesthetized and blood was collected via cardiac puncture. Results are the mean ± SEM of 6 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>FFA mmol/l</th>
<th>Cholesterol mmol/l</th>
<th>Triglycerides mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.56 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>CoCl₂-treated</td>
<td>0.55 ± 0.1</td>
<td>1.8 ± 0.25</td>
<td>1.1 ± 0.07</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

i) Plasma, liver and body lipids

Table 4.6 shows the plasma lipid levels of CoCl₂-treated and control rats. Plasma FFA and cholesterol showed similar values in both groups of animals. However, the plasma triglyceride values were doubled in CoCl₂-treated rats compared to their controls (p < 0.001).

Liver weights and lipid content values of CoCl₂-treated and control rats are shown in Table 4.7. There were no significant changes in liver weights, total liver lipids, liver cholesterol, and liver FFA in the two groups of animals. However, there was a significant difference in liver
Table 4.7  Liver weight and lipid content of CoCl$_2$-treated and control rats. Animals were fasted for 18hr. Lipids were estimated on the chloroform phase of the homogenized livers methanol-chloroform extract. Results are the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>Total liver lipid (mg/g)</th>
<th>Total cholesterol (mmol/g)</th>
<th>Liver FFA (mmol/g)</th>
<th>Liver triglycerides (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>268±4</td>
<td>12.1±1</td>
<td>20.0±3</td>
<td>0.022±0.002</td>
<td>0.09±0.01</td>
<td>0.036±0.002</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>251±5</td>
<td>12.8±0.8</td>
<td>26.6±4</td>
<td>0.025±0.003</td>
<td>0.11±0.02</td>
<td>0.056±0.003</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 4.8  Body Lipids of CoCl$_2$-treated and control rats. Animals were fasted for 18hr. Lipids were estimated on the chloroform phase of the methanol-chloroform extract of dried and minced rats. Results are the mean ± SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Body wt (g)</th>
<th>Total body lipid (g)</th>
<th>Total body cholesterol (g)</th>
<th>Total body FFA (g)</th>
<th>Total body triglycerides (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>230±5</td>
<td>20.3±1.2</td>
<td>0.11±0.015</td>
<td>1.9±0.15</td>
<td>18±1.2</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>225±6</td>
<td>15.6±1.3</td>
<td>0.10±0.010</td>
<td>1.8±0.12</td>
<td>14±1.0</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>&lt; 0.01</td>
<td>ns</td>
<td>ns</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
triglycerides between the CoCl$_2$-treated and the control animals. Liver triglycerides in the CoCl$_2$-treated group showed a significant elevation of 55% compared to the lean p < 0.01.

Values for the total body lipid in CoCl$_2$-treated and control animals are shown in Table 4.8. Total body lipid in the CoCl$_2$-treated animals is lowered by 23% compared to their controls. This is accompanied by a 22% decrease in the total body triglycerides (p < 0.001), in the CoCl$_2$-treated rats compared to their controls. However, total body cholesterol and FFA were shown to be the same in the two groups of rats. There was no significant difference in the body weights between the CoCl$_2$-treated and control animals.

i') Hormone-sensitive lipase

Table 4.9 Basal and stimulated levels of hormone-sensitive lipase in CoCl$_2$-treated and control rat adipose tissue. Epididymal adipose tissue was prepared and treated as for the Zucker Rats (please see Legend to Fig. 4.4). Results are expressed as the mean ± SEM of 4 animals in each group. Activities are expressed as nmol FFA released/min/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>+ Glucagon (5µg/ml)</th>
<th>+ Insulin (5µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5 ± 0.5</td>
<td>6.0 ± 0.7</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>4.0 ± 0.3</td>
<td>5.6 ± 0.4</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 4.9 shows basal and stimulated levels of hormone-sensitive lipase in the adipose tissue of CoCl$_2$-treated and control rats. Basal activity and hormone activated levels showed no significant difference between the two groups of rats.

Lipoprotein lipase

Table 4.10 shows the plasma post-heparin lipoprotein lipase activity. CoCl$_2$-treated rats showed a significant decrease in this activity compared to their controls. 32% of this activity remained after the addition of protamine sulphate in the control and the CoCl$_2$-treated rats.

Table 4.10 Plasma post-heparin lipoprotein lipase activity in CoCl$_2$-treated and control rats. After anaesthesia following 18hr fast, heparin was given through the heart in a dose of 200 IU/kg body weight. Ten minutes later blood was collected in EDTA tubes. Lipoprotein lipase activity was determined. Activity is expressed in nmol FFA released / ml plasma/min. Results are the mean ± SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>+ 2 mg/ml Protamine Sulphate</th>
<th>+ 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>250 ± 21</td>
<td>80 ± 12</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>190 ± 20</td>
<td>60 ± 10</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.01</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 4.11 Adipose tissue lipoprotein lipase activity in CoCl$_2$-treated and control rats. n-Butanol extracts were prepared from epididymal fat pads. After extraction in 5mM Tris-HCl buffer, lipoprotein lipase activity was determined. Activity is reported as nmol FFA released/min/mg protein. Results are the mean + SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>+ 2 mg/ml Protamine Sulphate</th>
<th>+ 0.5M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.1 ± 0.16</td>
<td>0.74 ± 0.1</td>
<td>0.70 ± 0.2</td>
</tr>
<tr>
<td>CoCl$_2$-treated</td>
<td>2.5 ± 0.12</td>
<td>0.70 ± 0.2</td>
<td>0.68 ± 0.2</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.01</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Similar values (18% of plasma post-heparin lipoprotein lipase activity remained after the addition of 0.5M NaCl in both groups of rats). From the protamine sulphate results nearly one-third of the total activity of this enzyme system is contributed by the liver. This value was decreased as judged by results following NaCl inhibition. Approximately one-fifth was the contribution of the liver to this enzyme system from the NaCl results. However, the difference after inhibition by either protamine sulphate or NaCl was found to be statistically insignificant between the two groups of animals. This indicates that lipoprotein lipase of adipose tissue origin is decreased in the CoCl$_2$-treated rats compared to their controls.

Table 4.11 shows the activity of adipose tissue lipoprotein lipase activity in CoCl$_2$-treated and control rats. These results show that the activity of this enzyme is significantly decreased ($p < 0.01$) in the CoCl$_2$-treated rats compared to their controls.
4.4 Discussion:

The present series of studies indicate that the Zucker fatty rat is a model of marked hypercholesterolaemia and hypertriglyceridaemia associated with gross obesity.

The hormone-sensitive lipase is nearly halved in the fatty rats compared to the lean. The insulin resistance state of this strain of rat is further demonstrated by the lack of response of this enzyme to the action of exogenous insulin (Fig. 4.1). There appears to be a reciprocal relationship between the activities of hormone-sensitive lipase and lipoprotein lipase as demonstrated by the present study. This is in agreement with previous published studies on rat adipocyte (180).

Liver triglyceride lipase is significantly reduced in total liver homogenate as well as all liver subcellular fractions examined (Fig. 4.2) in the fatty rat compared to the lean. Of special interest is the microsomal and soluble fractions. The triglyceride lipase of these two fractions are highly reduced in the fatty rat compared with the lean. It has been shown that the triglyceride lipase activity of these two fractions are both inhibited by NaCl and protamine sulphate (181).

It is possible that the liver contribution of PHLP activity is of two types 1) NaCl and protamine sulphate sensitive (similar to extrahepatic PHLP) and contributed by the microsomal and soluble fractions; 2) NaCl and protamine sulphate resistant and contributed by the lysosomal fraction as the mitochondrial fraction has been shown to possess no triglyceride lipase activity (182). The mitochondrial activity of this enzyme shown in Fig. 4.2 is probably due to the contamination of this fraction by other subcellular fractions (182).
As has previously been shown the lipoprotein lipase in the fatty rats plasma is highly elevated compared to the lean. This increased activity is mobilised probably partly from the liver microsomal and soluble fractions and partly from the extrahepatic tissue. This increase is most probably due to the constant hypertriglyceridaemic state present in the fatty rats. This depletes the liver lipoprotein lipase reserve and thus the low activity of this enzyme in the microsomal and soluble fractions.

The CoCl$_2$-treated rat is an animal model of induced hypertriglyceridaemia. The hypertriglyceridaemia may be governed by the rate of triglyceride production, by the rate of triglyceride removal, or both. The present series of studies indicates that the hypertriglyceridaemia of this rat is due to a reduction in the triglyceride removal rate. This is shown by a) reduced post-heparin lipoprotein lipase activity, b) reduced adipose tissue lipoprotein lipase activity.

As a direct result of lower activities of lipoprotein lipase which regulates the rate of uptake of plasma triglycerides by adipose tissue, total body lipid as well as total body triglycerides are significantly reduced in the CoCl$_2$-treated rat (Table 4.8).
CHAPTER FIVE

A LONG-TERM STUDY OF PLASMA HORMONE CONCENTRATIONS
AND COBALT DISTRIBUTION IN THE CoCl₂-TREATED RATS
5.1 Introduction

Previous studies on the effect of cobalt chloride on experimental animals were of short duration ranging from a few hours to a few days. This did not allow enough time to assess the long-term effect of cobalt chloride treatment on these animals, nor the correlation between cobalt chloride levels and the duration of hyperlipaemia and polycythemia.

In the last chapter it was shown that cobalt chloride treatment of the rat resulted in an increase in liver triglyceride turnover rate coupled with a decrease in the clearance of plasma triglycerides.

In this chapter a long-term study of the effect of cobalt chloride treatment on the rat was carried out. The following studies were performed after 1, 4, 12 and 15 months of cobalt chloride treatment on the rat:

1) Insulin and glucagon plasma levels,
2) Cobalt levels in different tissues and the intracellular distribution in the hepatocyte,
3) Haemoglobin and packed cell volume (PCV),
4) Plasma lipid levels,
5) The in vitro effect of cobaltous ion on plasma lipoprotein lipase activity compared to other divalent cations (Fe^{++} and Zn^{++}) in Wistar Albino rats.

5.2 Methodology

For the present long-term study 50 Wistar Albino rats were treated with CoCl$_2$ as described in Chapter 2. Treated rats and their controls were housed (10 rats to a cage) and fed ad libitum until time of experiment. Rats were used for the present experiments after 1, 4, 12, and 15 months of last CoCl$_2$ injection.
CoCl₂-treated rats and their controls were fasted for 18hrs. Following anaesthesia with Nembutal (6 mg/100g body weight) blood was withdrawn via cardiac puncture. Blood for hormone levels was collected into heparinized tubes containing Trasylol (2,000 KIU per ml of blood). Blood for haematological studies was collected into EDTA tubes. Plasma was used for lipid estimations after blood was collected in heparin tubes.

Haemoglobin was estimated by the cyanmethaemoglobin method (172).

Different tissues were isolated and their cobalt content was estimated. Liver subcellular fractions were prepared by differential centrifugation technique, as previously described, and their Cobalt content was measured.

The effect of Co⁺⁺, Fe⁺⁺ and Zn⁺⁺ on post-heparin lipoprotein lipase activity was measured in plasma collected from Wistar Albino rats following heparin injection as described in the previous chapter.

Statistical analysis was carried out using the Mann-Whitney U-test (169).

5.3 Results

i) Plasma insulin and glucagon

Table 5.1 Plasma insulin levels of CoCl₂-treated and control rats after ½, 4, 12 and 15 months of last CoCl₂ injection in mU/l. Results are the mean ± SEM of 4 animals in each group

<table>
<thead>
<tr>
<th></th>
<th>½ month</th>
<th>4 months</th>
<th>12 months</th>
<th>15 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26 ± 5</td>
<td>37 ± 6</td>
<td>40 ± 10</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>23 ± 4</td>
<td>39 ± 5</td>
<td>42 ± 10</td>
<td>58 ± 10</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns
Table 5.1 shows plasma insulin levels in CoCl₂-treated and control rats. No statistically significant difference was seen between the two groups of rats after ¼, 4, 12 and 15 months of last CoCl₂ treatment in the plasma insulin levels. However, there was an increase in the absolute levels with increasing age in both groups of rats in the plasma insulin.

Table 5.2

<table>
<thead>
<tr>
<th></th>
<th>¼ month</th>
<th>4 months</th>
<th>12 months</th>
<th>15 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 ± 5</td>
<td>40 ± 6</td>
<td>50 ± 10</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>CoCl₂-treated</td>
<td>27 ± 4</td>
<td>43 ± 7</td>
<td>47 ± 9</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 5.2 shows the plasma glucagon levels of CoCl₂-treated and control rats. As for insulin glucagon showed no statistically significant difference in both groups of rats after the four periods examined of CoCl₂ treatment. Once again as for insulin glucagon seemed to increase with increase in age in the CoCl₂-treated and control rats.

ii) Haemoglobin PCV

Table 5.3

<table>
<thead>
<tr>
<th></th>
<th>¼ month</th>
<th>4 months</th>
<th>12 months</th>
<th>15 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>13.2</td>
<td>12.7</td>
<td>12.7</td>
<td>12.2</td>
</tr>
<tr>
<td>PCV</td>
<td>41±1</td>
<td>35±1</td>
<td>35±1</td>
<td>34±1.1</td>
</tr>
<tr>
<td></td>
<td>+0.8</td>
<td>±0.5</td>
<td>±0.6</td>
<td>±0.9</td>
</tr>
<tr>
<td>CoCl₂-treated</td>
<td>17.1</td>
<td>12.5</td>
<td>12.7</td>
<td>12±0.5</td>
</tr>
<tr>
<td>treated</td>
<td>+0.9</td>
<td>+1.2</td>
<td>+0.6</td>
<td>+0.8</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Haemoglobin is expressed as g/100 ml blood and PCV as % of whole blood.
Table 5.3 show blood haemoglobin and PCV values for CoCl\textsubscript{2}-treated and control rats after $\frac{1}{2}$, 4, 12 and 15 months of last CoCl\textsubscript{2}-injection. As shown in this table, Hb and PCV levels were significantly increased after 14 days of cobalt chloride treatment. However, the values returned to normal levels after 4 months of last CoCl\textsubscript{2} injection and remained so until the last experiment i.e. after 15 months of CoCl\textsubscript{2} treatment.

iii) Plasma lipids

Fig. 5.1 shows plasma FFA, cholesterol and triglyceride levels in CoCl\textsubscript{2}-treated and control rats. There was no significant difference between the two groups in either FFA or cholesterol levels after $\frac{1}{2}$, 4, 12 and 15 months of last CoCl\textsubscript{2} injection. However, values for both FFA and cholesterol increased with age in both groups. Plasma triglyceride levels showed a significant increase in the CoCl\textsubscript{2}-treated rats compared to their respective controls after $\frac{1}{2}$, 4 and 12 months of last CoCl\textsubscript{2} injection. The difference in the increment decreased with time. After 15 months of last CoCl\textsubscript{2} treatment this increment had no statistical significance.

iv) Cobalt tissue distribution

Table 5.4 shows cobalt concentration of different tissues of CoCl\textsubscript{2}-treated rats and controls. The liver appeared to have the highest concentration of this metal followed by the kidney, pancreas, brain and muscle in that order after 15 days of last CoCl\textsubscript{2} treatment. After 4 months of last treatment the kidney appeared to have the highest concentration of cobalt followed by the liver. This may give an indication of the cobalt excretion route. However, after 12 months of last treatment the liver was the site for the highest cobalt concentration followed by the pancreas then the kidney. After 15 months of last treatment the cobalt
Fig. 5.1  Plasma FFA, cholesterol and triglycerides in CoCl₂-treated (■) and control rats (□), after 1/2, 4, 12 and 15 months of last CoCl₂ injection. The results are the mean ± SEM of 5 animals in each group.

** = <0.001
<table>
<thead>
<tr>
<th>Time (months)</th>
<th>Liver (µg/g)</th>
<th>Kidney (µg/g)</th>
<th>Pancreas (µg/g)</th>
<th>Brain (µg/g)</th>
<th>Muscle (µg/g)</th>
<th>Plasma per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 month</td>
<td>111</td>
<td>43</td>
<td>17</td>
<td>5</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>4 months</td>
<td>0.13</td>
<td>0.22</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
<td>ND</td>
</tr>
<tr>
<td>12 months</td>
<td>0.08</td>
<td>0.04</td>
<td>0.05</td>
<td>0.015</td>
<td>0.02</td>
<td>ND</td>
</tr>
<tr>
<td>15 months</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Controls</td>
<td>0.015</td>
<td>0.005</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 5.4 Cobalt concentration in µg/g tissue of CoCl₂-treated rats after time shown of last CoCl₂ injection and their controls. Results are the means of 3 rats. ND - non-detected
concentration remained the same in the liver and pancreas. After 15 months of last treatment brain and muscle appeared to be free of this metal. However, liver, kidney and pancreas after the same period had higher concentrations of cobalt compared with the control rats. Of special interest is the comparatively high concentration of this metal in the pancreas of the treated rats even after 15 months of last treatment.

It is possible that there is some correlation between hepatic cobalt concentrations and plasma triglyceride levels, but this could not be established in the present series of experiments. Plasma triglyceride levels reached a maximum level of $1.2 \pm 0.2$ mmol/l after 15 days of last CoCl$_2$ injection when cobalt concentrations were at a maximum level of 111 $\mu$g/g liver. After a 4 months laps plasma triglycerides had dropped from $1.2 \pm 0.2$ to $0.88 \pm 0.1$ and cobalt concentration in the liver had dropped to $0.13 \mu$g/g liver. After 12 months there was a further drop in the plasma triglycerides to $0.8 \pm 0.1$ mmol/l which was accompanied by a further drop in hepatic cobalt concentration to $0.08 \mu$g/g liver. After 15 months nearly all the cobalt had been cleared from the liver. This was accompanied by a return to "normal" values of plasma triglycerides concentration in the CoCl$_2$-treated rats as compared to their controls.

Fig. 5.2 shows the intracellular distribution of cobalt in the liver. As shown this cation seemed to be distributed in all subcellular fractions examined. The mitochondrial fraction contained the highest level of cobalt followed by the nuclear and microsomal fractions. The lysosomal and cytosol fractions appeared to contain the lowest levels of this metal.

v) Heavy metal ions effect on lipoprotein lipase activity

To evaluate the in vitro effect of cobalt and other metal ions on the activity of lipoprotein lipase, post-heparin plasma was incubated with
Fig. 5.2 Intracellular distribution of Cobalt in the Liver

Subcellular fractions were prepared by differential centrifugation technique and cobalt was estimated by atomic absorption method. Results are the mean ± SEM of 4 rats after 15 days of last CoCl₂ injection.
CoCl$_2$, FeCl$_2$ and ZnCl$_2$ and lipoprotein lipase activity was determined. Results are shown in Fig. 5.3. As shown, all three metals had some effect on post-heparin lipoprotein lipase activity. They all reduced this activity by varying degrees. Zn$^{++}$ had the highest effect in reducing lipoprotein lipase activity followed by Co$^{++}$ and Fe$^{++}$.

5.4 Discussion

Studies carried out in this chapter demonstrate no significant difference in either insulin or glucagon levels in the CoCl$_2$-treated rats compared to their controls, following CoCl$_2$ treatment after periods examined (Table 5.1 and 5.2). However, there is an increase in the level of both hormones with increase in age.

Cobalt is taken up by all tissues examined with the highest levels being taken up by the liver. Intracellular distribution of the cobalt ion in the liver show that this metal is distributed throughout the subcellular fractions examined. Highest levels are seen in the mitochondrial fraction.

The two main features of cobalt treatment are polycythemia and hypertriglyceridaemia. The polycythemia appears to be of shorter duration, lasting probably up to 2 months following chronic CoCl$_2$ treatment. The hypertriglyceridaemia on the other hand is of much longer duration, lasting up to one year. There seems to be some correlation between the duration of hypertriglyceridaemia and the presence of cobalt ion particularly in the liver.

In vitro cobalt as well as iron and zinc can inhibit lipoprotein lipase activity. This suggest non-competitive inhibition, the type generally recognised with -SH group-containing enzymes. However, this inhibition can only be detected at high metal concentration in the enzyme incubation mixture. This will be further discussed in conjunction with results from previous studies in this thesis in Chapter 8.
Fig. 5.3  Effect of Heavy Metal Ions on Post-Heparin Lipoprotein Lipase Activity.

Post-heparin plasma was obtained from 3 W/A rats after 200 IU heparin/kg bodyweight. Plasma were pooled and incubated at room temperature with ZnCl$_2$ (●), CoCl$_2$ (■) and FeCl$_2$ (▲) in concentrations shown. Lipoprotein lipase activity was then determined. Results are the mean of 3 determinations for each point shown.
CHAPTER SIX

INSULIN AND GLUCAGON LIVER AND ADIPOSE TISSUE RECEPTOR-INTERACTION IN THE ZUCKER AND CoCl₂-TREATED RATS
### 6.1 Introduction:

In recent years it has become increasingly apparent that certain clinical disorders and metabolic states are associated with insulin resistance. Generally, insulin resistant states are characterized by an increase in basal levels of circulating insulin, excessive plasma insulin responses to insulin secretory stimuli and reduced effectiveness of endogenous and exogenous insulin both in vivo and in vitro. Glucose intolerance may or may not be present. The Zucker fatty rat, as has been shown earlier (Chapters 1 and 3) is a good example of an insulin resistant animal.

The recent demonstrations that insulin initiates its effects by interacting with "specific" sites located on the plasma membrane of hormone-sensitive tissue (Chapter 1) has provided the investigative tools for exploring the possibility that altered insulin-receptor interaction is responsible for hormone resistance. This was made possible by the specific insulin binding systems developed with monoiiodinated insulin and purified plasma membranes of target cells (Chapter 2), which enabled the direct study of insulin-receptor interaction in pathological states of altered sensitivity to insulin. The same could also be applied to glucagon resistance studies.

Decreased insulin receptors in insulin-resistant states have been reviewed in Chapter 1. The aim of this Chapter is to investigate the possibility of decreased insulin receptors in the liver and adipose tissue of the Zucker fatty rat being responsible for its insulin resistant state and to investigate the role of the liver and adipose tissue receptors in the glucagon-resistant hypothesis suggested \((66, 67)\) for the hypertriglyceridaemia of the Zucker fatty and the CoCl₂-treated rats.
6.2 Methodology

Twelve week-old genetically fat (fa/fa) male Zucker rats and their lean littermates were used for the present study. Animals had access to food and water up to the time the experiment began. Animals were sacrificed by cervical dislocation and livers and epididymal fat pads were collected. Purified plasma membranes of liver and adipose tissue were prepared.

Mg	extsuperscript{++}-stimulated ATPase (EC 3.6.1.3), 5'-mononucleotidase (EC 3.1.3.5) and adenylate cyclase (EC 4.6.1.1.) on the purified plasma membranes were determined.

Iodinated monocomponent \textsuperscript{125}I-labelled glucagon and insulin were prepared and insulin and glucagon-receptor interaction studies were carried out. Non-specific binding (NSB) tubes (the percentage of \textsuperscript{125}I-radioactivity bound to membrane pellets in the presence of 50\,\mu g of unlabelled insulin and glucagon) were included for each test. The NSB values were subtracted from each of the test results. All tests were carried out in duplicate. Methods are described in detail in Chapter 2.

The insulin and glucagon degradation and inactivation by plasma membranes during a 30 min. incubation at 30\,^\circ\text{C} was determined by the loss of binding of labelled hormones preincubated with membranes to fresh membranes as described by Freychet et al (170).

CoCl\textsubscript{2}-treated rats and their controls were similarly investigated. Statistical analyses were carried out using the Mann-Whitney U-test (169).
6.3. Results:

a) Zucker rat

i) Yield and liver plasma membrane marker enzymes.

The plasma membrane yield and activities of the membrane bound enzymes: 5'-mononucleotidase, Mg$^{++}$-stimulated ATPase and adenylate cyclase basal and stimulated levels were similar for liver cell membranes prepared for lean and fatty rats as shown in Table 6.1.

ii) Insulin-receptor interaction with liver plasma membranes

Table 6.2 Apparent affinity constants and binding capacities for the insulin receptor on liver plasma membranes from Zucker lean and fatty rats. Site 1 is the high affinity, low capacity site; site 2 is the low affinity high capacity site. The contribution of low affinity binding site was subtracted from the results of the high affinity binding site as described by Olefsky et al (173). Results were calculated from Fig. 6.2.

<table>
<thead>
<tr>
<th></th>
<th>Maximum binding capacity pmol·insulin/mg membrane protein</th>
<th>Apparent affinity constant L/M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site 1</td>
<td>Site 2</td>
</tr>
<tr>
<td>Zucker lean</td>
<td>3.4</td>
<td>32</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>1.2</td>
<td>10</td>
</tr>
</tbody>
</table>

Specific binding of $^{125}$I-insulin to liver plasma membranes from lean and fatty rats and its displacement by unlabelled insulin is shown in Fig. 6.1. Maximum binding of $^{125}$I-insulin by liver cell membranes from fatty rats was only 46% of that obtained with similarly prepared membranes from livers of the lean rats.
Table 6.1. Yield and plasma membrane bound enzymes of the liver of Zucker lean and fatty rats. Yield is expressed as mg plasma membrane protein /g wet liver weight. 5'-mononucleotidase and Mg$^{++}$-stimulated ATPase activities are reported as μmol P released/30 min/mg protein. Adenylate cyclase activity is reported as p mol cAMP/15 min/mg membrane protein. Results are the mean ± SEM of 5 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Yield</th>
<th>5'-mononucleotidase</th>
<th>Mg$^{++}$-stimulated ATPase</th>
<th>Adenylate Cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Zucker lean</td>
<td>2.5 ± 0.5</td>
<td>18 ± 2.5</td>
<td>9.8 ± 1</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>2.3 ± 0.5</td>
<td>16 ± 3</td>
<td>9.0 ± 1.2</td>
<td>170 ± 18</td>
</tr>
<tr>
<td>P</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Fig. 6.1 Specific binding of $^{125}$I-insulin (75 pg/ml) to partially purified liver plasma membranes from lean (○) and fatty (■) Zucker rats and displacement by unlabelled insulin. NSB for lean rats was 5% and for fatty rats 4.5% which were subtracted from their respective results shown. Results are the mean ± SEM of 4 animals in each group.
Results from Fig. 6.1 were analysed by Scatchard analysis (174) in Fig. 6.2 and calculated apparent affinity constants and binding capacities for the insulin receptor of the Zucker rat liver plasma membranes are shown in Table 6.2.

Scatchard analysis of insulin receptor binding shown in Fig. 6.2 indicates the complexity and heterogeneity of the insulin receptors on liver membranes. For comparative purposes these receptors can be looked upon as consisting of two different types; a high affinity, low capacity site (site 1) and another of low affinity, high capacity (site 2). The data revealed a marked reduction in the maximum binding capacity of liver plasma membranes obtained from the Zucker fatty rats compared to those from the lean particularly in site 2. However, the reduction in site 2 appears to be slightly compensated for by higher affinity of the receptor to insulin in the fatty animals compared to the lean.

iii) Glucagon-receptor interaction with liver plasma membranes

Specific binding of $^{125}$I-glucagon to liver plasma membranes from lean and fatty rats and its displacement by unlabelled glucagon is shown in Fig. 6.3. As can be seen maximum binding of $^{125}$I-glucagon by liver cell membranes obtained from lean and fatty rats were similar in both groups of rats.

Due to the overlapping of experimental values in Fig. 6.3 one Scatchard plot sufficed to represent the two groups of animals. This is shown in Fig. 6.4, the calculated apparent affinity constants and maximum binding capacities for the glucagon liver receptor are shown in Table 6.3.
Fig. 6.2 Scatchard plot of the data in Fig. 6.1. The apparent affinity constant of the high affinity site is obtained from the slope of the straight line at low insulin levels after correction for the contribution of the low affinity site at higher insulin concentrations. The apparent affinity constant of the low affinity site is obtained from the slope of the straight line at higher insulin concentrations. The maximum binding capacities were calculated from the intercepts of the two lines on the horizontal axis after converting the value to mol/l. (*) represent values from lean rats, (■) represent values from fatty rats.
Fig. 6.3 Specific binding of $^{125}$I-glucagon (82 pg/ml) to partially purified liver plasma membranes from lean (•) and fatty (■) Zucker rats and displacement by unlabelled glucagon. NSB for lean rats was 3.2% and for fatty rats 2.8% which were subtracted from their respective results shown. Results are the mean ± SEM of 4 animals in each group.
Fig. 6.4 Scatchard plot of the data in Fig. 6.2 for glucagon-receptor interaction in the Zucker lean and fatty rats. The apparent affinity constant of the high affinity site is obtained from the slope of the straight line at low glucagon levels after correction for the contribution of the low affinity site at higher glucagon concentrations, (dotted line). The apparent affinity constant of the low affinity site is obtained from the slope of the straight line at higher glucagon concentrations. The maximum binding capacities were calculated from the intercepts of the two lines on the horizontal axis after converting the value to mol/l.
Table 6.3: Apparent affinity constants and binding capacities of the glucagon receptor on liver plasma membranes from Zucker lean and fatty rats. Site 1 is the high affinity, low capacity site; Site 2 is the low affinity, high capacity site. The contribution of the low affinity binding site was subtracted from the results of the high affinity binding site. Results were calculated from Fig. 6.4.

<table>
<thead>
<tr>
<th></th>
<th>Maximum binding capacity</th>
<th>Apparent affinity constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/glucagon/mg membrane protein</td>
<td>L/M⁻¹</td>
</tr>
<tr>
<td>Site 1</td>
<td>10.6</td>
<td>6.6 x 10⁸</td>
</tr>
<tr>
<td>Site 2</td>
<td>63</td>
<td>5.2 x 10⁷</td>
</tr>
<tr>
<td>Zucker lean and fatty rats</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In contrast to the complexity and heterogeneity of the insulin receptor, glucagon-receptor interaction results when analysed by a Scatchard plot show two distinct types of receptors. Site 1 receptors having high affinity and low capacity; and site 2 receptors of low affinity high capacity.

iv) Plasma membrane marker enzymes of adipose tissue:

Table 6.4 shows the plasma membrane marker enzymes of adipose tissue from lean and fatty rats. There was no significant difference between 5'-mononucleotidase, Mg⁺⁺ stimulated ATPase and adenylate cyclase basal as well as stimulated levels between the two groups of animals.

v) Insulin receptor-interaction with adipose tissue plasma membrane:
Table 6.4  Plasma membrane bound enzymes of the Zucker lean and fatty rats adipose tissue.  5'-mononucleotidase and Mg\(^{++}\)-stimulated ATPase activities are reported as \(\mu\)mol P released/30 min/mg protein. Adenylate cyclase activity is reported as pmol cAMP/15 min/mg membrane protein. Results are the mean \(\pm\) SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>5'-mononucleotidase</th>
<th>Mg(^{++})-stimulated ATPase</th>
<th>Adenylate Cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td>12.5 ± 0.5</td>
<td>10.5 ± 0.6</td>
<td>Basal 120 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ NaF (10 mM) 620 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ Glucagon (+ (\mu)g/ml) 810 ± 70</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>13.2 ± 0.8</td>
<td>11.2 ± 0.5</td>
<td>135 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>678 ± 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>790 ± 55</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 6.5: Apparent affinity constants and binding capacities for the insulin receptor on adipose tissue plasma membranes from Zucker lean and fatty rats. Site 1 is the high affinity, low capacity site; site 2 is the low affinity, high capacity site. The contribution of low affinity binding site was subtracted from the results of the high affinity binding site. Results were calculated from Fig. 6.6.

<table>
<thead>
<tr>
<th>Max binding capacity pmol insulin/mg membrane protein</th>
<th>Apparent affinity constant L/M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>Site 2</td>
</tr>
<tr>
<td>Zucker lean 2.6</td>
<td>19</td>
</tr>
<tr>
<td>Zucker fatty 1.0</td>
<td>15</td>
</tr>
</tbody>
</table>

Specific binding of ¹²⁵I insulin to adipose tissue plasma membranes from lean and fatty rats and its displacement by unlabelled glucagon is shown in Fig. 6.5. Maximum binding of ¹²⁵I-insulin by adipose tissue plasma membranes from fatty rats was only 58% of that obtained with similarly prepared membranes from adipose tissue of lean rats.

Results from Fig. 6.5 were analysed by Scatchard analysis in Fig. 6.6 and calculated data are presented in Table 6.5.

Scatchard analysis indicates a marked reduction in the maximum binding capacity of adipose tissue plasma membranes obtained from the Zucker fatty rat compared to the lean in both binding sites extrapolated.

vi) Glucagon-receptor interaction with adipose tissue-plasma membranes

Fig. 6.7 shows the specific binding of ¹²⁵I-glucagon to adipose tissue plasma membranes for lean and fatty rats and its displacement by unlabelled glucagon. No detectable difference was obtained in binding capacities by adipose tissue plasma membrane between the two groups of rats.
Fig. 6.5  Specific binding of $^{125}$I-insulin (78 pg/ml) to adipose tissue plasma membranes from lean (•) and fatty (□) Zucker rats and displacement by unlabelled insulin. NSB for lean rats was 2.9% and for fatty rats 3.2% which were subtracted from their respective results shown. Results are the mean ± SEM of 4 animals in each group.
Fig. 6.6  Scatchard plot of the data in Fig. 6.5 for insulin-receptor interaction in the Zucker lean (●) and fatty rats (■) adipose tissue plasma membranes. Apparent affinity constants and maximum binding capacities were calculated as in the Legend to Fig. 6.2.
Fig. 6.7 Specific binding of $^{125}$I-glucagon (80 pg/ml) to adipose tissue plasma membranes from lean (●) and fatty (■) Zucker rats and displacement by unlabelled glucagon. NSB for both lean and fatty rats was 2.2% which was subtracted from values shown. Results are the mean ± SEM of 4 animals in each group.
Due to overlapping of data in Fig. 6.7 from the two groups of rats, one Scatchard plot sufficed to represent the two groups. This is shown in Fig. 6.8 and the calculated apparent affinity constants and maximum binding capacities for the glucagon adipose tissue receptors are shown in Table 6.6.

Table 6.6  Apparent affinity constants and binding capacities for the glucagon receptor on adipose tissue plasma membranes from Zucker lean and fatty rats. Site 1 is the high affinity, low capacity site, site 2 is the low affinity, high capacity site. The contribution of the low affinity binding site was subtracted from the results of the high affinity binding site. Results are calculated from Fig. 6.8

<table>
<thead>
<tr>
<th></th>
<th>Maximum binding capacity pmol glucagon/mg membrane protein</th>
<th>Apparent affinity constant L/M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site 1</td>
<td>Site 2</td>
</tr>
<tr>
<td>Zucker lean and fatty rats</td>
<td>0.43</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Compared to the liver, the adipose tissue of the Zucker rat bound much less glucagon at both "calculated" sites. The liver plasma membranes bound 25 times more glucagon than adipose tissue plasma membranes per mg protein at both sites, and in both the Zucker lean and fatty rats. The higher binding was accompanied by a greater affinity for the hormone by liver receptors particularly site 1, compared to the affinity of the adipose tissue receptors.

vii) Other possible causes of reduction in binding

In addition to a true alteration in the insulin-receptor interaction, two other major causes of an apparent reduction in insulin binding to the liver and adipose tissue plasma membranes in the Zucker fatty rat were considered. To evaluate the effect of the marked increase in circulating insulin levels in the fatty rat on its receptor binding a group of lean rats were given
Fig. 6.8 Scatchard plot of the data in Fig. 6.7 for glucagon-receptor interaction in the Zucker lean and fatty rats. Results extrapolated and calculated as shown in the legend of Fig. 6.4.
exogenous insulin to raise the circulating insulin level to those observed in the fatty rats. 3 µg of insulin was given by direct injection into the heart to lean rats. This was followed by sacrificing the animals after 10 min. Liver and adipose tissue plasma membranes were prepared from these treated animals as well as fatty untreated rats. Insulin-receptor interaction study was then carried out. Results obtained were similar to those shown in Fig.6.1 i.e. the reduction in binding in the fatty rats was not due to high circulating insulin levels.

The second possibility that the reduced insulin binding observed in the fatty rats is probably due to increased insulin degradation was also considered. Results are shown in Table 6.7.

Table 6.7 Insulin and glucagon degradation by the liver and adipose tissue plasma membranes of lean and fatty Zucker rats. Degradation was determined by the loss of binding expressed as percentage to fresh membranes after preincubation with liver and adipose tissue plasma membranes (0.33 mg/ml) for 30 min. at 30°C. Hormone concentration was 0.15 mg/ml. Results are expressed as the mean ± SEM of 4 animals in each group.

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Glucagon</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Adipose Tissue</td>
<td>Liver</td>
<td>Adipose Tissue</td>
</tr>
<tr>
<td>Zucker lean</td>
<td>43 ± 4%</td>
<td>48 ± 5%</td>
<td>37 ± 5%</td>
<td>43 ± 5%</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>35 ± 4%</td>
<td>42 ± 3%</td>
<td>20 ± 3%</td>
<td>38 ± 5%</td>
</tr>
</tbody>
</table>

Direct measurement of insulin degradation revealed that like the insulin binding this was also decreased in the plasma membranes of both the liver and adipose tissue of fatty rats. Glucagon degradation was also decreased in the plasma membranes of both the liver and adipose tissue of fatty rats compared to the lean but with no apparent reduction in glucagon binding to either plasma membranes as shown earlier.
viii) CoCl₂-treated rats

Fig. 6.9 shows the specific binding of ¹²⁵I-insulin to liver and adipose tissue plasma membranes of CoCl₂-treated and control rats. There appears to be no apparent difference in binding of insulin to liver and adipose tissue plasma membranes in the two groups of animals.

Fig. 6.10 shows the specific binding of ¹²⁵I-glucagon to liver and adipose tissue plasma membranes of CoCl₂-treated and control rats. As with insulin, glucagon binding to these membranes show no difference between the two groups of animals.

6.4 Discussion:

The data presented in this series of studies suggests that in the Zucker fatty rat there is an impairment of the insulin-receptor interaction in both the liver and adipose tissue due to a decrease in the number of receptors at the plasma membranes of these tissues, compared to the lean rat. Control studies suggest that the alteration is not due to the high circulating endogenous insulin levels, to an increased degradative processes or to some major difference in membrane purification.

Apart from the difference in the insulin receptor population, there appears to be no difference in the liver and adipose tissue plasma membrane function as shown by the similarity of the membrane marker enzymes 5'-mononucleotidase, Mg²⁺-stimulated ATPase and adenylate cyclase in fatty rats and their lean counterparts.

The decrease in insulin receptors in these animals may explain their apparent insulin resistance discussed in Chapter 1. This decrease is much smaller in the fatty (fa/fa) rat than in obese (eb/ob) mouse which also exhibits marked hyperglycaemia. Liver and adipose tissue cells isolated from the obese mouse bind only 20-25% of insulin at physiological
Fig. 6.9 Specific binding of $^{125}$I-insulin (88 pg/ml) to partially purified plasma membranes from liver and adipose tissue of CoCl₂-treated and control rats and displacement by unlabelled insulin.

(♦ O) Control rats, (■ □) CoCl₂-treated rats.

NSB for liver plasma membranes binding was 6.4% for CoCl₂-treated and control rats. NSB for adipose tissue plasma membranes binding was 3.5% for treated and control rats which were subtracted from their respective values shown. Results are the mean ± SEM of 4 animals in each group.
Fig. 6.10 Specific binding of $^{125}$I-glucagon (92 pg/ml) to partially purified liver and adipose tissue plasma membranes of CoCl$_2$-treated and control rats, and displacement by unlabelled glucagon. NSB for liver plasma membranes binding was 4% for both group of rats and 3% for adipose tissue membranes of both groups of rats which were subtracted from their respective values shown. Results are the mean ± SEM of 4 animals in each group. (●) Control, (□) CoCl$_2$-treated, (○) CoCl$_2$-treated and control rats.
concentrations per mg of membrane protein compared to their lean controls (30-34), while the Zucker fatty rat binds 40-50% compared to their lean controls. Greater reduction in the insulin receptors of the obese mouse may contribute to the greater hyperglycaemia and low glucose tolerance found in this species compared with the Zucker fatty rat in which they are usually very mild or absent.

In contrast to the difference in insulin receptors, glucagon, liver and adipose tissue receptors in the Zucker fatty rat showed no detectable differences compared to the lean.

The above findings in the fatty rats conflict with a previous report (176) that the binding of insulin to isolated hepatocytes was the same in the cells obtained from fed homozygous Zucker fatty rats as that from their lean siblings. However, the same report showed that the specific binding of insulin was lower in the fasted fatty than in the fasted lean rats. This discrepancy may be due to the use of whole liver cells as compared to isolated liver plasma membranes employed for this study. This report was published after the present studies were completed.

The CoCl₂-treated rats showed no significant difference in either insulin or glucogen binding to the liver and adipose tissue plasma membranes than those of their controls.

Site-site interactions:

In the present work the interpretation of Scatchard analysis where curvilinear plots were obtained depends upon certain assumptions. One of these is that sites extrapolated from such curves act independently of each other and thus the receptor is assumed to be univalent. Recent studies have suggested that for some hormone-receptor interaction, this is not the case and that the receptor behaves as a multisubunit protein
in which binding of a ligand to one subunit alters the affinity of another subunit for its ligand. Since binding of the first ligand reduces the affinity of the receptor for the subsequent ligands, the phenomenon is called "negative cooperativity".

Recently De Meyts et al. (177, 178) have presented evidence for site-site interactions among insulin receptors exhibiting negative cooperativity. This effect was demonstrated by showing that the rate of dissociation of $^{125}\text{I}$-insulin produced by "infinite" dilution was further increased in the presence of unlabelled insulin in the dilution medium. They have further suggested that all receptor systems for which non-linear Scatchard plots have been obtained are potential candidates for negative cooperativity for example glucagon receptors. However, such interpretations have been challenged by Cuatrecasas et al. (179, 180) who have shown that the negative cooperativity phenomenon was not applicable to glucagon receptors and that negative cooperativity shown by insulin receptors could be due to insulin dimerization and self-aggregation which enhances dissociation \textit{in vivo} by the addition of unlabelled hormone.

However, leaving aside the interpretation of such data the fact still remains that insulin binding to liver and adipose tissue of the Zucker fatty rat is impaired compared to the lean and it could account for its insulin resistance. Considering all the available evidence for and against the site-site interactions explanation of Scatchard analysis I tend to favour the interpretation as put forward in the present studies that insulin and glucagon receptors consist of multiple sites of different binding capacities and affinities.
CHAPTER SEVEN

THE EFFECT OF INDUCED HYPERGLUCAGONAEMIA IN THE ZUCKER FATTY RAT
7.1 Introduction:

Glucagon is well recognized to have lipolytic activity both in vivo and in vitro. The in vitro activity is readily demonstrated in tissue derived from both mammals and birds. In vivo activity is, however, more difficult to demonstrate in mammals due to the concomitant stimulation, by glucagon, of insulin secretion, where lipogenic action more than counter-balances the lipolytic effect of glucagon. Nevertheless, when the insulinotropic effect of glucagon is abolished by prolonged fasting, glucagon can be shown to possess lipolytic activity in man (57), probably as in other species through activation of adenylate cyclase activity, and increased production of cAMP.

Glucagon has a hypolipaemic (hypolipoproteinaemic) action in man, dog, fowl and rat (57-61) and when administered to hyperlipaemic patients whether for a short time (63) or for longer periods (62), it produces a moderate to profound fall in plasma triglyceride and cholesterol levels. The hypolipaemic effect has often been attributed to reduction in hepatic triglyceride output (64) or to decreased apoprotein synthesis (65).

In order to further elucidate the mechanism of the hypolipaemic action of glucagon, its short and long term effects were studied in the present chapter in the Zucker fatty rat, also in the CoCl₂-treated rat.

7.2 Methodology:

a) Short-term effect:

After an overnight fast, 15-week old male Zucker lean and fatty animals were anaesthetized with Nembutal (6 mg/100g body weight) and fasting blood samples were collected via cardiac puncture. Blood samples for plasma glucose were collected in small fluoride tubes. Plasma
for lipid estimations were collected in heparinized tubes. A dose of 100 μg glucagon was then given intraperitoneally. 1 ml blood samples were then collected after 10, 30 and 60 min of glucagon administrations via cardiac puncture. Plasma, glucose and triglycerides were measured.

The same procedure was repeated on CoCl₂-treated and control rats.

b) Long-term effect:

Ten-week old genetically fatty male Zucker rats and their lean controls were used for this experiment. These animals were treated with long-acting glucagon (zinc protamine glucagon). A dose of 50 μg of long-acting glucagon was administered by subcutaneous injection into the abdominal region to each rat twice a day at 10 a.m. and 3 p.m. for a period of two weeks. The dose of glucagon was reduced to one injection a day for a third and final week because by the end of the first two weeks of glucagon treatment, the animals had become lethargic and inactive. Reduction in the dosage of glucagon was associated with restoration of full activity. Controls were set up for each of the fatty and lean groups of experimental animals and treated exactly the same as the experimental animals, except that saline was injected subcutaneously instead of glucagon.

Twenty-four hours after the last injection the animals were anaesthetized by i.p. injection of Nembutal (animals had access to food and water up to the time the experiment began). Blood was collected via cardiac puncture into a 10 ml syringe containing Trasylol, 2,000 KIU/ml of blood and added to a plastic pot containing 10 IU heparin/ml of blood and centrifuged at 4°C. Aliquots (0.5 ml) of each plasma sample were pipetted into small oxalate fluoride tubes for glucose determination. The remainder was stored at -20°C until assayed for hormones and lipid levels.
Rats were then sacrificed by cervical dislocation and livers were collected. Partially purified plasma membranes were prepared. Plasma membrane marker enzymes were determined as well as hormone-receptor interaction studies. All methods employed are shown in detail in Chapter 2.

7.3 Results:

a) Zucker rats.

i) Short-term effect of glucagon:

Fig. 7.1 shows the effect of a single glucagon dose (100 μg) on the plasma glucose and triglyceride levels of Zucker lean and fatty rats. After glucagon was given intraperitoneally, lean and fatty rats showed a rise in their plasma glucose. The rise in the Zucker fatty rats was much more pronounced. This was also true for the values after 30 min. Following 60 min. of glucagon administration plasma glucose values for the lean rats returned to the fasting level. However, after the same period the fatty group maintained approximately the same level of plasma glucose as that after 30 min.

Following the same dose of glucagon the plasma triglyceride levels showed negligible decrease in the lean rats after 10, 30 and 60 min. However, there was an appreciable drop in plasma triglycerides in the fatty rats after the same periods.

ii) Long-term effect of glucagon:

Long-term glucagon treatment had no effect on the rate of weight gain by either the lean or fatty experimental animals compared with their controls (Table 7.1).

Plasma glucose levels did not differ significantly in the treated and control lean animals. In the fatty rats there was a significant
Fig. 7.1 Effect of a single glucagon dose (100 μg) on the plasma glucose and triglycerides of Zucker lean (●) and fatty (■) rats. Results are the mean ± SEM of 4 animals in each group of rats.
Table 7.1  Average weights of the Zucker rats before and after glucagon treatment with their controls, plasma glucose insulin and glucagon levels. Results are the mean + SEM of 4 rats in each group. * = P < 0.01

<table>
<thead>
<tr>
<th>Zucker</th>
<th>Average Weight - g</th>
<th>Plasma glucose mmol/l</th>
<th>Plasma insulin mU/l</th>
<th>Plasma glucagon ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>lean</td>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>337 ± 5.6</td>
<td>372 ± 16</td>
<td>7.9 ± 0.5</td>
<td>47.5 ± 9</td>
</tr>
<tr>
<td>Treated</td>
<td>338 ± 10</td>
<td>376 ± 14</td>
<td>8.88 ± 0.9</td>
<td>152.5 ± 31*</td>
</tr>
<tr>
<td>fatty</td>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>470 ± 9.5</td>
<td>527 ± 7</td>
<td>11.4 ± 1.3</td>
<td>806 ± 98</td>
</tr>
<tr>
<td>Treated</td>
<td>469 ± 3.5</td>
<td>517 ± 6</td>
<td>7.66 ± 0.3*</td>
<td>750 ± 139</td>
</tr>
</tbody>
</table>


lowering \( p < 0.01 \) of plasma glucose as a result of chronic glucagon treatment, but this may be due to the unexpected high levels observed at the commencement of the experiment and was not observed in other experiments on fatty rat.

Plasma insulin levels were markedly elevated in fatty rats as compared with their lean controls. Plasma glucagon levels were also elevated but to a lesser degree. An insulinotropic effect of glucagon was demonstrated in the lean rats by a three-fold increase in plasma insulin concentration after glucagon treatment, compared with the saline treated controls. The glucagon treated fatty rats did not show a significant increase in plasma insulin concentration compared with their controls in whom plasma insulin levels were already very high. It has been shown (175) that sodium pentobarbital slightly inhibits insulin released from pancreatic islets of obese mice in vitro when incubated in a medium containing physiological levels of glucose and calcium. Consequently, the absolute values shown in Table 7.1 and elsewhere may be an underestimate of the concentration in unanaesthetized animals. Nevertheless, assuming that the anaesthesia had the same effect on both the lean and fatty rats, the insulin levels reported in this study are suitable for comparative purposes.

Mean plasma glucagon levels were below the lower limit of accurate measurement in the lean controls but were measurable in the fatty controls. This was especially marked in the fatty rats in which terminal plasma glucagon levels were more than six times those of their untreated controls and over three times as high as in the glucagon treated lean rats.

Chronic administration of glucagon produced significant reductions in plasma triglycerides and cholesterol levels (Table 7.2) in both the lean and fatty animals. Treated lean rats showed a 42.4% reduction in plasma triglycerides compared with their controls; treated fatty rats, on the other hand, showed only a 27.2% reduction despite much higher
Table 7.2  Plasma FFA, cholesterol and triglycerides of the Zucker lean and fatty rats and the change in their levels expressed as percentages after glucagon treatment and their controls. Results are the mean ± SEM of 4 animals in each group.

* = p not significant, ** = p < 0.01, + = p < 0.001 and † = p < 0.02.

<table>
<thead>
<tr>
<th></th>
<th>FFA mmol/l</th>
<th>% Change</th>
<th>Cholesterol mmol/l</th>
<th>% Reduction</th>
<th>Triglycerides mmol/l</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zucker lean</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.39 ± 0.04</td>
<td>41</td>
<td>1.67 ± 0.08</td>
<td>22</td>
<td>1.10 ± 0.07</td>
<td>42.4</td>
</tr>
<tr>
<td>Treated</td>
<td>0.55 ± 0.02**</td>
<td></td>
<td>1.30 ± 0.09*</td>
<td></td>
<td>0.64 ± 0.05+</td>
<td></td>
</tr>
<tr>
<td><strong>Zucker fatty</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.52 ± 0.01V</td>
<td></td>
<td>4.68 ± 0.2</td>
<td>25.6</td>
<td>4.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>0.48 ± 0.03*</td>
<td></td>
<td>3.48 ± 0.3**</td>
<td></td>
<td>2.9 ± 0.3</td>
<td>†</td>
</tr>
</tbody>
</table>
levels initially. Glucagon treatment reduced plasma cholesterol levels more or less equally in the lean and fatty rats (22 and 25.6% respectively). Glucagon treatment increased plasma FFA levels by 41% in the lean rats and reduced them by 7.6% in the fatty ones.

Values obtained for the plasma membrane marker enzymes, 5' mononucleotidase and Mg$^{++}$-stimulated ATPase are shown in Table 7.3. Both enzymes demonstrated a slight, but statistically non-significant, increase in activity after glucagon treatment in both lean and fatty rats.

Membrane-bound adenylate cyclase activities after treatment are shown in Fig 7.2. The control lean and fatty rats did not differ significantly from each other (statistical values not shown). Glucagon treatment produced a significant increase in basal and stimulated levels, in both groups. The increase was greater in the fatty animals both in relative and absolute terms when compared to their own untreated controls and the treated lean animals.

Specific binding of $^{125}$I-glucagon to liver plasma membranes prepared from lean and fatty Zucker rats and their controls and its displacement by unlabelled glucagon is shown in Fig 7.3. There were no differences in glucagon binding characterisation between the glucagon treated and untreated lean animals or the untreated fatty animals. Because of overlapping of the experimental values one displacement plot sufficed to represent the three groups of animals (control and treated lean and control fatty). There was, however, a marked and significant drop in glucagon binding by liver cell membranes prepared from glucagon treated fatty rats compared with their untreated controls.

Scatchard analysis (174) was carried out on the glucagon radio-receptor assay data shown in Fig 7.3 and represented diagramatically in Fig 7.4. The affinity and kinetic constants derived from the two plots
Table 7.3  Plasma membrane bound enzymes of the glucagon treated Zucker lean and fatty rats and their controls.  *p = not significant.

<table>
<thead>
<tr>
<th></th>
<th>5'-Mononucleotidase</th>
<th>Mg++-Stimulated ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol phosphate released/30 min/mg membrane protein</td>
<td>μmol phosphate released/30 min/mg membrane protein</td>
</tr>
<tr>
<td>Zucker lean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18 ± 2.5</td>
<td>9.7 ± 1.2</td>
</tr>
<tr>
<td>Treated</td>
<td>23.5 ± 4.5*</td>
<td>10.8 ± 1.6*</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.5 ± 2.0</td>
<td>8.8 ± 1.0</td>
</tr>
<tr>
<td>Treated</td>
<td>15.3 ± 2.2*</td>
<td>10.2 ± 1.4*</td>
</tr>
</tbody>
</table>
Fig. 7.2  Adenylate cyclase levels of the Zucker lean and fatty rats after glucagon treatment and their controls, basal (□), + 10 mmol/l NaF (□□□) and + 10 μg/ml glucagon (□□□) stimulated levels. Results are the mean ± SEM of 4 rats in each group.
Specific binding of $^{125}$I-glucagon (75 pg/ml) to partially purified liver plasma membranes from lean and fatty Zucker rats after glucagon treatment and their controls, and displacement by unlabelled glucagon. NSB for the treated lean and controls was 2.2% and for treated fatty and controls 2.6% which were subtracted from all results shown. (■) Lean treated and controls and fatty controls. (○) ''Fatty''-treated. Results are the mean ± SEM of 4 rats in each group. $p (*) < 0.05$, $p (***) < 0.001$. 

Fig. 7.3
Fig. 7.4 Scatchard plot of the data in Fig. 7.3. Results were calculated as shown in the legend to Fig. 4. (□) represents values from treated lean and control rats, (○) represents values from treated fatty and control rats.
are shown in Table 7.4. In both plots there appear to be two types of glucagon receptors; site 1 receptors having high affinity and low capacity; and site 2 receptors a low affinity and high capacity. There was a significant decrease in the binding capacity of both sites in membrane prepared from treated fatty rats compared with their untreated controls, the values being nearly halved by glucagon treatment. The reduction in capacity was accompanied by an increase in the affinity constant of site 2 in the fatty treated animals compared with their controls without significant change in site 1.

Specific binding of $^{125}$I-insulin to liver plasma membranes from lean and fatty rats and their controls and its displacement by unlabelled insulin is shown in Fig.7.5. Glucagon treatment did not alter insulin binding to liver receptors in either the treated lean and fatty animals as compared with their controls. However, maximum $^{125}$I-insulin binding by liver cell membranes from both control and treated fatty rats was only 50% of that obtained with the membranes prepared from livers of either control or glucagon treated lean rats.

Scatchard analysis of the insulin displacement studies are shown in Fig.7.6 and the calculated affinity and kinetic constants are shown in Table 7.4. Due to the overlapping of the results from the treated and control lean groups and the treated and control fatty groups, one Scatchard plot sufficed to describe the radioreceptor assay data obtained from the glucagon treated lean rats and their controls and another Scatchard plot to describe the data obtained from the glucagon treated fatty rats and their controls. For the sake of convenience two types of insulin receptors for each group of animals were extrapolated from the Scatchard plots, a high affinity low capacity (site 1) and a low
Table 7.4  Apparent affinity constants and binding capacities of the Zucker lean and fatty rats after glucagon treatment and their controls. Site 1 is the low capacity high affinity site; site 2 is the high capacity low affinity site. The contribution of the low affinity binding site was subtracted from the results of the high affinity binding site as described by Olefsky et al. (173). Results were calculated from Fig.7.4 and Fig. 7.6 for glucagon and insulin Scatchard analyses respectively.
Fig. 7.5  Specific binding of $^{125}$I-insulin (75 pg/ml) to partially purified liver plasma membranes from lean and fatty Zucker rats after glucagon treatment and their controls, and displacement by unlabelled insulin. NSB for the treated lean and controls was 5.6% and for treated fatty and controls 4.9%, which were subtracted from their respective results shown. (■) Treated lean, (○) lean controls, (△) treated fatty and (□) fatty controls. Results are the mean ± SEM of 4 rats in each group.
Fig. 7.6 Scatchard plot of the data in Fig. 7.5. The ratio of the bound to free insulin is on the vertical axis and bound insulin is on the horizontal axis. The association constant of the high affinity site is obtained from the slope of the straight line at low insulin concentrations after correction for the contribution of the low affinity site at higher insulin concentrations. The association constant of the high affinity site is obtained from the slope of the straight line at higher insulin concentrations. The maximum binding capacities were calculated from the intercepts of these two lines on the horizontal axis after converting the values to mol/l. (■) represents values from treated lean rats and their controls, (○) represents treated fatty rats and their controls.
affinity high capacity (site 2). The calculated data shown in Table 7.4 reveals a marked reduction in the maximum $^{125}$I-insulin binding capacity of liver plasma membranes obtained from the fatty rats compared with membranes from lean rats. This reduction in binding capacity affects both sites and is probably compensated for by an increased binding affinity at both sites, particularly site 1.

Table 7.5 shows insulin and glucagon degradation by the liver plasma membranes of the lean and fatty Zucker rats. These results indicate that degradation of both hormones is higher in the lean rats than from their fatty counterparts.

None of the glucagon-treated animals developed $^{125}$I-glucagon binding glucagon antibodies during the course of these experiments.

<table>
<thead>
<tr>
<th>Zucker lean</th>
<th>Insulin</th>
<th>Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>43%</td>
<td>37%</td>
</tr>
<tr>
<td>Zucker fatty</td>
<td>35%</td>
<td>20%</td>
</tr>
</tbody>
</table>

b) $\text{CoCl}_2$-treated rats

The short-term effect of glucagon on $\text{CoCl}_2$-treated and control rats is shown in Fig.7.7. In contrast to results shown in Fig.7.1 for the
Fig. 7.7 Effect of a single glucagon dose (100 µg) on the plasma glucose and triglycerides of CoCl₂-treated (■) and control (●) rats. Results are the mean ± SEM of 4 animals in each group of rats.
Zucker rat CoCl$_2$-treated and control rats showed no increase in their plasma glucose level following glucagon administration. This is probably due to the lower hepatic glycogen content W/A rat exhibit compared to the Zucker strain (Chapter 3).

Plasma triglycerides following glucagon administration showed a decrease in both the CoCl$_2$-treated and control rats after 10, 30 and 60 min.

7.4 Discussion:

The present study indicates that in the Zucker fatty rats as in most other conditions associated with hyperinsulinaemia, there is an impairment of insulin interaction. This impairment on the surfaces of the liver cell membranes appears to consist mainly of a decrease in the number of insulin receptors. This confirms previous results (Chapter 6) that insulin resistance in this strain of rat could be accounted for by reduced insulin receptors on its target cells.

Of special interest is the difference in hypolipidaemic and hyperglycaemic potency of glucagon in fatty and lean rats. Although greater in fatty than in lean rats when expressed in terms of an absolute reduction in plasma triglyceride levels, the situation is reversed when the data is expressed in terms of percentage reduction from pretreatment values. Plasma glucose levels were lower in glucagon treated than untreated fatty rats despite the fact that plasma glucagon was increased and plasma insulin remain unchanged.

Prolonged exposure of Zucker rats to exogenous glucagon led to a marked rise in total adenylate cyclase activity through which the hormone is believed to exert its metabolic effect. The effect both upon basal adenylate cyclase levels and its activity after stimulation in vitro
was much greater in the fatty rats than the lean ones. However, if the relative effectiveness of glucagon in vitro as expressed by the percent increase above the basal activity is considered, it appears that glucagon increases the enzyme activity by only about 170% in the fatty treated, vs 370% in the fatty control and 400-450% in the lean treated and control.

The relative decreased responsiveness in the treated fatty rats as demonstrated by their decreased adenylate cyclase activity following in vitro glucagon stimulation may be used to explain the above discrepancies between the two groups. To overcome this relative decreased responsiveness, higher glucagon levels would be required by the fatty animals compared to the lean in order to achieve similar level of glucagon response. This is shown in Table 7.1 by the high plasma glucagon levels in the treated fatty rats compared to the lean. This decreased relative responsiveness to glucagon also explains the lower relative decrease in plasma triglyceride levels in the treated fatty rats than in treated lean. The same reasoning could also explain the decrease in plasma glucose in the face of unchanged plasma insulin levels (Table 7.1) and insulin binding (Fig.7.5).

The high levels of circulating exogenous glucagon caused the 'down regulation' or the decrease of its own plasma membrane receptors shown in Fig.7.3 similar to that observed with insulin (Fig.7.5).

Alternatively, the decreased lipolytic activity as well as the lowering of plasma glucose shown by the glucagon treated fatty rats could be explained on the basis of the gross hyperinsulinaemia present in the fatty rats more than counter balancing the effect of the induced hyper-glucagenaemia. However, this explanation is made less likely if the reduction in the number of insulin receptors shown in Fig.7.5 is taken into account as probably indicative of increased insulin resistance.
In the present study the liver was studied as the probable main site of glucagon and insulin action. However, it is worth considering that other pools of triglyceride and glucose exist in the body where the two hormones might exert their effect. They could possibly differ in Zucker fatty and lean rats which may put a different explanation in the above discussed discrepancies.

In the short term effect of glucagon on the lean and fatty Zucker rats, it was shown that the fatty rats demonstrated a more marked fall than the lean in their plasma triglyceride levels. They showed no evidence of resistance to the hypotriglyceridaemic action of glucagon as was demonstrated with longer period of glucagon treatment. Similar results were also shown by the CoCl$_2$-treated rats compared to their controls.

Plasma glucose levels following a single dose of glucagon in the Zucker rats showed higher elevation in the fatty compared to the lean. This difference could be explained by the fact shown in Chapter 3 that the hepatic glucogen reserve in the fatty rats was much higher than the lean. Glucagon had no effect on the plasma glucose levels of the CoCl$_2$-treated rats or their controls. There was no rise following glucagon administration in the plasma glucose levels in either group. This is probably due to the lower hepatic glycogen reserve W/A rats possess compared to the Zucker strain shown in Chapter 3, bearing in mind that these animals were also fasted prior to commencement of the experiment.

These results will be the subject of further discussion in the light of other results of previous studies which will be presented in Chapter 8.
CHAPTER EIGHT

FINAL DISCUSSION AND CONCLUSIONS
8.1 The Zucker Fatty Rat:

The Zucker fatty rat is one of a group of animals that inherit obesity as an autosomal Mendelian recessive trait. These rats as shown in the present series of studies are fatty, hyperphagic and hyperinsulinaemic associated with insulin resistance, but their blood glucose remains at normal levels. The hypertriglyceridaemia which characterises these animals, is probably due to the increased hepatic production of triglycerides (Chapter 4).

In this chapter two important aspects which emerged from the present work will be discussed in some detail in the hope of further elucidating the pathogenesis of this type of inherited obesity and hyperlipaemia: a) obesity and insulin resistance of the fatty Zucker rat, b) hypertriglyceridaemia and glucagon-resistance.

a) Obesity and insulin resistance of the Zucker fatty rat

In discussing the relationship between obesity of the Zucker fatty rat and its insulin resistance, several pathogenic mechanisms leading to the insulin resistant state must be mentioned. These are hyperphagia, hyperinsulinaemia, and changes in the sensitivity of the target tissues of insulin leading to an insulin resistant state.

i) Hyperphagia and the role of the hypothalamus:

It is well established that the hypothalamus plays a fundamental role in the regulation of food intake (183). More specifically it has been shown that two opposing mechanisms modulate this process: a mechanism located in the ventromedial area of the hypothalamus (VMH) brings about satiation of appetite; the "satiety centre", whilst another mechanism located in the ventrolateral area (VLH) initiates feeding i.e. "feeder centre" (184). It has been further shown that the VMH exerts an inhibitory influence upon the VLH; thereby modulating food intake.

Since the Zucker fatty rat is hyperphagic like most animals with
spontaneous obesity, it has been hypothesised that defect(s) in hypothalamic regulation of food intake may be present (183). This hypothesis gains support from parabiosis experiments (185). Following parabiosis of one particular type of obese mouse (ob/ob) with a normal mouse, an improvement of the obese syndrome, in particular, a normalisation of the food intake is observed. In other instances, the parabiosis of another type of obese mouse (db/db) with either normal or abnormal mice results in the premature death of either one of the latter, a death apparently due to aphagia. On the basis of these experiments at least two different anomalies of the hypothalamus can be envisaged in the obese animals. The first anomaly would be an insufficient production by the ob/ob mouse of a "satiety factor" with consequent hyperphagia. Upon parabiosis with a normal mouse, the latter would supply the ob/ob mice with the lacking "satiety factor". This would be an example of a functionally altered but otherwise responsive hypothalamus. The second anomaly would be the excessive production of a "satiety factor" by the db/db mice to which it will not respond, hence producing hyperphagia. Following parabiosis of the db/db mice either with a normal or an obese mouse, the overproduction of "satiety factor" would be sufficient to turn off the feeding centre of either one of the parabionts, with consequent aphagia and death by starvation. This would be an example of a functionally altered and, in addition, unresponsive hypothalamus (186). Such studies with obese and normal mice have established that food consumption and rate of weight gain was regulated in the obese partner by "something" coming from the normal partner. This suggested that the obese mouse cannot produce "satiety factor" in sufficient amounts to control its appetite.

Studies by Strautz (187) have shown that transplantation of normal pancreatic islets to obese mice stabilised rate of weight gain and reduced both the hyperglycaemia and hyperinsulinaemia. These studies imply that
the missing "satiety factor" may be pancreatic in origin. However, similar islet transplant studies undertaken by others (185) with obese mice have not been able to repeat these results and this hypothesis until confirmed must be considered with caution.

The above studies were mainly carried out on obese mice implicating a defective "satiety centre" as a possible primary cause for its hyperphagia. The same argument for the hyperphagia of the obese mouse can be applied to the Zucker fatty rat as these two strains of animals show great resemblance in their inherited abnormalities.

ii) Hyperinsulinaemia

In laboratory animals hyperphagia may result in a reversible syndrome characterised by obesity and hyperinsulinaemia (188). Hyperphagia and hyperinsulinaemia are often concomitant in animal obesities, and it is possible that hypersecretion of insulin is simply a consequence of hyperphagia. Hyperphagia could initiate changes in the B-cells causing hyperinsulinaemia or, alternatively, a primary defect in the B-cell could cause excessive insulin secretion thereby causing the hyperphagia. The question of which comes first is probably academic since neonatal genetically obese rodents are hyperphagic from birth (186).

The role of hyperinsulinaemia in the development of obesity is suggested by the observation that the increase in depot fat which follows lesions of the VMH, is prevented when B-cells are destroyed by the B-cell cytotoxic agent streptozotocin (189). Moreover, a good correlation between insulinaemia and subsequent obesity has been reported in animals with VMH lesions (190). Basal insulin secretion as well as that induced by glucose and other stimuli, is excessive in most obese animals (183). Hyperinsulinaemia or hyperphagia would ultimately lead to B-cells
hyperactivity causing either compensatory hypertrophy and hyperplasia of the β-cell or in some strains B-cell exhaustion and atrophy (186).

Morphologically the islets of all obese animals are characterised by varying degrees of hyperplasia and hypertrophy, increased insulin content, increased vascularisation and varying signs of B-cell hyperactivity (183).

Since the Zucker fatty rat is both hyperphagic and hyperinsulinaemic, the obesity of this animal could be a result of these changes, or be an independent primary expression of the gene defect. This latter suggestion would seem appropriate since an increase in body fat in this animal has been reported to occur prior to any detectable rise in insulin or hyperphagia (191, 192). However, it has been demonstrated that fatty acid synthesis in both liver and adipose tissue, and the serum insulin in this strain of rats are close to the levels in lean rats pair-fed on identical meal eating regime. This suggests that the excess lipogenesis in the Zucker fatty rats is secondary to the hyperphagia, hyperinsulinaemia and increased tissue mass (193).

iii) Insulin resistance

With the persistence of hyperinsulinaemia in the Zucker fatty rat, the target organs for insulin become maximally stimulated. Insulin resistance intervenes, probably to protect the animal against the undesirable metabolic consequences of hyperinsulinaemia. The pancreatic output of insulin is either sustained, thus maintaining grossly elevated concentration of insulin and producing a massive obesity, which is typified by the Zucker fatty rat, or islet atrophy occurs causing insulinopaenia, severe diabetes and premature death such as the case in the diabetic mouse (db).

The resistance to insulin appears to be due to several factors either alone or in combination:
1) changes in the intracellular metabolism of target tissues; 2) changes in the state of target tissue insulin receptors. As was shown in Chapter 7 and 8 the insulin resistance of the Zucker fatty rat is most probably due to the last possibility, and will be further discussed here. However, there is no reason to exclude the participation of the other possibility.

A question which puzzled workers in this field is whether the insulin concentration regulates the number of its receptors at the target tissue level or does the insulin receptor number dictate the insulin concentration? I, favour the first possibility. Chronic stimulation of the pancreatic B-cell results in hyperinsulinaemia and that peripheral insulin resistance as manifested by the "down regulation" of insulin receptors is merely an adaptive phenomenon secondary to chronic exposure to high insulin concentration. Considerable information exists in support of this view (34, 41, 43), nevertheless the alternative hypothesis cannot be totally excluded.

The defect in insulin binding in the Zucker fatty rat shown by the experiments presented in this thesis can account for its insulin resistance. The decreased capacity to bind insulin in the tissues examined can be fully accounted for by an actual diminution of the number of receptors per mg membrane protein, the basal kinetics of the receptors remaining otherwise unaltered. Since normalisation of hyperinsulinaemia results in correction of the insulin receptor defect, hyperinsulinaemia has been incriminated, through a mechanism not yet understood in the reduction of receptor sites per cell (194). The abnormality of insulin resistance would thus be secondary, not primary in nature.

The above discussed mechanism for insulin resistance in the Zucker fatty rat is summarized in Fig. 8.1.
Primary Hypothalamic Defect(s)  
(diminished satiety factor?)

↓

Hyperinsulinaemia ⇆ Hyperphagia → Obesity

↓

Changes in the Sensitivity of Insulin Target Tissues

↓

Decreased Insulin Receptors

↓

Insulin Resistance

Fig. 8.1. Proposed mechanism for insulin resistance in the Zucker fatty rat
Recently the concept of one gene/one enzyme has been adapted to explore the genetic transmitted form of obesity in the obese mouse by York, et al. (195). Their work was based on the mechanism of thyroid hormone action in the regulation of energy balance proposed by Edelman, et al. (196). This mechanism of thyroid hormone action involves the sodium pump activity and its stimulation by thyroxine causing an increase in energy expenditure in active sodium transport. This process is apparently controlled by the activity of an enzyme, called NaK-ATPase, which catalysis the reaction of oxygen consumption by various tissues, which in turn is controlled by hormones produced by the thyroid gland.

York, et al. have proposed that a defect in the T3-induced NaK-ATPase may be the basis for the inherited obesity of the obese mice. They have further suggested that this inherited defect could explain many, if not all, of the metabolic changes that have been reported in the obese mice. These include defective thermogenesis, that results in a lower body temperature of these mice, their susceptibility to cold and their increased efficiency of food utilisation. These abnormalities could lead to obesity by reducing the animals ability to burn off extra carbohydrate in the tissues and at the same time would reduce the animal's ability to generate extra heat in cold conditions. They have also proposed that the low activity of NaK-ATPase in the pancreatic islets might be the basis of the hyperinsulinaemia of this strain of mice, without actually measuring its activity within the pancreas.

Although this discovery was considered of great value in the understanding of the aetiology of inherited animal obesity, there still remains several questions to be answered by this proposed mechanism. 1) The precise defect in the thyroid dependent ATPase system in the obese mice still awaits further clarification. 2) The exact role of such a defect in the development of hyperphagia and hyperinsulinaemia in the obese mice. 3) The NaK-ATPase-sodium
pump mechanism for thyroid hormone action on which this proposal is based, is merely one of several mechanisms proposed for the action of this hormone. There are at least six others with equally convincing supporting data. These include nuclear transcription, mitochondrial activation, tyrosine incorporation, adrenergic receptor sensitivity, membrane action or more likely a combination of one or more of these proposed mechanisms (197).

Although the Zucker fatty rat shares many abnormalities with the obese mouse, it still remains to be seen whether the above discussed proposed mechanism for the obese mouse may be applicable in the case of the Zucker fatty rat. This is made unlikely by the light of experiments carried out on the Zucker fatty rat by Powley, et al. (198). These workers have shown by hypophysectomy experiments on the Zucker rat that the pituitary and the major endocrine pathways it controls (which include the thyroid) are not the locus of the primary genetic lesion responsible for maintaining the Zucker fatty rat's obesity. They have concluded that the obesity of the Zucker rat is much more massive than the modest adiposity occasionally attributed to hypothyroidism. This conclusion for the Zucker fatty rat would indirectly add a further point against the above mentioned proposed mechanism for inherited obesity. Until these points are fully clarified, I still hold the view that animal, as well as human, obesity is most likely not to be found in a single enzymatic or metabolic defect nor is it the result of any single psychogenic factor. I further believe that a defect in the NaK-ATPase found in the obese mouse is only one more addition to the already existing long list of many enzymatic and metabolic defects found in this and other strains of obese animals.

b) Hypertriglyceridaemia and glucagon resistance in the Zucker fatty rat

Obesity in the Zucker fatty rat is associated with marked hypertriglyceridaemia. As was previously mentioned, the concentration of plasma triglycerides theoretically may be governed either by the rate of triglyceride
production, by the rate of triglyceride removal or by both.

i) Triglyceride turnover rate

Hyperlipaemia is a major consequence of obesity and the incidence of hypertriglyceridaemia is high in human obesity (5). It is possible that triglyceride turnover rate in the fatty rat is increased compared to its lean litter mate. Insulin plays an important role in triglyceride metabolism. It has clearly demonstrated that insulin enhances triglyceride formation from its-precursors (19-25). The Zucker fatty rat is hyperinsulinaemic. However, this hyperinsulinaemia is associated with insulin resistance both in the liver and adipose tissue. The diminished responsiveness to insulin may result in increased flux of FFA and glucose precursors to the liver leading to over production of triglycerides.

ii) Triglyceride removal rate

The present study demonstrates a more than four-fold increase in lipoprotein lipase activity in the fatty rat compared to its lean control. If this activity is taken to reflect the triglyceride removal rate, then this animal should show signs of hypotriglyceridaemia rather than hypertriglyceridaemia which actually prevails. This discrepancy could be explained as follows:

1) Lipoprotein lipase is the enzyme system believed to regulate the rate of uptake of plasma triglycerides by adipose tissue. It was measured in the present study in vitro using a commercial pure triglyceride substrate. This may not necessarily reflect the true conditions in vivo, where inhibitors may exist to this enzyme. However, this is made unlikely due to the presence in excess of lipoprotein lipase activators (apolipo proteins) rather than inhibitors in these fatty rats (89).
2) It may be that these alterations in lipoprotein lipase activity and its activators levels represent attempts to adapt to increased levels of triglyceride in the fatty rats, which is probably insufficient to cope with such high triglyceride increases. Saturation of such removal system by excess triglycerides could result in impaired disposal and accumulation of triglycerides leading to hypertriglyceridaemia.

3) It is suggested from the present series of studies that mechanism 2 is operating in this type of fatty rat coupled by a probable enhanced triglyceride synthesis and is proposed here to account for the Zucker fatty rat hypertriglyceridaemic syndrome.

Eaton et al. (66, 67) have put forward a hypothesis which could account for inherited and induced hyperlipaemia. This hypothesis as previously mentioned (Chapter 1) involves reduced glucagon secretion, reduced glucagon responsiveness, and thus resistance to the action of both endogenous and exogenous glucagon. They have presented evidence from work carried out on the Zucker fatty rat and the CoCl$_2$-treated rat, both of which are the subject of the present study.

In one study (66) they reported high levels of glucagon in patients with endogenous hyperlipaemia as well as in some experimental animals. They
concluded that glucagon resistance may result in the inappropriate increase in serum glucagon concentration. However, in another report (52) when testing obese patients with endogenous hyperlipaemia, they reported decreased plasma glucagon levels.

In the Zucker fatty rat the same group reported (144) reduced basal plasma glucagon, in response to fasting and in response to pharmacologic glucagon stimulation (arginine). In the CoCl$_2$-treated rat they reported increased glucagon secretion and a resistance to the action of this hormone both in vivo and in vitro (67, 200). They suggested from these studies that the loss of the normal hypolipaemic action of glucagon in conditions of decreased glucagon activity could be responsible for the development and/or maintenance of endogenous hyperlipaemia.

Evidence from the present study as well as from similar studies carried out by other workers do not lend support to such a hypothesis. Elkeles and Hambley (63) did not find glucagon resistance after glucagon administration to hyperlipaemic subjects. Bryce, et al. (201) showed no difference in the basal plasma levels of glucagon between Zucker lean and fatty rats. Laburthe, et al. (98) reported increased plasma glucagon levels in the Zucker fatty rat compared to the lean as I did.

In insulin resistant states as demonstrated by the present study, such resistance is associated with decreased insulin receptors at the target tissue level. Liver and adipose tissue plasma membranes glucagon receptors showed no difference in their binding capacity or association constant between fatty and lean rats. The same observation was also made for the CoCl$_2$-treated and control animals. The glucagon resistance hypothesis, cannot, therefore be explained on the basis of reduction in the number and avidity of both liver and adipose tissue glucagon receptors as was the case in the insulin resistant state. Furthermore, adenylate cyclase levels, the system
by which glucagon is believed to exert its action showed no difference in either basal or stimulated levels in liver and adipose tissue plasma membranes of both animal models used for the present study. However, decreased responsiveness to glucagon as measured by adenylate cyclase activity was observed in the fatty rat (Chapter 7). This was however, only evident after subjecting these rats to external high doses of glucagon, which is unlikely to occur under normal circumstances.

Neither the Zucker fatty rats nor the CoCl$_2$-treated rats showed any significant changes either at basal or arginine stimulated levels of glucagon compared to their respective controls.

In conclusion it can be said that there is little evidence in support of the "glucagon resistance" hypothesis that can be drawn either from the present study or other studies carried out by other workers. The decreased activity of glucagon, if it exists in hyperlipaemic syndromes, particularly when associated with obesity, could simply be explained by the hyperinsulinaemic states associated with such syndromes, e.g. Zucker fatty rat and the obese mouse, more than counterbalancing the lipolytic action of glucagon. In such cases it is advisable to consider the activities of the lipogenic hormones, e.g. insulin on one hand versus the activities of the lipolytic hormones, e.g. glucagon on the other, rather than to consider the activity of each hormone independently.

8.2 The Cobalt-Chloride Treated Rat:

The present work has shown that treatment of animals with CoCl$_2$ gives rise to a polycythemic state of shorter duration than the accompanying hypertriglyceridaemic state, which is in agreement with previous reports (100-110). The polycythemic affect has been attributed to the action of cobalt on the erythropoietic system (202) by initiating a state of histotoxic (tissue) hypoxia (203).
The mechanism of CoCl\textsubscript{2}-induced hyperlipaemia in these experimental animals is poorly understood (107-109). The hypothesis that prevailed prior to the Eaton, et al. hypothesis of glucagon resistance (discussed under Section 8.1) was based on the work of Van Compenout and Cornelis (110) and assumed that cobalt selectively destroyed the A-cells (the glucagon producing cells) of the pancreatic islets. This causes acute glucagon release followed by chronic glucagon deficiency which then gives rise to elevated plasma glucose and lipid levels. It is obvious that all the cobalt effects cannot simply be explained by such mechanism.

From the results of the present study it can be seen that no insulin or glucagon abnormalities can be detected in the CoCl\textsubscript{2}-treated rat compared to its control. Glucagon levels either in the basal state or after arginine stimulation in the CoCl\textsubscript{2}-treated rat are found to be within normal range as set by the control rats. The present study suggests that the hypertriglyceridaemia of the CoCl\textsubscript{2}-treated rat can be accounted for by decreased triglyceride removal rate from the plasma caused by decreased lipoprotein lipase activity. Further supporting evidence and a possible mechanism is presented as follows:

i) The role of cobalt in enzyme activity

Metal ions play important roles in the biological function of many enzymes. The various modes of metal-protein interaction include metal-ligand and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators (204). While certain metals are essential as co-enzymes for the activity of certain enzyme systems, other metals when present interfere and inhibit many enzymes. For example organo-phosphorus compounds can form covalent bonds to serine-containing enzymes such as trypsin, cholinesterase and phosphoglucomutase, organic mercurials form covalent mercaptide bond to cysteine-containing enzymes, trivalent arsenicals form As-mercaptides with disulphide containing enzymes such as dehydrogenase
enzymes, heavy metals such as \( \text{Ag}^+ \), \( \text{Hg}^+ \), etc. form covalent metal salts with histidine-containing enzymes. Metal-complexing agents and metal-chelating agents can all inhibit enzymatic activity (216).

Apart from its presence in vitamin \( \text{B}_{12} \), cobalt, has to date been found to be a component of only one enzyme, the biotin-dependent zinc-containing oxaloacetate transcarboxylase (205).

Cobalt has been found both to enhance and to inhibit the activity of many enzymes, thus affecting the metabolic routes these enzymes control. Webb (206) has shown that cobalt under aerobic conditions, irreversibly chelates with the two reduced disulphide groups of lipoic acid, a co-enzyme required for the oxidative decarboxylation of pyruvate to acetyl Co-A and \( \alpha \)-keto glutarate to succinate. Cobalt inhibits both glycolysis and respiration by forming a complex between the ion and \(-\text{SH}\) group of lipoic acid (150).

Cobalt elevates the activity of plasma protease and cathepsins A and B, hydrolytic lysosomal enzymes involved in the erythropoietin system (202). Cobalt also induces the activity of haem oxygenase (207). Cobalt inhibits liver haem synthesis \textit{in vivo} by inhibiting the activity of 5-amino-laevulinate synthetase (208). It has also been reported to increase the activity of arginine esterase, decrease plasma bradykininase and increase plasma protease activity (203).

In common with many other hydrolytic enzymes, lipases are inhibited by several heavy metals. Milk lipase is strongly inhibited by \( \text{Cu}^{++} \) and less effectively by \( \text{Fe}^{+++} \), \( \text{Ni}^{++} \), \( \text{Co}^{++} \), \( \text{Cr}^{++} \) and \( \text{Zn}^{++} \) (209). Pancreatic lipase (EC. 3.1.1.3) has been a subject of lengthy investigations. It has been shown that this enzyme is inhibited by \( \text{Cu}^{++} \), \( \text{Hg}^{++} \) and \( \text{Zn}^{++} \) (209) through the oxidation of \(-\text{SH}\) groups.
By the use of several specific sulphydryl reagents, many attempts have been made to establish whether lipases in general are -SH enzymes. Early studies showed that pancreatic lipase was unaffected by 0.01 M iodoacetic acid (209). Singer and Baron (210) found that although pancreatic lipase was unaffected by $10^{-3}$ M iodoacetamide, it was inhibited by p-chloromercuri-benzoate and trivalent arsenicals (210). Wills (211) found that versene, a metal complexing agent strongly inhibited pancreatic lipase while most oxidising agents which affect -SH groups had little or no effect. It was concluded that the enzyme possess -SH groups that are not part of the active centre but lie within close proximity of it. Sites other than -SH groups must, therefore, be involved and histidine was suggested as part of the active site of pancreatic lipase. This was later confirmed by Sermeriva, et al. (212) and Verget, et al. (213).

ii) Cobalt and lipoprotein lipase

Lipoprotein lipase has the same numbering system (EC. 3.1.1.3) of international enzyme nomenclature as that of pancreatic lipase, because it is very similar in action and probably in structure to pancreatic lipase. Recently it has been obtained in pure form from post-heparin plasma (214) and rat heart (215) which facilitated studies on its molecular, chemical and physical-properties.

Histidine (217) and cysteine (218) have been implicated in the active site of lipoprotein lipase. The imidazole group of histidine and sulphydryl group of cysteine are nucleophilic (electron-donating), whilst cobalt is an electrophile (electron-seeking). Nucleophilic groups readily react with electrophilic groups and vice versa. There are two possible mechanisms by which cobalt can interfere with the activity of lipoprotein lipase:

a) Cobalt being an electrophile can combine reversibly with the
nucleophilic sulphydryl group or the imidazole group of the active site of lipoprotein lipase (non-competitive inhibition).

b) Lipoprotein lipase like many other enzymes, is probably a metalloenzyme which contains a metal as an integral part of the protein molecule. Although the nature of the metal still remains to be established, cobalt may compete and displace it thus causing reduced activity as demonstrated in the case of CoCl$_2$-treated rats. For example, carboxypeptidase A is a classic zinc metalloenzyme (204). Removal of the metal atom by dialysis or by treatment with chelating agents gives a totally inactive enzyme. Activity can be restored by addition of zinc or a number of other metal divalent ions. The cobalt enzyme, for example, has twice the peptidase activity of the zinc enzyme while the nickel and manganese enzymes are much less active. Whilst cobalt enhances the activity of zinc peptidase it may act to reduce the activity in case of lipoprotein lipase.

iii) The role of cobalt in triglyceride turnover rate:

Besides its action on plasma triglyceride removal rate, cobalt may have a role in plasma triglyceride turnover rate. This role can be accounted for as follows:

Webb (206) and Dingle, et al. (150) have shown that cobalt irreversibly chelates with the two reduced disulphide groups of lipoic acid, a co-enzyme required for the oxidative decarboxylation of pyruvate to acetyl CoA and $\alpha$-ketoglutarate to succinate. By such interference with cell respiration at the tricarboxylic acid cycle, cobalt causes a state of tissue hypoxia.
The glycolytic pathway is directly linked to the tricarboxylic acid cycle, so that a metabolic block of the latter will result in accumulation of intermediary metabolites at the former. Such increased metabolites have been shown to occur in the CoCl₂-treated rat. Dihydroxyacetone phosphate, fructose diphosphate, glyceraldehyde-3-phosphate and α-glycerophosphate have been shown to be increased in liver extracts of CoCl₂-treated rats (147, 219). It has been further shown that the myocardium of rats treated with cobalt is unable to oxidise fatty acids (148). Both α-glycerophosphate and fatty acids are prerequisites for triglyceride formation.

With such metabolic block and the availability of these triglycerides precursors, the mechanisms of triglyceride formation are activated, resulting in increased hepatic triglyceride turnover rate.

These and previously discussed abnormalities leading to the state of hypertriglyceridaemia in the CoCl₂-treated rat is summarised in Fig. 8.2.

8.3 Conclusions:

1) The Zucker fatty rat is a model of inherited obesity associated with hyperlipaemia, hyperinsulinaemia and insulin resistance. Insulin resistance can be accounted for by a decrease in insulin receptors at its target tissues in an attempt by the animal to combat the harmful effects of hyperinsulinaemia.

Hypertriglyceridaemia of this strain of rats is associated with a probable high triglyceride output and increased lipoprotein lipase activity. This high activity if taken as a measure of triglyceride removal rate is insufficient to cope with the very high plasma triglyceride levels.
2) The CoCl$_2$-treated rat is a model of induced polycythemia associated with hypertriglyceridaemia. The hypertriglyceridaemia can be accounted for by a probable increase in triglyceride turnover rate coupled with decreased triglyceride removal rate. The probable increase in turnover rate is due to the toxic effect of cobalt on the tricarboxylic acid cycle, blocking it at two sites, causing tissue hypoxia and accumulation of intermediary metabolites of the glycolytic pathway. Two of these metabolites are dihydroxyacetone phosphate and glyceraldehyde 3-phosphate which can be converted to $\alpha$-glycerophosphate, a precursor for triglyceride synthesis. The decreased triglyceride removal rate is believed to be due to the inhibition of lipoprotein lipase by the reaction of cobalt with the imidazole group of histidine and/or the sulphhydryl group of cysteine, the two amino acids believed to form part of the active site in this enzyme.

3) Glucagon plays very little, if any role either in obesity of the Zucker fatty rat or its hyperlipaemia. Neither does it have a role in the hypertriglyceridaemia of the CoCl$_2$-treated rat as demonstrated in the present series of studies.
Effect of cobalt on intermediary metabolism

Fig. 8.2


