ASPECTS OF LIPOLYSIS IN SHEEP

A THESIS PRESENTED IN PARTIAL
FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF AGRICULTURAL SCIENCE
IN ANIMAL SCIENCE AT
MASSEY UNIVERSITY

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1983

ABSTRACT

1. Treatments which might be expected to produce lipolytic responses were applied to ewes. These treatments included fasting and the administration of ACTH, noradrenaline and BGH. Twelve experiments are reported, 3 of which involved indirect calorimetry and 9 which involved measuring the responses of blood hormones and metabolites including GH, insulin, FFA's and glucose.

2. Two groups of ewes were used. The first group consisted of 2 Romneys and 2 Southdowns. Each breed pair comprised a short, fat and a long, lean phenotype which may have been genotypically different in their propensity to be overfat. The second group of ewes comprised 4, 5 year cull Romney ewes.

3. Calorimetry showed that there were no differences in the normal fed metabolic rate of the first group of sheep sufficient to explain their differences in fatness.

4. Fasting (2-3 days) resulted in significantly elevated mean afternoon plasma FFA levels in the long, lean ewes.

5. The fat ewes had steeper regressions of RQ on time when fasted than the lean ewes, which probably indicated a greater rate of lipolysis.

6. The injection of ACTH into ewes produced inconclusive results in terms of plasma hormones and metabolites.

7. Injection of the catecholamine, Bronkephrine into the cull Romney ewes, did not produce any significant effects on blood hormones and metabolites in one study. In a second study N.A. resulted in transitory peaks in GH insulin and FFA's. These results were confused with concurrent responses to feeding.

8. Fasting cull Romney ewes resulted in higher mean afternoon levels of GH and FFA. Insulin levels were lower than in fed sheep but differences were not statistically significant.

9. Administration of ACTH and N.A. did not produce detectable changes in heat production or respiratory exchange ratios. However these studies were complicated by ruminal CO_2 production following feeding.

10. Both BGH and PBS infusions produced marked declines in plasma insulin and increases in FFA levels.

11. It is concluded that the prime control of lipolysis in sheep is probably the autonomic nervous system through the release of N.A. at sympathetic nerve endings. GH and insulin secretion are neurally mediated and these hormones have important roles in directing the transfer and utilisation of metabolites between tissues. GH potentiates lipolysis, defends tissue protein stores and promotes the transfer to and utilization of FFA in productive tissues such as muscle and mammary gland. Insulin is primarily anabolic and antilipolytic in adipose tissue. It antagonises GH action in adipose tissue yet supports the anabolic role of GH in the productive tissues.

ACKNOWLEDGEMENTS

I am grateful to my supervisors Prof. D.S. Flux and Dr R. Purchas for their help and guidance.

Thanks also to; Dr C.W. Holmes for expert help with the calorimetry, Dr D.D.S. MacKenzie for helpful discussions and advice on biochemical and physiological matters, and to other members of the department for advice and discussions.
Dr R.M. Greenway for glucose analyses and other analytical information.
Dr J.M. Gooden for discussions on fatness and advice on the Dole analysis.
Dr S.N. McCutcheon for help and company on the night shift, as well as A.K. Gibson, J. Lockyear and B. Parlane for attending the sheep.
Prof. R.E. Munford for SORD programmes and G.S. Stinson for sort programmes. Christine Aldridge, Veronica McKeay-Christie, Alison Wright, Robin Christienson, Janis Frain and Wendy Nelson for technical assistance.
Veronica and Mark Fieldsend for typing the script.
My friends for their encouragement, threats and support and my parents for instilling the ambition.

"What I do is me, for that I came" G.M. Hopkins

CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER ONE

RATIONALE

1.1 THE PROBLEM OF OVERFATNESS IN MEAT ANIMALS

Kirton (90) stated that intensive animal production appeared to be encouraging the conditions described as the major prerequisites for obesity in man. The problem of overfatness in farm animals has been described by Allen et al (1), Barton (4), Bauman (16) and Kirton (90). The major problem is that consumers prefer lean meat for a variety of reasons which include palatability, cost, and the belief that excessive intake of animal fats may be detrimental to human health. According to Frazer (59) one British researcher commented; "at the heart of the changes in market requirements is the increasing consumer demand for lean meat and reluctance to pay for fat...". Results of a Meat Research Institute survey (59) indicated that 65% of children and 45-51% of adults cut off all lamb fat and leave it on their plates. Broad (28) indicated that the pressure to eliminate excessive fatness in meat producing animals will increase and that the rising cost of energy makes animal fat a luxury we are less able to afford.

Overfat carcasses incur additional processing costs as they must be trimmed. Allen et al (1) reported that 4 billion pounds of excess fat were trimmed annually from carcasses in the U.S.A. while Bauman (16) quoted a weight of 1 billion kg trimmed from beef alone representing a cost of US$1.15 billion.

1.2 GRADING SYSTEMS AND DISINCENTIVES TO OVERFATNESS

As a disincentive to production of overfat animals, meat industry authorities penalise overfat carcasses through grading systems. The New Zealand Meat Producers Board have an F grade for lamb and an MF grade (114) for hogget, wether and ewe mutton carcasses which are regarded as excessively fat for all markets and carcasses in

these grades may not be exported without cutting and trimming (90).

The number of lamb carcasses graded F in the 1980-81 season was 308,414 (0.99%) and the MF grade contained 201,349 (2.85%) (114). However a recent survey of British wholesalers and retailers indicated that they had a strong preference for lambs with a subcutaneous fat cover over the rib eye area of no more than 4mm (59). Frazer (59) stated that "if we were to have fully met this requirement within our 1982 export production, then around 25% would not qualify".

1.3 THE PRESENT STUDY

Accordingly the present study was set up as a pilot study to investigate the factors controlling lipid metabolism in N.Z. sheep and to attempt to discover differences in lipid metabolism between genetically fat and lean ewes, with a view to identifying genetically superior sheep for use in breeding programmes.

Whereas aspects of lipid metabolism in sheep have been studied no published work has attempted to define the metabolic and endocrine differences between genetically fat and lean ewes. In any case the factors controlling lipid metabolism in sheep in general were not clear at the outset of this work (and indeed are still far from perfectly understood).

The objectives of this study then were: -

1. to investigate and discover differences in lipid metabolism between genetically fat and lean ewes by administering treatments designed to stimulate lipolysis.

2. to characterise the effects of the treatments in terms of plasma hormones and metabolites and calorimetric measurements.

Owing to the loss of experimental animals and their replacement with ewes of unknown genetic background objective 1 was modified as follows:

3. to identify metabolic differences between individual ewes which may correlate with possible differences in fat metabolism.

An overview of the experimental design is presented in section 3.9.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 INTRODUCTION

Adipose tissue performs two major metabolic functions; the synthesis of lipids and the mobilization of lipids. For both ruminents and other species, the relative importance of these processes is dependent upon the balance between energy intake and energy requirement for maintenance and productive functions (16). Fatness is the result of the relative rates of lipogenesis and lipolysis. The balance between lipogenesis and lipolysis is altered by both short-term (acute) controls and long-term (chronic) controls. Acute changes are noticed on an hourly basis, while chronic changes occur over a period of weeks or even months. Intuitively lipogenesis must involve chronic controls whereas lipolysis must involve acute overiding mechanisms (though not exclusively).

Because of the time limits imposed upon this study it was decided to investigate the acute controls of lipolysis rather than attempt to examine all the controls of fat metabolism. This has necessarily restricted the scope of conclusions reached but since the control mechanisms of lipolysis and lipogenesis are most likely to be closely interrelated, some discussion of lipogenic factors was inevitable.

Initially the metabolic pathways of lipogenesis and lipolysis are described in secton 2.2. The control of lipogenesis is reviewed in section 2.3 followed by the control of lipolysis in section 2.4 where the major emphasis is directed to endocrine factors. Evidence for genetic differences in fat metabolism is reviewed in section 2.5 followed by a brief summary of the whole review.

2.2 PATHWAYS OF OVINE LIPID METABOLISM

2.2.1 Introduction

The pathways of ruminant lipid metabolism are relatively well established (16) and have been reviewed extensively in recent years (3, 16, 17, 51, 52, 146, 156). The following review summarizes the salient features with a view to identifying the likely points of control. The pathways are described for ruminants in general with specific reference to ovine metabolism where necessary. Lipogenesis and lipolysis are considered separately.

2.2.2 Lipogenesis

2.2.2.1 Precursors; their sources and transport

Fatty acids which are stored with glycerol as triglycerides in adipose tissue arise from two sources, the uptake of preformed fatty acids (predominantly from gut absorption) and de novo synthesis of fatty acids from other metabolites (16, 146).

Fatty acids released into the blood by adipose and other tissues are transported bound to albumin (137) and other blood proteins with a small amount of fatty acid in true solution (51). The synthesis and mobilisation of these fatty acids is described later in this chapter.

Cells of the intestine receive fatty acids from blood (endogenous) and from the digesta (predominantly exogenous). These are mostly 16-18 carbons in length and are esterified to form triglycerides, phospholipids, and cholesterol esters (51). Apolipoproteins are complexed with them and the lipoprotein is secreted into the intestinal lymph and transported to the blood (51). About 75 percent of the lipid in lymph is associated with the very low density lipoproteins (VLDL) and 25 percent with the chylomicrons (95). The lipid they contain is mainly in the form of triglyceride (95, 146) (though normally a smaller proportion than in non-ruminants (51)) and is predominantly saturated (16, 95).

The terminology used to describe the lipoproteins (including chylomicrons) in the literature is confusing. For example Havel (74) reserved the term chylomicron for lipid entering blood from the lymph, while VLDL

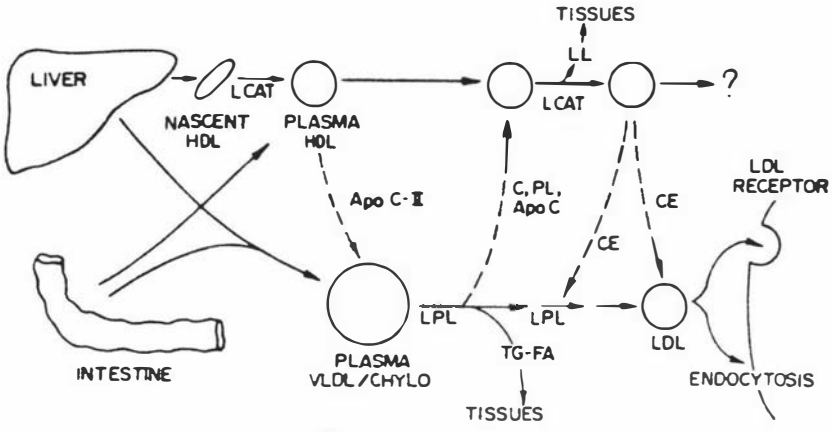

Figure 2.1 Outline of lipoprotein metabolism.
Hydrolysis of the chylomicron and VLDL core is shown with concomitant transfer of surface components, cholesterol (C),phospholipid (PL),and apoC to HDL, with subsequent uptake of triglyceride fatty acid (TG-FA) into tissues.The LCAT reaction poduces lyso lecithin (LL) and cholesterol ester (CE).Broken lines (----) indicate nonenzymatic net transfer of lipoprotein components.

(after Nilsson-Ehle et al (115))

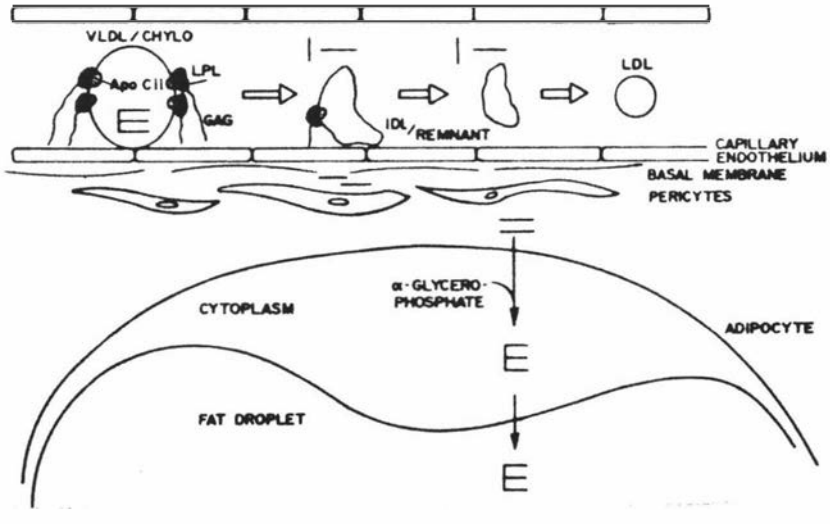

Figure 2.2 Scheme for triglyceride assimilation.
LPL located on glucosaminoglycan (GAG) residues catalyses the hydrolysis of lipoprotein triglyceride(E) producing fatty acid (—) and glycerol (|).

(after Nilsson-Ehle et al (115))

referred to lipoprotein entering blood from the liver (74). However, as mentioned above others describe both chylomicrons and VLDL in lymph (95). This confusion may in part be due to differences between human and ruminant lipoproteins and the techniques used to study them (51). In their recent review Nilsson-Ehle, Garfinkle, and Schotz (115) indicated that the intestine is a source of chylomicrons and VLDL as well as HDL (high density lipoprotein) although they did not specify species (see Figure 2.1). Other terms such as the classification of lipoproteins as A, B and C as described by Emery (50) appear to be redundant now, so the review of Nilsson-Ehle et al (115) is used as the basis for the terminology used to describe lipoproteins in this thesis.

The lipoprotein classes are designated as HDL, LDL and VLDL. Chylomicrons are large lipoproteins of extremely low density derived from the intestine. All plasma lipoproteins share the same fundamental structure i.e. a hydrophobic core (mainly triglyceride and cholesterol esters) surrounded by a surface film of phospholipid cholesterol and specific proteins (115). The density and lipid content of these lipoproteins depends partially on their apolipoprotein content (51). Eight major apolipoproteins have been identified and are distributed among the lipoproteins of different density classes. In addition to their role in maintaining lipoprotein structure, some apolipoproteins serve as cofactors for key enzymes in lipid metabolism (115).

VLDL and chylomicrons transport triglyceride from the liver and intestine for utilisation or storage in peripheral tissues. Both of these triglyceride-rich lipoproteins are delipidated by the action of lipoprotein lipase (LPL) (described later) to produce triglyceride-poor LDL particles. The LDL is eliminated from the circulation in peripheral tissues by endocytosis that may or may not be receptor mediated. The function of HDL is exceedingly complex. It serves as a reservoir for apolipoproteins necessary for the catabolism of triglyceride-rich lipoprotein and in concert with LCAT (lecithin cholesterol acyl transferase) participates in the metabolism of the lipoprotein components, cholesterol and phospholipid. HDL is also believed to transport cholesterol to the liver for excretion (115).

Since lymph lipids enter the vena cava rather than the hepatic portal circulation they are available to the peripheral tissues before they reach the liver. Following the initial hydrolysis of chylomicrons and VLDL triglyceride by LPL in peripheral tissues, the particles produced may be modified on return to the liver. Nilsson-Ehle et al (115) reviewed evidence for possible actions of hepatic lipase. Liver also receives fatty acids from the blood, some of which are then esterified and complexed with apoliproprotein to reform lipoproteins which are secreted into the hepatic venous blood (51).

In the plasma of adult sheep 70 percent of the lipid is transported as HDL and 17 percent as LDL. VLDL and chylomicrons account for less than 5 percent of the total lipoprotein (95) (following peripheral metabolism and liver modification).

Since typical ruminant diets contain only 2-5 percent fat (16, 139), a major portion of the fat deposited in adipose tissue (or secreted into milkfat) must be synthesised (de novo synthesis) (51). There are marked differences between ruminant and non-ruminant tissues with respect to the kinds of substrates used for fatty acid synthesis (17). Once the ruminant has acquired a functional rumen, acetate becomes the principal carbon source for fatty acid synthesis in adipose tissue (16, 17, 51, 156). β-hydroxybutyrate can also be incorporated into fatty acids as the initial four-carbon primer unit (17). Acetate and β-hydroxybutyrate along with propionate are the major products of carbohydrate digestion in the reticulo-rumen, so in the fed sheep these precursors should be freely available.

Fatty acid esterification requires a source of glycerol 3-phosphate which in sheep adipose tissue is mostly synthesised from glucose via the glycerol-3-phosphate dehydrogenase reaction as the activity of glycerol kinase is very low (156). Since little glucose (if any) is obtained from digestion it must be produced by gluconeogenesis (in the liver) primarily from propionate (17).

2.2.2.2 Uptake of preformed fatty acids: Lipoprotein Lipase

Uptake of circulating triglycerides by adipose tissue requires hydrolysis of lipoprotein triglyceride at or near the adipocyte membrane, passage of the resulting fatty acids into the cell and, finally, esterification of these acids with glycerol 3-phosphate generated from glucose (1).

Circulating VLDL and chylomicrons bind to capillary walls where the physiologically active LPL is localised on glucosaminoglycan chains anchored to the lumen of the endothelial cells (115) (see Figure 2.2). The binding is probably due to an interaction between apoC-II on the lipoprotein particle and LPL (51, 115). In addition interactions between the glucosaminoglycan chains and lipoprotein surface structures and between LPL and lipoprotein lipid may aid immobilisation of the lipoprotein particle. It is thought that the triglyceride may be hydrolysed during a series of attachments and detachments from LPL at a number of sites along the capillary endothelium (115).

The contact between enzyme (LPL) and triglyceride results in the rapid production of fatty acids, some of which may escape into the circulation (51, 115). Most of the glycerol is released into the blood for utilisation elsewhere (51). However most of the fatty acids are transported through the endothelial layer into tissue cells (115) following the increased local concentration (50, 51). The mechanism of transport is unknown although it has been suggested that they may move by lateral diffusion in cell membranes (115).

The uptake of FFA's from plasma sources other than lipoproteins does not appear to have been described in the literature. Presumably this is because the lipoproteins are the major source of fatty acids for adipose tissue (156). It is probably fair to assume that FFA's are transferred in the same way as fatty acids from lipoproteins.

2.2.2.3 De novo fatty acid synthesis

As mentioned earlier a major portion of the triglyceride deposited in adipose tissue must be produced by de novo synthesis (51). Furthermore it has been suggested that in the non-lactating sheep, adipose tissue

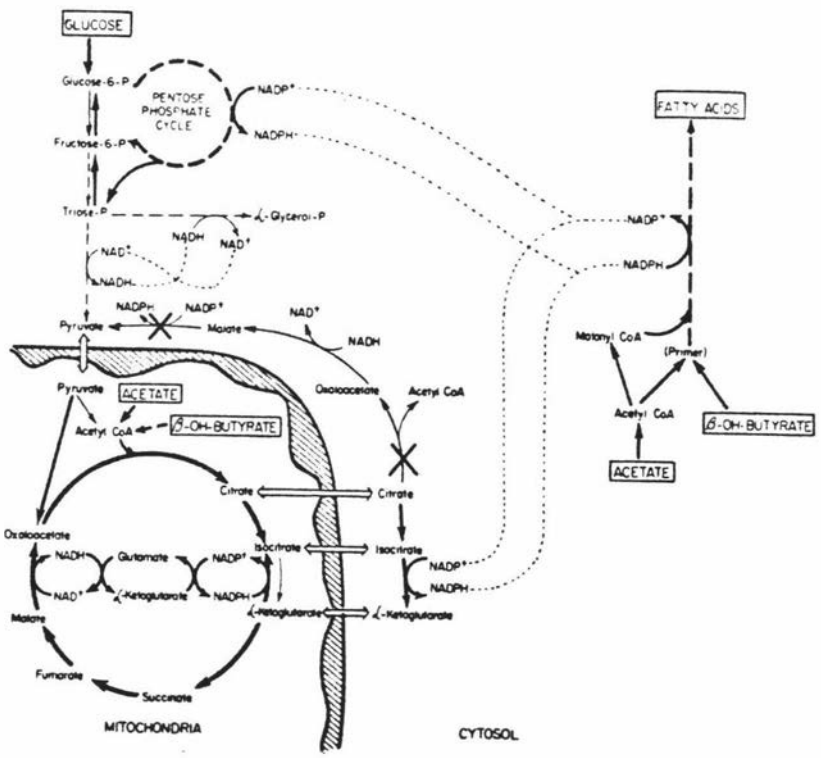

Figure 2.3 Pathways of fatty acid synthesis in ruminant adipose tissue.

The negligible activities of ATP citrate-lyase and NADP-malate dehydrogenase are denoted by X.

(after Bauman, D.E. and Davis, C.L. (17))

is responsible for more than 90 percent (17, 156) of fatty acid biosynthesis i.e. adipose tissue is the only important site of fatty acid synthesis (156). Certainly it appears to be the major site (3, 16, 17, 51) with liver playing only a minor role in this respect (17).

According to Allen et al (1) at least three intracellular sites of fatty acid synthesis are recognised:

(i) cytoplasm,
(ii) mitochondria and
(iii) microsomes.

The cytoplasm is responsible for de novo synthesis of fatty acids while the mitochondrial and microsomal systems are responsible for elongation of absorbed or de novo synthesised fatty acids (1).

It is well established that ruminant adipose tissues utilises acetate but very little glucose for fatty acid synthesis (1, 16, 17, 156). Essential to the utilisation of glucose for fatty acid synthesis is the presence of an active citrate-cleavage pathway for the translocation of mitochondrial acetyl-CoA to the cytoplasm for incorporation into fatty acids. In sheep adipose tissue the activities of ATP-citrate lyase and NADP-malate dehydrogenase are so low (compared to rat adipose enzymes) that the pathway is virtually absent (3, 16, 17, 156) (see Figure 2.3). While Bauman and Davis (17) and Bauman (16) said that this is the cause of low glucose utlisation for fatty acid synthesis, Vernon (156) suggested that it may be the result rather than the cause of such low glucose utilisation.

Since mitochondrial acetyl-CoA cannot be translocated to the cytoplasm, de novo fatty acid synthesis utilises acetate in the cytoplasm derived from rumen fermentation. In addition to acetate, β-hydroxybutyrate is used in fatty acid synthesis but only as the initial four-carbon primer (17, 156) because β-hydroxybutyrate dehydrogenase, the first enzyme in the conversion of β-hydroxybutyrate to acetyl-CoA is located almost exclusively in the mitochondria (17).

The source of reducing equivalents (as NADPH) for fatty acid synthesis in ruminant adipose tissue is the pentose phosphate pathway and the isocitrate cycle (16, 17, 156). The advantage of the isocitrate cycle to ruminants is that acetate can be used to generate NADPH (see Figure 2.3) (16). The malate transhydrogenation cycle plays only a very minor role in ruminants because of the low activity of NADP-malate dehydrogenase (16, 17).

Glucose is at a premium in ruminants and originates almost entirely from gluconeogenesis (16, 17, 156). The utilization of acetate as the major carbon source for fatty acid synthesis and in the production of a proportion of the required NADPH (via the isocitrate cycle) represents a distinct physiological advantage for ruminants in that it spares glucose. In fact, the glucose that is metabolised by ruminant adipose tissue is oxidised primarily in the pentose phosphate pathway with extensive recycling occurring (16). The ruminant has another physiological adaptation which is important in sparing glucose. The liver has primary responsibility for gluconeogenesis while adipose tissue carries out the role of excess energy conversion to fatty acids. The fact that these two important synthetic processes occur in separate tissues eliminates the competition for carbon, reducing equivalents and energy (17).

The enzyme sequences involved in the conversion of precursors to fatty acids in ruminant tissues are thought to be the same as in non-ruminants (156). The process has been adequately reviewed by Vernon (156). A point worth noting is that the major energy source of adipose tissue is probably acetate oxidation (156). So acetate provides both the source of energy and the major precursor for fatty acid synthesis.

2.2.2.4 Synthesis of Glycerol 3-Phosphate

As mentioned earlier, glycerol 3-phosphate is mostly synthesised from glucose via the glycolytic intermediate dihydroxyacetone phosphate and the glycerol 3-phosphate dehydrogenase reaction in ovine adipose tissue (156).

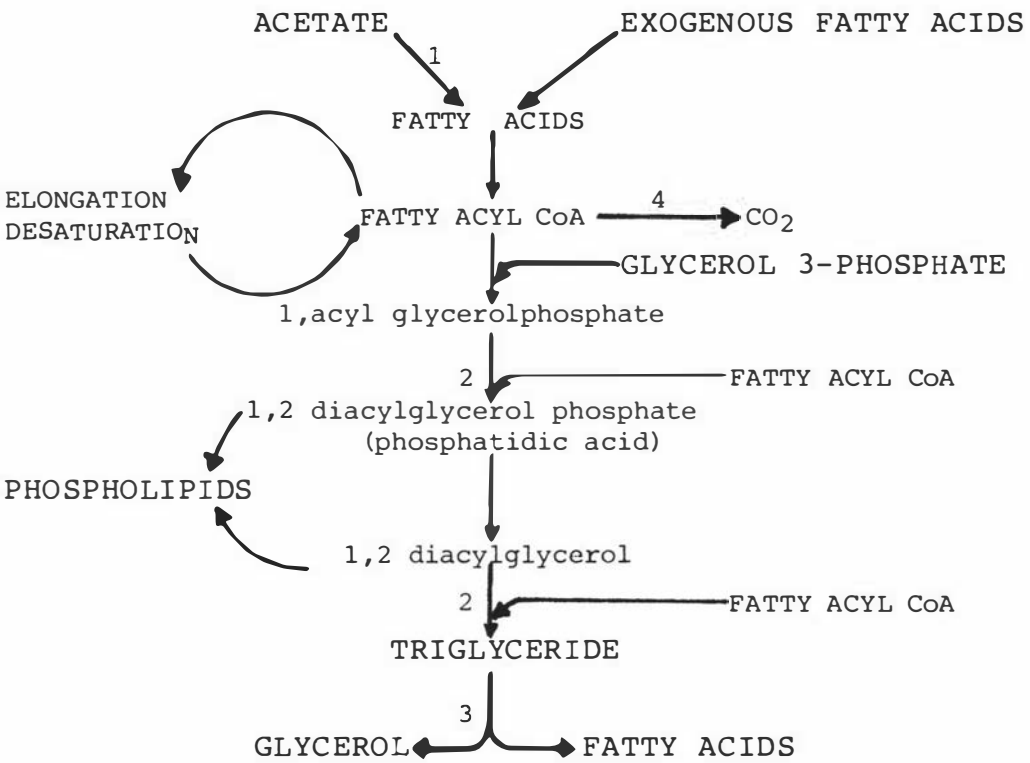

Figure 2.4 Pathways of fatty acid metabolism and lipid synthesis and breakdown.

1 fatty acid synthesis
2 fatty acid esterification
3 lipolysis
4 oxidation

(after Vernon (156))

Dihydroxyacetone phosphate + NADH + H+ = L-glycerol 3-phosphate + NAD+

Dihydroxyacetone phosphate is reduced to glycerol 3-phosphate by the NAD-linked glycerol-3-phosphate dehydrogenase of the cytosol (97). Very low activities of glycerol kinase were reported in ovine adipose tissue and negligible rates of glycerol incorporation into glyceride glycerol or oxidation to carbon dioxide were observed (3, 16, 156). While it has been shown that glyceride glycerol can be synthesised from lactate by the reversal of the glycolytic pathway in ovine adipose tissue slices the rate was only 15 percent of the rate of synthesis from glucose (156). Thus it appears that glucose is the major precursor of glycerol 3-phosphate in ovine adipose tissue.

2.2.2.5 Esterification

The final step in the synthesis of triglyceride in adipose tissue is the esterification of the fatty acids (16). Relatively little is known, however, of the details of the esterification process and its regulation in ruminants (156). It is generally assumed that the glycerol 3-phosphate pathway (see Figure 2.4) is the major, if not the only, pathway of acylglycerol synthesis in ruminant adipose tissue (3, 16, 156).

Glycerol 3-phosphate is acylated to form phosphatidic acid, phosphate is removed by hydrolysis and the resulting diglyceride is subsequently acylated by a third acyl transferase to form triglyceride (51).

2.2.3 Lipolysis

Lipolysis is the hydrolytic cleavage of triglyceride to FFA and glycerol in adipose tissue. The initial step is the cleavage of triglyceride to diglyceride which is catalysed by hormone-sensitive lipase(s) (HSL). The diglyceride is then hydrolysed by further lipases, usually resulting in the complete hydrolysis of the triglyceride to FFA and glycerol (156). This is the process in the non-ruminant and it is thought to be the same in ruminants (156). Apparently no definitive work has been done

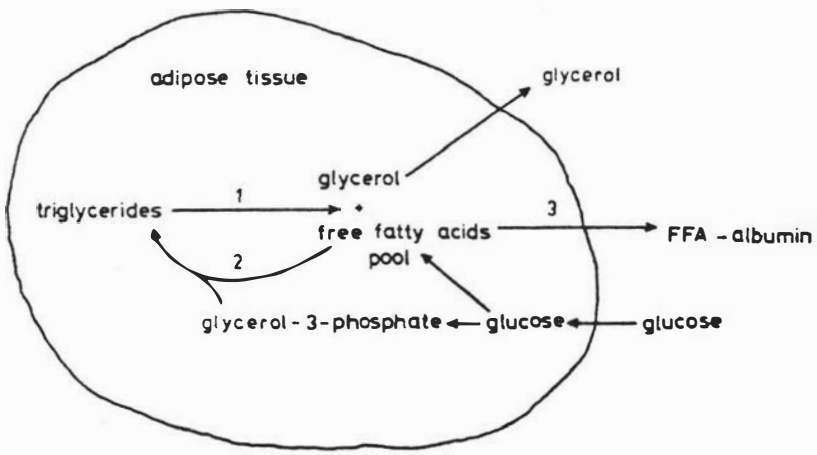

FIGURE 2.5 Lipolysis, re-esterification and FFA release in adipose tissue.

1. Lipolysis
2. re-esterification
3. FFA release

(after Metz and Van Den Berg (111))

in ruminants but there is no evidence to suggest that the mechanisms differ.

The literature contains various names for the enzyme(s) involved in lipolysis, including HSL (1, 16, 156), triglyceride lipase (1) and Fatty Acid Mobilising Lipase (FAML) (51). Most authors use one or more of these terms quite loosely (i.e. non-specifically) and since it can be inferred from Vernons review (156) that there are several lipases involved it is probable that the generalisations in many cases result from a dearth of knowledge in this area. In this thesis the term hormone-sensitive lipase(s) (HSL) will be used specifically to refer to the enzyme(s) catalysing the cleavage of the first fatty acid as this is believed to be the rate limiting step in lipolysis (16, 156).

Partial hydrolysis of triglyceride may occur in sheep during certain stimuli (156). A question that is apparently unanswered is whether or not partially hydrolysed acylglycerides may be reesterified (see Figure 2.5). i.e. while lipolysis is occurring can concurrent esterification complete a futile cycle in which no net release of lipid components from adipose tissue occurs? If so this might represent a metabolic inefficiency that may differ between sheep. Yang and Baldwin (162) reported high FFA : glycerol release ratios from isolated cow adipocytes and suggested that intense partial hydrolysis of triglyceride does occur. However, according to Vernon (156), partial hydrolysis probably does not occur in ruminants, though this opinion was based upon a comparison of FFA and glycerol entry rates and does not clarify the intracellular situation.

Lipolysis has been assessed both in vitro and in vivo. The in vivo measurements have involved determining the FFA entry rate which makes the assumption that essentially all FFA are derived from lipolysis. However some will be derived from the gut and some will be released by LPL so that FFA entry rates do not give an absolute measure of the rate of lipolysis in vivo. Nevertheless changes in FFA entry rate may indicate changes in the rate of lipolysis as may glycerol entry rate.

Changes in plasma FFA and glycerol levels have also been used to assess the rate of lipolysis. Of specific interest is the observation that plasma concentrations of palmitic acid (and oleic but not stearic acid) are proportional to the respective entry rates (156). This may be of importance since the Dole method of FFA analysis (46) used in the present study (see chapter 3) utilises palmitic acid as the standard fatty acid for calibration of the titration system. Plasma FFA concentration is also affected by the rate of FFA utilisation, so changes in plasma FFA levels are but tentative indicators of changes in the rate of lipolysis, but very rapid changes in FFA levels might be expected to reflect changes in the rate of lipolysis (156).

2.3 CONTROL OF LIPOGENESIS

2.3.1 Introduction

Having outlined the metabolic pathways, this section considers factors affecting lipogenesis and probable sites of regulation. External factors such as diet will not be considered directly since they are expected to exert their efforts through the mechanisms described herein.

2.3.2 Supply of Precursors

The metabolism of ruminant adipose tissue is markedly influenced by both the quantity and the quality of the diet. Weekes and Godden (159) recently reviewed the effects of diet on metabolic hormones, Broad (28) reviewed the effects of nutritional factors on fatness and Vernon (156) reviewed the effects of diet on lipid metabolism in general.

The continuous flow microbial digestion of the ruminant is characterised by slow rates of passage compared to monogastrics, so one would not expect rapid changes in lipid metabolism even in response to infrequent feeding (3,16).

While dietary factors are important they would be expected to ultimately affect the factors discussed in the following sections.

2.3.3 Regulation of Fatty Acid Uptake

Fatty acid supply to adipose tissue cells is proportional to serum fatty acid concentration (51), which is locally controlled by LPL. There is strong evidence that the hydrolysis of lipoprotein triglyceride is the rate limiting step in uptake of plasma triglyceride fatty acids (115). LPL is sensitive to diet and to hormones (1, 50, 51, 156) and the activity increases as the animal enters a more positive energy balance (16, 115). Nilsson-Ehle et al (115) reviewed the mechanism of activation of LPL. The activity of LPL was low in adipose tissue of lambs fed at maintenance but increased when they were fed ad libitum (1). There are differences between adipose tissue depots. In particular, LPL activity increased very dramatically in lamb subcutaneous tissue during the finishing period (1, 16).

Factors which regulate LPL activity in the ruminant have received little attention. However, glucose and insulin stimulated bovine LPL activity both in vivo and in vitro (50, 131). The activity of LPL can be modulated by the proportion of lipids and type of protein in the lipoprotein substrate which themselves are modulated by diet and hormones (50). Mammary gland LPL increases in activity at the initiation of lactation while adipose tissue LPL activity decreases, and these changes are probably mediated by prolactin (156).

LPL activity reflects propensity of adipose tissues to deposit triglycerides but is subject to deficiencies as a predictor of propensity to fatten as discussed by Emery (51) and by Allen et al (1). A major reason for this is that fatty acids can be transferred among adipose tissue depots or mobilised for use by other organs (3, 51).

2.3.4 Regulation of Fatty Acid Synthesis

The rate of fatty acid synthesis is influenced by a variety of factors including substrates, products, metabolites, cofactors and hormones, all of which may have a key role in the regulation of the process in vivo, by altering either the activity or the amount of key enzymes (156).

This topic has been extensively reviewed by Vernon (156) and the following brief review draws heavily upon his conclusions.

The chronic regulation of fatty acid synthesis involves adaptation of the enzymes involved to changing physiological conditions (17, 156). Although Vernon (156) stated that the mechanisms responsible for these adaptations have not been investigated, Baldwin et al (3) briefly summarised chronic hormone actions on adipose function but did make the point that few studies had been undertaken. In any case both agree that insulin is involved.

The acute regulation has been studied extensively. Plasma acetate concentrations are likely to provide a vital influence, since normal values do not saturate the capacity of the synthetic pathway (156).

The role of glucose in the regulation of fatty acid synthesis appears far from clear. While glucose is not used in significant amounts for de novo fatty acid synthesis it has been shown to have profound effects on the rate of synthesis. In ruminants with a functional rumen, glucose incorporation into fatty acids and activities of relevant enzymes can be increased substantially by infusing glucose postruminally or intravenously. The infusion of glucose into lambs dramatically increased glucose utilisation for lipogenesis relative to acetate with a 44- and 9-fold increase in activities of ATP citrate-lyase and NADP-malate dehydrogenase (16).

Vernon (156) stated that addition of glucose increased by 3-10-fold the rate of fatty acid synthesis from acetate in ovine adipose tissue. He attributed the effect to increased NADPH and glycerol 3-phosphate production, however in the same review he claimed that it is not possible to say whether NADPH production becomes rate-limiting for fatty acid synthesis. He further concluded that glucose availability may become rate limiting, but that the evidence for this is less definitive (156). The effect of increased glycerol 3-phosphate production is clearer as it will promote fatty acid esterification, which in conjunction with a decreased rate of lipolysis should decrease the concentration of long-chain fatty acyl CoA and hence activate acetyl CoA carboxylase (156).

Bauman and Davis (17) stated that the enzymes involved in NADPH generation apparently play no major role in the regulation of fatty acid synthesis in ruminants. They, and Bauman (16), suggested that in ruminant adipose tissue the enzymes involved in NADPH production undergo only very minor changes in activity during fasting and refeeding or with a prolonged fast of 3-4 weeks. However, they did not actually specify whether NADPH per se could become rate limiting.

Of further concern is the statement by Bauman and Davis (17) that when the animal develops a functional rumen it loses the ability to utilise glucose carbon for fatty acid synthesis, yet in the same paragraph they stated that in adult ruminants glucose incorporation into fatty acids and the enzymes in the citrate cleavage pathway can be induced to substantial levels by infusing glucose post-ruminally or intravenously. Combined with the increase in NADP-malate dehydrogenase activity cited by Bauman (16) this seems to indicate that increased glucose supply can in fact increase fatty acid synthesis both by increased glucose carbon incorporation and by increased supply of NADPH.

Studies on the fate of glucose in adipose tissue have led to speculation on other metabolic factors affecting glucose utilisation for fatty acid synthesis. In ruminant adipose tissue most of the glucose metabolised in glycolysis is converted to lactate and small amounts of pyruvate (156). Vernon (156) postulated that pyruvate dehydrogenase rather than ATP-citrate lyase is the rate-limiting step in the utilisation of glucose for fatty acid synthesis. Prior and Jacobsen (126) similarly postulated either low activity of key glycolytic enzymes or a limitation to glucose uptake by ruminant adipose tissue.

Both lactate and pyruvate can stimulate the rate of fatty acid synthesis from acetate and inhibit the rate of synthesis from glucose (156). Furthermore lactate can be transported to the liver to be resynthesised into glucose (2) and in sheep it has been reported that 19-20 percent of the body glucose pool was derived from lactate, and that 11 percent of lactate was converted to glucose (124, 125). Results of recent in vivo studies have shown that lactate is an important fatty acid precursor in ovine adipose tissue (123, 124). In fact lactate and pyruvate are

better fatty acid precursors than glucose, but it is not known why (156). The pathway of lactate utilisation for fatty acid synthesis is not clear (124), but the ability of adipose tissue to utilise lactate and pyruvate provides a mechanism for the utilisation of (surplus) glucose and presents a possible role for lactate in the adaptation of lipogenic pathways to increased feed intake.

The concentrations of fatty acids or more probably their acyl CoA esters clearly have a role as potential inhibitors of acetyl CoA carboxylase (51, 156). Catecholamines inhibit fatty acid synthesis probably due to an increased rate of lipolysis which would result in increased levels of long-chain fatty acyl CoA esters in the tissue. This could result in the inhibition of acetyl CoA carboxylase (16, 51, 156). Likewise the stimulatory effects of insulin on fatty acid synthesis are primarily due to a decreased rate of lipolysis (156) removing inhibition of acetyl CoA carboxylase. Catecholamines can modify this action by suppression of insulin (64) and stimulation of glucagon secretion (163). The role of a glucagon is uncertain and the effect of insulin is slight compared to that of the non-ruminant. Even in the ruminant, the insulin : glucagon ratio is low and similar to that found in the underfed non-ruminant. The significance of this in the regulation of fatty acid synthesis is unclear (156).

Of the various lipogenic enzymes, it is thought that acetyl CoA carboxylase is the rate-limiting enzyme, although with sub-optimal acetate concentrations, acetyl CoA synthetase could well become rate-limiting (1, 156). Activities of other lipogenic enzymes respond to changes in the rate of fatty acid synthesis but do not cause the changes in it (156).

2.3.5 Regulation of Glycerol 3-Phosphate Synthesis

The rate of glycerol 3-phosphate synthesis may be determined by the availability of glucose, insulin and long chain fatty acyl CoA esters (156). Catecholamines stimulate the incorporation of glucose into glyceride glycerol, probably through their stimulation of lipolysis which increases the concentration of long-chain fatty acyl CoA esters

in the tissue. This could result in inhibition of acetyl CoA carboxylase and hence decreased glucose oxidation. Increased concentration of long-chain fatty acyl CoA esters would stimulate glycerol 3-phosphate synthesis and possibly deflect glucose from oxidation via the pentose phosphate pathway. Greater metabolism of glucose by glycolysis to glyceride glycerol and lactate could also be due to activation of phosphofructokinase by cAMP (156).

Vernon (156) and Baldwin et al (3) discussed the possibility that the supply of NADH might limit synthesis of glycerol 3-phosphate, and Vernon suggested an error in the assumptions made by the latter group. Vernon claimed that Baldwin et al had postulated an active pyruvate dehydrogenase. Vernon thought it unlikely to be active (156). This author cannot follow Vernon's reasoning as Baldwin et al did not actually mention pyruvate dehydrogenase in their paper. Whatever the basis for the apparent disagreement, it is concluded by this author that the supply of NADH and consequently the enzymes involved in its production probably do not limit glycerol 3-phosphate availability for lipogenesis.

2.3.6 Regulation of Esterification

Emery (51) and Vernon (156) indicated that the regulation of glyceride synthesis had not been extensively studied in ruminant adipose tissue and that little was known of the regulation of esterification.

As one might expect the rate of esterification is increased by higher fatty acid concentration and improved glucose availability. Addition of glycerol 3-phosphate stimulated the rate of fatty acid esterification in homogenates of ovine adipose tissue by more than 6-fold (156). The relative contribution of exogenous fatty acids, lipolysis and de novo fatty acid synthesis were discussed by Vernon (156). He suggested that the bulk of fatty acids esterified were provided by lipolysis due to the constant turnover of triglyceride, however net esterification will depend upon the supply of exogenous fatty acids and de novo synthesis (156). Estimates of the relative contribution of each source to esterification are of little value because all three sources are variable and interdependent. Neither adrenaline nor insulin were found to influence the rate of esterification in ovine adipose tissue in vitro (117).

2.3.7 Summary of Major Factors Affecting Lipogenesis

LPL regulates the supply of circulating fatty acids to adipose tissue cells and is itself regulated by glucose and insulin concentrations.

Fatty acid synthesis is also responsive to changes in glucose and insulin. The supply of reducing equivalents might become rate-limiting and lactate may be an important adaptive precursor for lipogenesis. The concentrations of fatty acids and their acyl CoA esters are potential inhibitors of acetyl CoA carboxylase which is the rate-limiting enzyme in lipogenesis.

Glycerol 3-phosphate synthesis also depends upon the availability of glucose, insulin and long-chain fatty acyl CoA esters.

Esterification depends upon the supply of glucose and fatty acids.

The key factors regulating lipogenesis at almost all levels are glucose, insulin and fatty acids.

2.4 CONTROL OF LIPOLYSIS

2.4.1 Introduction

In their 1975 review Bauman and Davis (17) stated that regulation of lipolysis in adipose tissue of ruminants had received little attention and that the mechanisms were poorly understood simply because few studies had dealt specifically with the problem. In his more recent review Vernon (156) confirmed that our knowledge of many aspects is still slight and attributes this to the cost of working with ruminants and to the difficulty of studying adipose tissue in vivo. The following review will attempt to summarize the current (1982) state of knowledge in the field, with emphasis on the hormones which are considered to be the major endocrine factors controlling lipolysis in sheep.

Initially the mechanism of hormone action in adipose tissue will be described along with evidence for the roles of individual hormones in modulating that mechanism.

This will be followed by a review of the effects of each hormone on fat mobilization and related metabolism. The effects of factors other than hormones will also be considered. Diurnal variation in plasma hormone levels will be described as will responses to fasting where these factors have a bearing on the studies described in this thesis.

Evidence for genetic differences in control of lipolysis between animals will be discussed prior to a summary.

2.4.2 Mechanism of Hormone Action: cAMP

2.4.2.1 Introduction

A large number of hormones have been tested for lipolytic activity both in vivo and in vitro (156). Various workers have reviewed effective lipolytic hormones and as many as 11 have been listed (102). Hormones with antilipolytic activity have also been described. It is believed that the various hormones exert their effects in different ways. Frieden and Lipner (60) described four functions of hormones:

1) Regulatory or homeostatic function - in which hormones are directly involved in the regulation of levels of specific substances at various body sites.

2) Integrative function - in which hormones parallel or supplement the role of the nervous system.

3) Morphogenetic function - in which hormones control the growth and development of animal tissues, and

4) Permissive function - in which the presence of a certain hormone is required before cells can respond to the stimulus of another specific hormone.

More particularly one hormone may induce metabolic changes which activate other endocrine organs and certain hormones also have the ability to influence other endocrine organs. The in vivo metabolic response to certain hormones may thus be a complex one mediated by several mechanisms (102).

The control of lipid mobilization in non-ruminants involves cyclic AMP (cAMP) and the second messenger system (17, 102, 135, 156). There is evidence to suggest that a similar mechanism operates in ruminants (156). Sidhu, Emery, Parr and Merkel (141) isolated a cAMP-dependent protein kinase from bovine adipose tissue and reported that cAMP stimulated lipolysis in homogenates of lamb subcutaneous adipose tissue. Vernon (156) briefly reviewed pharmacological evidence for the role of cAMP. Luthman and Jonson (101) found that injection of cAMP or theophylline increased plasma FFA levels in non-fasted intact female sheep. In the same and a later paper (102) on the effects of hormones in female sheep they stated that "lipolysis in adipose tissue ... (is) regulated by the intracellular level of cyclic 3', 5' andenosine monophosphate (3', 5' AMP)" (101, 102). Although they did not specify species, the reader might assume that they believed this to be so in sheep. So it appears that the small, but growing body of evidence will confirm that the mechanisms described for the non-ruminants are also active in the ruminant.

The mechanism believed to operate is summarized in Figure 2.6. Firstly a hormone binds with a receptor at the adipocyte membrane, activating adenyl cyclase which raises the intracellular concentration of cAMP. C.AMP activates a protein kinase which in turn activates HSL. Since HSL is the rate-limiting reaction in triglyceride hydrolysis, the cellular levels of cAMP indirectly regulate lipolysis (17).

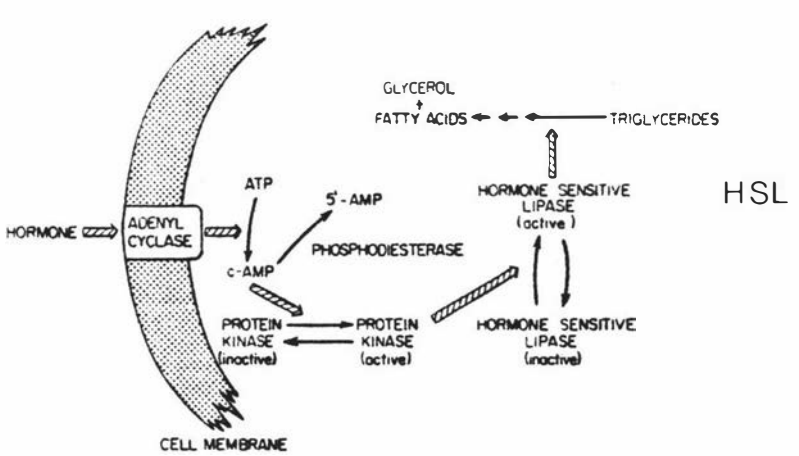

Figure 2.6 Mechanism of hormone action in adipose tissue.

(after Bauman and Davis (17))

cAMP is synthesised from ATP in a reaction which requires magnesium and which is catalysed specifically by adenyl cyclase (101, 135). Inactivation of cAMP by hydrolysis to adenosine 5' monophosphate (5' AMP), is catalysed by a specific phosphodiesterase (101, 134). The intracellular level of cAMP is thus dependent on the activity of at least two enzyme systems (101).

Both of these enzyme systems may be regulated by various factors. A number of lipolytic hormones have been shown to increase the intracellular concentration of cAMP of the adipocyte (33). There is evidence that the hormones affect the adenyl cyclase system (101). On the other hand it has been shown that the methyl xanthines (theophylline, caffein and theobromine) inhibit cAMP phosphodiesterase (33, 101, 134) thus increasing lipolysis by increasing cAMP levels. The antilipolytic action of nicotinic acid may be due to activation of cAMP phosphodiesterase (101).

The regulation of lipolysis through cAMP and intracellular acting substances, and evidence for the roles of hormones in affecting these intracellular mediators has been reviewed extensively by Butcher et al (33), Robison et al (135), Luthman and Jonson (101), Fain (54) and Fain and Shepherd (55).

The following review will briefly summarize the evidence for the roles of lipolytic and antilipolytic hormones in modulating cAMP levels in adipocytes. Much of the work cited has been carried out in chickens and rats. Rat fat cells contain adenyl cylase which is sensitive to many hormones (134). It must be emphasised that little of the work refers to fat cells in sheep. In his 1976 review Bauman (16) Bauman stated that adenyl cyclase and phosphodiesterase activities and cAMP levels had not been measured in ruminant adipocytes.

2.4.2.2 Adrenocorticotropic hormone (ACTH) - ACTH activates adenyl cyclase (55, 116) leading to increased levels of cAMP in isolated rat fat cells (33). This has been shown to be the mechanism by which ACTH stimulates lipolysis in rat adipocytes (116).

Garren et al (61) presented extensive evidence that ACTH acts extracellularly to stimulate the formation of cAMP in the adrenal cortex. There is apparently no evidence for the mechanism of action of ACTH in ovine adipocytes.

2.4.2.3 Catecholamines - the catecholamines activate adenyl cyclase (54, 55, 80, 111, 135) resulting in rapidly increased cAMP levels (18, 33) and consequent lipolysis (53, 54, 55, 80).

β-Adrenergic antagonists block the elevation of cAMP and of lipolysis due to catecholamines (54, 135). Compounds that inhibit the hydrolysis of cAMP, such as theophylline, potentiate the action of catecholamines (55, 80). Catecholamines are also capable of lowering cAMP levels through α-adrenergic receptors (134). Robison (134) proposed a system whereby β-adenergic receptors activate adenyl cyclase while α-adenergic receptors activate the breakdown of cAMP.

Etherton, Bauman and Romans (53) found that theophylline stimulated the rate of lipolysis in the presence of adrenaline in subcutaneous and perirenal adipose tissue slices of sheep and dairy steers. This supports the view that in sheep catecholamines stimulate lipolysis by activation of adenyl cyclase.

2.4.2.4 Glucagon - glucagon activates adenyl cyclase (54, 55, 116, 134, 135) resulting in increased cAMP levels (33).

2.4.2.5 Corticosteroids - the lipolytic action of corticosteroids is sensitive to inhibitors of RNA and protein synthesis and involves a lag period of 1-2 hours (55, 98). Corticosteroid addition to fat cells does not appear to affect cAMP accumulation. According to Fain and Shepherd (55) incubation of fat cells with corticosteroids increased cAMP-dependent kinase activity, suggesting that corticosteroids might act by potentiating the action of cAMP, i.e. a permissive action, increasing the sensitivity of fat cells to lipolytic agents such as catecholamines. Leung and Munck (98) reviewed the evidence of involvement of cAMP in the action of corticosteroids and pointed out that the situation was confusing.

2.4.2.6 Growth Hormone (GH) - The involvement of cAMP is not clear. GH increased cAMP accumulation of rat fat cells after a lag period of about 2 hours and these effects were blocked by inhibitors of protein synthesis. Fain and Shepherd (55) suggested that the lipolytic action of GH involved the synthesis of protein(s) which increased the sensitivity of fat cell adenyl cyclase to activation by catecholamines. This suggests a permissive action of GH in lipolysis.

2.4.2.7 Insulin - The mechanism by which insulin inhibits lipolysis remains to be established (55). While there is evidence that insulin can activate cAMP phosphodiesterase and lower cAMP (55, 134, 135) there is no clear proof that these effects explain the antilipolytic action of insulin (55). Fain and Shepherd (55) reviewed the evidence and presented a model illustrating six possible effects of insulin on fat cell metabolism and suggested that all are the result of a primary effect on membrane-bound enzymes (55). (see Figure 2.7).

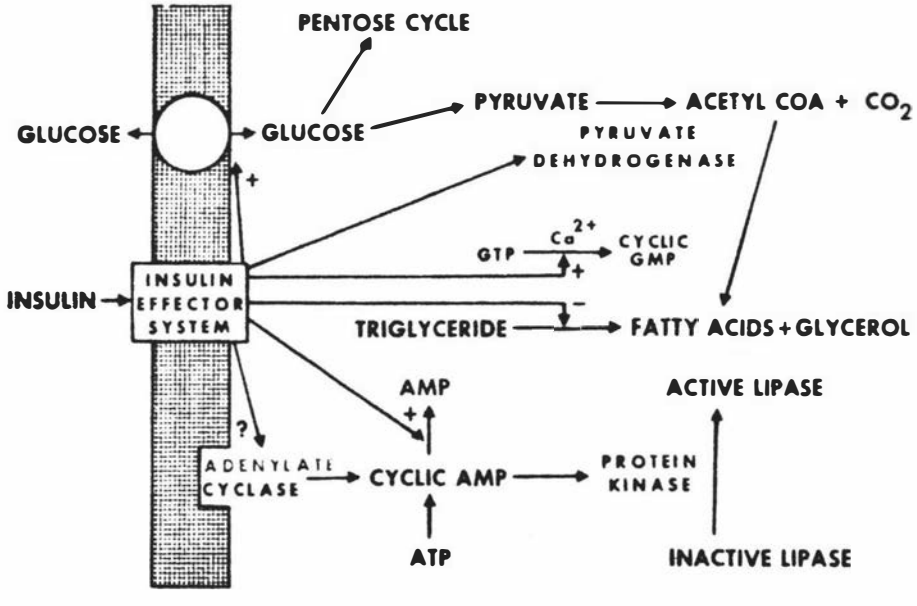

Figure 2.7 Insulin action on fat cell metabolism.
The figure illustrates six possible effects of insulin on fat cell metabolism and suggests that all are the result of a primary effect on membrane-bound enzymes. It is quite possible that some of the effects are more interrelated than is depicted in the model. The six effects of insulin are ;
(1) stimulation of glucose transport
(2) activation of pyruvate dehydrogenase
(3) increase in cGMP via increase in intracullular Ca^{2+}
(4) inhibition of triglyceride lipase
(5) activation of cAMP phosphodiesterase,and
(6) inhibition of adenyl cyclase.

(after Fain and Shepherd (55))

As a result of studies on isolated nuclei from rat liver Goldfine & Smith (62) have suggested that the nucleus may be a site of insulin action.

2.4.2.8 Luteinizing hormone (LH) - LH is capable of stimulating the adenyl cyclase system in isolated (rat) fat cells and does not appear to affect the phosphodiesterase (33).

2.4.2.9 Prostaglandins - The prostaglandins are extremely potent as antilipolytic agents (135). Prostaglandin E_1 (PGE_1) decreased the effects of lipolytic hormones on both cAMP levels and FFA release; but alone caused increased intracellular levels of cAMP (sufficient to activate lipolysis fully) and yet had no effect on FFA release. This stimulatory effect is dependent upon the presence of other cell types around the adipocyte (32, 33). So, like the catecholamines, prostaglandins are capable of both raising and lowering the level of cAMP (134).

2.4.2.10 Thyroid hormones - it has been reported that homegenates of adipose tissue from hyperthyroid rats contained more adenyl cyclase activity than normals and that a very high concentration of triiodothyronine inhibited the phosphodiesterase activity in extracts (33). Thyroid hormone administration enhanced the sensitivity of adipose tissue to the action of lipolytic hormones (135). Nunez et al (116) summarised evidence that thyroid hormones do not activate the adenyl cyclase system but rather inhibit the cAMP phosphodiesterase. They suggested that the action of thyroid hormones is a permissive one and their effect on the lipolytic response to several hormones depends on a modification of the rate of degradation of cAMP (116). It is not known whether the action of thyroid hormones involves protein synthesis and RNA (55).

2.4.2.11 Thyroid stimulating hormone (TSH) - TSH has been shown to activate adenyl cyclase (55, 135).

The effect on cAMP of factors other than hormones, e.g. methyl xanthines and adenosine have been reviewed by Fain and Shepherd (55). While their actions are important in experimental procedures it has not been established that they are important factors in normal in vivo physiological controls, so they will not be considered in detail in this study.

2.4.2.12 Problems in Establishing the Role of cAMP

A major problem in interpreting the role of cAMP in mediating lipolysis is that the relationship between levels of total fat cell cAMP and the rate of lipolysis has not been rigorously established (22, 54, 55). Fain and Shepherd (55) reviewed evidence that maximal activation of lipolysis can be observed without detectable rise in cAMP and that rises in cAMP can be abolished without affecting lipolysis. Fain (56) presented data suggesting that only a small increase in cAMP over the basal level is all that is necessary for maximal stimulation of lipolysis. He stated that the correlation between cAMP and lipolysis occurs over such a small range that it is difficult to see (54). Birnbaum and Goodman (22) found cAMP levels were only transiently elevated peaking 3 min following exposure to adrenaline then returning to basal levels by 9 min. although glycerol production increased and leveled out at a constant rate. Protein Kinase activity also peaked at 3 min then declined to a level about 25% greater than basal activity. They also showed a dose-dependent correlation (coefficient not stated) between the peak level of cAMP, activation of protein Kinase and lipolysis. They suggested that the spike in cAMP may be necessary to trigger the activation of lipolysis but that continued activation of protein Kinase (perhaps by another mechanism) may be essential to sustained lipolysis (22). Fain and Shepherd (55) suggested that an unknown factor could act synergistically with cAMP or that multiple pools of cAMP may allow activation of lipolysis without detecting overall changes in intracellular cAMP levels.

According to Birnbaum and Goodman (22) several laboratories have reported that cAMP was only transiently elevated while others have reported that cAMP pateaued at a level which was maintained for sustained periods. It remains unclear, therefore, whether the initial rapid rise in tissue cAMP triggers the activation of LPL or whether a sustained increase in levels of the nucleotide is necessary for lipolysis (22).

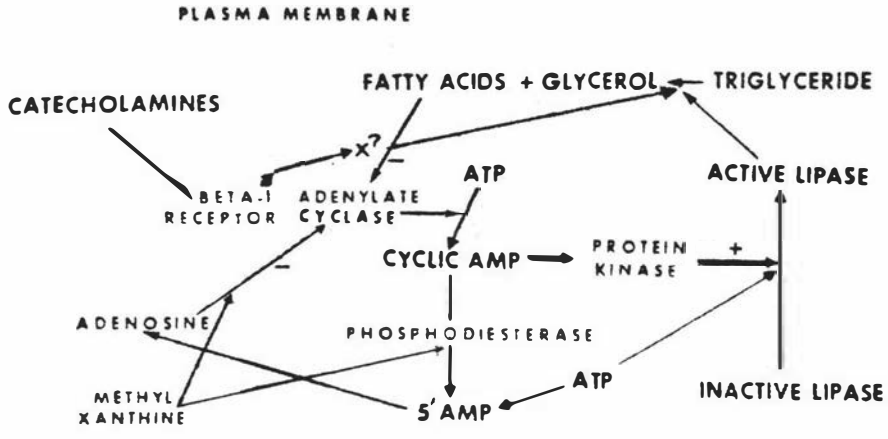

Figure 2.8 Model for the activation of lipolysis by catecholamines and and its modulation by methyl xanthines , fatty acids and adenosine.

(after Fain and Shepherd (55))

Fain and Shepherd (55) and Fain (54) presented a model for the activation of lipolysis by catecholamines and its modulation by various intracellular factors (see Figure 2.8). This model is an expanded version of that presented by Bauman and Davis (17) shown in Figure 2.6. While not a complete model of lipolysis it does in the opinion of this author summarize the key features of the mechanism. The hormones described above which alter adenyl cyclase activity presumably act in a manner analagous to catecholamines, and those which alter the phosphodiesterase may be represented by methyl xanthine in Figure 2.8.

The role of fatty acids in the model are important. During the activation of lipolysis, the fatty acids released into the medium appear to be the major feedback regulators of adenyl cyclase. The important factor is the ratio of FFA's to albumin within the medium. If the primary binding sites on the albumin were saturated, a further increase in FFA's would inhibit adenyl cyclase (54). Fain and Shepherd (55) have reviewed the role of adenosine as a potential feedback inhibitor of adenyl cyclase. They concluded that adenosine does not play a key role in feedback regulation but the large elevation of cAMP seen in the presence of methyl xanthines may result from blockade of adenosine inhibition of adenyl cyclase (55).

2.4.2.13 Briefly summarizing, lipolysis is directly activated by catecholamines, and the sensitivity to catecholamines released at nerve endings is modulated by thyroid hormones, corticosteroids, GH and insulin. Insulin inhibits fatty acid release both by inhibiting lipolysis and by accelerating fatty acid re-esterification. Fatty acids are the major feedback regulator of adenyl cyclase.

2.4.3 Hormones Affecting Lipolysis in Sheep

2.4.3.1 Introduction

In contrast to the extensive list of hormones and drugs which have been investigated for lipolytic effect with non-ruminant adipose tissue several authors agree that relatively few studies have been conducted with ruminants (3, 16, 53, 130, 155). This review will summarise studies of lipolysis in sheep, but results of work with cattle will be used where necessary. With a view to the practical application of the results of this study it is imperative that the important factors (regulating hormones and key metabolites) are relatively accessible for measurement. In particular it is hoped that the key factors can be identified in plasma samples. With this in mind, special emphasis will be placed upon circulating factors in vivo.

2.4.3.2 ACTH - while ACTH has been shown to be lipolytic in other species (137, 156) very few studies have investigated the effect of ACTH in sheep. Vernon (156) cited only four studies and one of these appears to be an inaccurate reference as the paper by Manns and Boda (105) does not refer to ACTH. In the remaining three studies administration of ACTH had no effect on plasma FFA levels (156). Luthman and Jonson (102) found that intravenous administration of ACTH did not change blood glucose or FFA's. Apparently plasma levels of ACTH have not been measured in relation to lipolysis. There are special difficulties in RIA of ACTH presented by the strong adsorption of ACTH to glassware, by susceptibility of ACTH to radio-iodination damage and by low plasma ACTH levels (56).

2.4.3.3 Catecholamines and the sympathetic nervous system

Many studies have shown that injections or infusions of the catecholamines, adrenaline and noradrenaline (N.A.) into ruminants result in an almost instantaneous increase in plasma FFA (6, 20, 76, 78) or glycerol (20, 22). According to Himms-Hagen (80) such effects on lipolysis are usually considered to be a direct effect on adipose tissue but an alternative explanation is the reduction by adrenaline and N.A. of the secretion of insulin. Bassett (6) has shown that adrenaline prevents rises in plasma insulin levels in sheep and Hertelendy et al (76) have shown that it inhibits the insulin secretory response to propionate, butyrate and to intravenous arginine infusion. However the synthetic catecholamine isoprenaline is a powerful lipolytic agent in sheep (6) even though it is a powerful stimulator of insulin secretion (6, 7). Furthermore studies in which blocking agents have been used to dissociate the lipolytic effect from inhibition of insulin secretion indicate that inhibition of insulin is not the major factor involved in the lipolytic action of adrenaline (80).

Both adrenaline and isoprenaline decrease plasma GH levels (7) and adrenaline inhibits the GH secretory response to intravenous arginine infusions in sheep (76). Himms-Hagen (80) stated that adrenaline decreased the concentration of amino acids in the blood but does not specify species. N.A. does not have this effect (80). This may be due to concurrent stimulation of gluconeogenesis since Young and Landsberg (163) stated that gluconeogenesis from lactate and from endogenous amino acids is increased by catecholamines. In his 1967 review (10 years earlier) Himms-Hagen (80) could cite no evidence for such action of catecholamines. The action of catecholamines in protein metabolism appears to be open to speculation.

Adrenaline has been shown to cause marked hyperglycaemia in sheep (6, 7, 78, 158). According to Himms-Hagen (80) adrenaline profoundly modifies carbohydrate metabolism in adipose tissue, but many of the effects result from the concurrent changes in lipid metabolism. Adrenaline accelerates glycolysis, increases glucose uptake, lactate formation, oxidation, the formation and utilization of glycerol 3-phosphate for triglyceride synthesis, glycogenolysis, decreases intracellular protein synthesis and increases re-esterification of FFA's

into triglyceride (80, 163). Fatty acid synthesis is not usually altered by catecholamines (80). These changes are probably due mainly to the increased availability of FFA from lipolysis (80). Overall, adrenaline increases glucose utilization by adipose tissue (80).

Catecholamines in conjunction with thyroid hormones determine the general level of metabolic activity (163). The increase in metabolic rate produced by the catecholamines is generally referred to as their calorigenic effect. This calorigenic effect must be associated with an increased oxidation of available substrate, which may be fatty acid (80). Himms-Hagen (80) concluded that the calorigenic action of catecholamines is poorly understood and presented a hypothesis involving accelerated mitochondrial movements and the associated energy cost.

Postganglionic fibres of the sympathetic nervous system bring about their effects by the liberation of N.A. Stimulation of the sympathetic nervous system also evokes the liberation of adrenaline from the adrenal medulla. The effects of adrenalin augment and complement those of sympathetic nerve stimulation. According to Young and Lansberg (163) a few but not all fat cells appear to possess sympathetic terminals on their surfaces. Conditions which stimulate the sympathetic nervous system lead to an increase in plasma N.A. levels and plasma FFA levels (102, 156).

Which system is primarily responsible for the observed lipolytic effects of catecholamines on adipose tissue; adrenaline from the adrenal medulla or N.A. from sympathetic nerve endings? Since conditions which lead to increased plasma N.A. levels also increase the level of adrenaline (156) conclusive evidence is difficult to obtain. Furthermore the extensive autonomic innervation of the islets of Langerhans in the pancreas (12) may mean that sympathetic motor activity can alter lipolysis through insulin.

While the role of the sympathetic nervous system is agreed to be of importance in regulating lipolysis (6, 55, 80, 156, 163) no one has clearly defined its importance relative to adrenaline. Vernon (156) concluded that "under conditions of stress the sympathetic nervous system is clearly the principal if not the only effector which

stimulates lipolysis". However the role of the sympathetic nervous system is not clear during fasting (156) when it probably does not initiate lipolysis according to Himms-Hagen (80). Himms-Hagen (80) proposed that tonic stimulation of lipolysis by the sympathetic nervous system probably provides a background against which lipid metabolism is further modified by other hormones and provides a mechanism by which lipolysis can be increased or decreased according to level of activity (e.g. sleep or exercise) or stress. Fain and Shepherd (55) however appeared to support strongly a direct control of lipolysis by catecholamines at nerve endings.

2.4.3.4 Glucagon - Bassett (7) found that plasma FFA concentration increased during the first 15 min of glucagon infusion; but that the magnitude of this increase did not appear related to the infusion rate. After 15 min there was a marked decrease in plasma FFA (during glucagon infusion). He concluded that either glucagon has little lipolytic activity in sheep or that any lipolytic action of glucagon is obscured by the antilipolytic effect of insulin secreted during glucagon infusion (7). In a later paper Bassett reported that during a prolonged fast plasma glucagon concentrations declined (8). This finding does not support a lipolytic role for glucagon. Luthman and Jonson (102) also found that intravenous glucagon administration to sheep caused a small initial increase in FFA followed by a depression. They indicated that this decline was probably due to hyperglycaemia and increased insulin secretion. Radloff and Schultz (130) made a similar observation in goats. Brockman (29) reported in his review that Glucagon infusions in vivo increased plasma FFA and glycerol when glucagon-stimulated insulin secretion was prevented. At one point he stated that an effect of glucagon on ruminant adipose tissue is equivocal but in his summary said that glucagon increases lipolysis (29). In a later review Brockman (30) stated that in vivo studies by Bassett (7) and by himself and co-workers, suggested that high concentrations of glucagon may be marginally lipolytic.

Etherton, Bauman and Romans (53) found that glucagon did not enhance the lipolytic rate in sheep subcutaneous adipose tissue slices. Bauman (16) reported glucagon had no effect on in vitro lipolysis in ruminant

adipose tissue, citing Etherton's M.S. thesis. However, in an earlier review Bauman and Davis (17) stated that adipose tissue showed an increased rate of lipolysis in the presence of glucagon, citing unpublished results of Etherton, Bauman and Romans.

It appears that both in vivo and in vitro results are inconclusive and in disagreement. The effects of glucagon on carbohydrate metabolism are however better established. Glucagon is a potent hyperglycaemic hormone and stimulates both glycogenolysis and gluconeogenesis in sheep (29). It is apparently also ketogenic (29, 30). The net effect of glucagon is to promote gluconeogenesis at the expense of peripheral stores (29).

The stimuli to glucagon secretion were discussed by Bassett (8) in an intensive investigation of plasma glucagon regulation in sheep. He reported a highly significant inverse correlation

$$(r = -0.704;\ n = 36;\ P < 0.001)$$

between plasma glucose and glucagon levels in samples collected during the first 8 hours after feeding. However, in his review Brockman (29) cited Bassetts paper (8) and stated that glucagon concentrations correlate poorly with blood glucose. Nevertheless Bassett (8) and Brockman (29) agree that glucagon secretion is stimulated by hypoglycaemia, amino acids, VFA's and adrenaline.

Bassett (12) recently reviewed the regulation of glucagon and insulin secretion in ruminants. According to him, the α and β cells of the pancreas form part of a complex neuro-endocrine control system, the gastroenteropancreatic (GEP) system. The GEP system is concerned primarily with the acquisition, absorption and metabolic disposal of food nutrients and in the redistribution of metabolites within the body in response to changing physiological conditions. Pancreatic islets, as well as being innervated by adrenergic and cholinergic nerves are also extensively innervated by nerve fibres containing one or other of two peptidergic neurotransmitters, (VIP and CCK) both of which have potent stimulatory actions on islet cells (12). In addition islet cell secretion also involves the closely related endocrine and neural mechansims of the gut wall. For example somatostatin, released in

response to the presence of food in the tract, in addition to regulating metabolite absorption, also plays an important part in regulating associated glucagon and insulin secretory responses (12).

The regulation of glucagon secretion and the role of glucagon in adipose tissue require elucidation.

2.4.3.5 Corticosteroids - in their review Leung and Munck (98) stated that the most important direct effect of corticosteroids on (non-ruminant) adipose tissue in vivo appears to be enhancement of fatty acid mobilization. They described the permissive action of corticosteroids on the lipolytic activity of catecholamines, GH, glucagon and ACTH, without which the activity of these hormones is drastically curtailed. However they doubt the importance of this effect with respect to GH, glucagon and ACTH in vivo.

At moderate to high concentrations corticosteroids may inhibit lipogenesis and certainly can reduce the sensitivity of adipose tissue to insulin (98). Leung and Munck (98) attribute this to inhibition of glucose uptake. More recently Kahn et al (87) found that corticosteroid excess resulted in a 50-60% decrease in insulin binding to its specific receptors in rat liver. It has also been reported that cortisone acts peripherally to inhibit the action of LPL (48). Little work has been done in this field with ruminants. According to Vernon (156) in his review administration of cortisol (hydrocostisone) or dexamethazone to sheep had no effect on plasma FFA levels. Certainly Luthman and Jonson (102) found no response to the latter two, but they did report that prednisolone injection slightly elevated FFA levels after 2 hours. The lack of response to cortisol in one study referred to by Vernon could not be confirmed since his reference to the work of Scott and Cook (139) was apparently in error. (They did not mention cortisol). However, Purchas (128) reported that plasma FFA levels were slightly suppressed in prefeeding samples as a result of the administration of cortisol acetate to mature wethers and that there was a "tendency for lambs receiving cortisol acetate to be fatter and to have lower levels of FFA's in their serum at slaughter". Treatment of ketotic cows with dexamethazone similarly resulted in a decrease in plasma FFA levels (102, 156).

Perhaps the strongest evidence for the action of cortisol in sheep was provided by Bassett and Wallace (13). They found that daily intramuscular injections of either 150mg or 500mg of cortisol acetate to adult wethers and ewes during a 6 day fast produced both hyperglycaemic and antilipolytic responses. Furthermore the lipolytic response to GH appeared to be slightly reduced by the cortisol treatment (13). However Kuhlemeier and Trenkle (93) reported a larger lipolytic response to GH in lambs receiving 50mg of cortisone daily than those treated with GH alone. The difference in results may be because of the age difference in the sheep and/or because the lambs were apparently not fasted.

It is apparent that the literature presents conflicting results. While it appears on balance that cortisol may be antilipolytic this is inconsistent with the finding that cortisol apparently antagonizes insulin action in sheep (14). However since high cortisol levels increase growth rate in lambs (128) and are associated with some types of obesity in humans (154) it seems that in some physiological states at least that cortisol may be antilipolytic.

2.4.3.6 Growth hormone - administration of GH has been reported to induce a lipolytic response in sheep (13, 42, 43, 93, 102, 105), calves (75), cows (92, 118) and goats (130). The increase in plasma FFA's is delayed for 1-2 hours and there may be an initial slight decrease in FFA levels (13, 42, 102). This initial effect of GH is referred to as the insulin-like effect of GH (102) and is sometimes associated with a concurrent depression in plasma glucose levels (13, 42) which is apparently not caused by insulin (102, 157). The early insulin-like action of GH has been confirmed in vitro by Goodman (64) in rat adipose tissue. Manns and Boda (105) however did not find such an effect in vivo and others (102, 157) reported an early hyperglycaemic response (the diabetogenic effect of GH (102, 105)). Such differences may have been due to differing GH preparations and differing state of the sheep.

Bassett and Wallace (13) reported that the hyperglycaemic effect of GH was greater in sheep previously fed ad libitum than in those fed

TABLE 2.1 SOME BIOLOGICAL EFFECTS OF GH

I Processes stimulated by GH

A. Cell division

1. numbers (measured as increased DNA or cell count); muscle, liver, spleen, mammary and other tissues.

2. DNA polymerase

B. Protein anabolism

1. N retention

2. Amino acid uptake

3. Incorporation of amino acids into protein

4. RNA polymerase

5. Messenger RNA elongation

6. Ornithine decarboxylase (polyamine synthesis)

C. Lipid metabolism

1. Fatty acid oxidation

2. Fatty acid release from adipose tissue

D. Carbohydrate metabolism

1. Tissue glycogen deposition

2. Pancreatic release of insulin in response to a variety of stimuli

3. Peripheral insulin resistance (glucose intolerance)

4. Plasma glucose levels

E. Mineral metabolism

1. Calcium and phosphate deposition in bone

2. Calcium turnover

3. Urinary calcium excretion

4. Retention of Na, K, P.

II Processes inhibited by GH

A. Fat synthesis and cell size

B. Mixed function oxidase (drug metabolism)

(after Hart (72))

a maintenance ration and was negligible in sheep fasted for 6 days.

The late phase of the biphasic response to GH is characterized by a rise in FFA's and glucose reaching peaks about 8-10 hours following injection (13, 42, 93). In vitro inhibitors of protein synthesis abolish lipolysis leading to the hypothesis that a protein, necessary for stimulating lipolysis, is synthesized during the lag phase (102).

The biological activities of GH are many and varied as can be seen from the summary presented in Table 2.1. It is inappropriate to consider the effects of GH on lipid metabolism alone. According to Hart (72) the secretion of GH in ruminants is closely related to the metabolic status of the animal, being elevated during periods of chronic energy deficit and suppressed when in energy surplus. Furthermore he indicated that the primary role of GH is to preserve protein reserves, particularly during periods of energy deficit, by stimulating the incorporation of amino acids into protein whilst diverting glucose away from tissue deposition and by possibly stimulating lipolysis. According to Hart (72), such a mechanism is somewhat at odds with the long known somatotrophic effect of GH (18) and its ability to stimulate milk production, body growth and wool growth under suitable conditions (72) since GH levels are depressed when there is surplus energy available.

Since GH is secreted as a result of energy substrate, being low, it is expected that plasma GH levels may be higher than normal during fasting. However Wallace and Bassett (158) reported no systematic change in plasma GH in sheep fasted 4 days and Machlin et al (104) reported that it caused GH secretion in the pig but not in sheep fasted for 7 days. Bassett (10) found that fasting sheep for 36 hours following a month of restricted intake did not cause any consistent increase in mean plasma GH levels, but that it caused a marked increase in the amplitude and frequency of oscillations in plasma GH levels. Fed sheep exhibit decreased size and frequency of GH secretory bursts (10, 11).

Responses to feeding have been reviewed by Bassett (11) and recently by Weekes and Godden (159). When sheep were fed once or twice daily at a restricted level, GH concentrations decreased rapidly on feeding and the episodic release of GH was suppressed for up to 4 hours (10). Mean GH levels were lower in sheep fed at high levels than those fed at maintenance level (10). Driver et al (47) found that GH levels also decreased when sheep fed ad libitum ate spontaneous meals and confirmed that short-term fasting (10 hours) produced increases in the size and frequency of GH peaks which began 1-2 hours after the removal of food and continued to increase until the food was returned. It has been suggested that somatostatin may mediate the above effects (11) but this has not been tested (159).

While evidence in the literature indicates that fasting does not significantly increase mean plasma GH levels, it is evident from the same results that GH concentrations reach higher levels more often. Furthermore reduced levels of feeding have increased the mean and the variation in GH levels. Perhaps the mean value is not of prime importance in lipolysis but rather the size and/or frequency of peak levels. According to Weekes and Godden (159) GH appears to be elevated when availability of nutrients becomes limiting and metabolic demands are increased but at other times GH may be secreted in response to ultradian rhythms unrelated to plasma metabolites.

The secretion of GH is under the control of central nervous structures which exert both stimulatory and inhibitory influences on the GH secreting cells of the pituitary. These controls have been recently reviewed by Chiodini and Liuzzi (36). It is the general opinion that the hypothalamus synthesizes and releases two neurohormones into the portal vessels: GH releasing hormone (GRH) and GH release·inhibiting hormone (somatostatin). The secretion or activity of GRH and somatostatin are modulated by the neurotransmitters dopamine, noradrenaline and seratonin (and possibly also by GABA, histamine, substance P and endorphins).

Of interest in this study are the effects of circulating hormones and metabolites on GH release and their interactions with GH. GH has been shown to be released by high levels of certain amino acids in plasma. Hertelendy et al (77) found that intravenous infusion of arginine resulted in a prompt increase in plasma GH in sheep and cows. Abomasal infusion of casein and feeding of formaldehyde protected casein have achieved a similar effect in goats and cows respectively (72). Hyperglycaemia, produced by glucose infusion decreased the GH response to arginine in sheep (77) and in lambs (158) and there is some evidence that hypoglycaemia stimulates GH release in ruminants, according to Bassett (11). There is also evidence for an inverse relationship between FFA and GH as Hertelendy and Kipnis (78) found that FFA's as well as VFA's inhibit GH release in sheep. Given that GH secretion is neurally controlled it is hardly surprising that Hertelendy et al (76) found that adrenaline abolished GH secretion.

Administration of GH to sheep caused marked increases in plasma glucose and insulin concentrations in some studies (43, 157) but not in others (42, 105). Sirek et al (144) recently provided in vivo evidence for a direct trophic effect of GH on pancreatic islets in that physiological doses of GH produced a rise in insulin and pancreatic glucagon levels in dogs. This effect could not be produced in animals without a pancreas (144). GH may antagonize insulin action in vivo in sheep since Wallace and Bassett (157) indicated that GH inhibited the effect of insulin on glucose uptake (but not its repressive effect on FFA release). Kahn et al (87) reported that GH administration produced mild insulin resistance and a decrease in receptor concentration in rats.

It seems that GH has a central role in metabolism but the importance of its actions in lipolysis are somewhat hidden by the many and complex interactions between GH and key hormones and metabolites.

2.4.3.7 Insulin - insulin is generally thought to stimulate lipogenesis (3, 16, 29, 162) and to inhibit lipolysis (3, 16, 17, 29, 30, 33, 55, 78, 88, 102, 155, 162) in ruminants. Vernon (156) stated that "insulin is clearly the principal antilipolytic hormone in rumin-

ants". Insulin administration to sheep resulted in a rapid (within 5 min) decrease in plasma FFA (20, 78, 102, 160) and glycerol levels (20). There is usually a subsequent increase in the plasma FFA levels, but this is thought to be due to the actions of other hormones released as a result of the hypoglycaemia induced by insulin administration (102). Insulin has been shown to promote the activity of rat adipose tissue LPL (51) and of dairy cow LPL (127). Davis et al (43) stated that Fain, Kovacev and Scow (1966) had reported that insulin is capable of completely blocking the lipolytic effect of GH. Insulin treatment has been shown to decrease the output of glycerol and FFA by ovine adipose tissue (127).

Some workers (16, 29, 85) have stated their belief that ruminant adipose tissue is less responsive to insulin than adipose tissue from other species (in vitro). Furthermore, Prior and Smith (127) recently argued that "the limited available data in the ruminant" which suggested that insulin is lipogenic and antilipolytic, is not convincing. They reported that daily injections of insulin (1u kg^{-1}) for 8 days did not produce a sustained increase in lipogenesis in bovine adipose tissue. Metz and Van den Bergh (111) found that insulin had no effect on fat mobilisation from bovine adipose tissue (in vitro) with or without glucose. Conversely Yang and Baldwin (162) found that long term (4 weeks) daily insulin injections and high concentrate feeding depressed lipolytic activities of adipose tissue cells from lactating cows.

If insulin is lipogenic and antilipolytic then the association between fatness and high insulin secretion seen in non-ruminants (34, 132, 143) should equally apply to ruminants. Gregory et al (69) found that insulin-secreting ability was inversely related to the amount of dissectable fat between breeds in lean Friesian steers and fatter Herefords, but that there was a higher insulin-secreting ability in fatter steers within breeds.

Prior and Smith (127) reviewed other conflicting evidence and suggested that the primary effect of insulin on ruminant adipose tissue was to increase the glucose uptake and availability of glycerol 3-phosphate

and to stimulate LPL, thus favouring triglyceride deposition. Without directly affecting lipogenic enzymes they concluded that undue importance had been placed on the role of insulin in regulating glucose and lipid metabolism and that insulins major role may be in altering protein synthesis and deposition.

Gregory (67) similarly expressed doubts about the role of insulin in adipose tissue. He pointed out that insulin is the only hormone which influences lipolysis at physiological concentrations whereas the minimal effective doses for the apparently lipolytic hormones are usually at least 100 times greater than the concentrations normally found in the circulation. Nevertheless, the inverse relationship between fatness and insulin secreting ability in different breeds of cattle (69) and the inverse relationship between fatness and the perfusion rate of insulin in adipose tissue of lambs (67) caused him to doubt whether immunoreactive insulin was responsible for genetic differences in fat growth rate in ruminants.

So while a large body of evidence points to an antilipolytic role for insulin, Gregory and Prior, both well established researchers in this field, have reservations. Brockman (29) also reviewed conflicting in vitro and in vivo evidence for the role of insulin in lipid metabolism and concluded that in vivo studies supported a role for insulin in promoting net lipogenesis in nonhepatic tissues. With respect to protein metabolism he reported that insulin enhanced uptake of amino acids by muscle in 2 studies with sheep and that during starvation when insulin levels were low amino acids were released. He concluded that insulin appears to be anabolic directing the storage of certain metabolites into non-hepatic stores, i.e. muscle and adipose tissue. In its absence synthetic activity is reduced in these tissues, while proteolysis and lipolysis are promoted, thus providing substrate for gluconeogenesis and energy production.

The secretion of insulin is influenced in many ways and has been extensively reviewed by Bassett (11, 12) and briefly by Brockman (29). VFA's appear to be more potent than glucose with respect to insulin secretion in sheep (76, 86, 106, 107). Amino acids are also important

stimulators of insulin secretion in sheep (77, 151). Plasma insulin levels in sheep have been shown to increase significantly after feeding (8, 9). In addition the mean level of insulin correlated well with the digestible organic matter (DOM) intake (11, 15). The insulin response to feeding in sheep (9) and steers (35) is biphasic. The initial secretion is transient and is probably mediated by reflex vagal mechanisms, while the second, prolonged peak 3-6 hours after feeding is associated with hyperglycaemia and increasing absorption of the products of digestion (159). Fasting resulted in decreased plasma insulin levels in sheep (152).

Pancreozymin (CCK) can stimulate insulin release in sheep (11, 29, 151). Trenkle (151) found that secretion also stimulated insulin release in lambs fasted 48 hours. The involvement of gastointestinal hormones and the nervous system in the regulation of pancreatic secretion (the GEP system) was reviewed by Bassett (12) and was discussed earlier in this section in relation to glucagon secretion. Glucagon itself stimulates insulin secretion (29). Trenkle (151) suggested that intestinal hormones cause the late rise in insulin and that a combination of these hormones, along with metabolites may maintain insulin secretion for several hours after feeding. The regulation of insulin secretion and the role of insulin in the control of lipid metabolism in ruminants remain unclear.

2.4.3.8 Prolactin - prolactin administration did not affect plasma FFA levels in sheep (102, 105). In man prolactin shares some characteristics with HGH due to their similar structures, however Fraser and Blackard (58) showed that prolactin elevation following insulin was not inhibited by infusion of lipids (unlike HGH). According to Winkler et al (161) administration of ovine prolactin did not increase FFA in man or monkey but did so in the dog. However Bauman et al (18) cited evidence for increased FFA levels in humans and cows in response to prolactin (but did not indicate doses used). Emery (51) reported that prolactin did not significantly change LPL activity in rat adipose tissue in vitro but that it increased mammary LPL activity.

Bauman et al (18) recently postulated an homeorhetic role for prolactin in growth. Homeorhesis is the coordination of metabolism of body tissues in support of a dominant developmental or physiological process

(18). Homeorhetic mechanisms provide chronic regulation whereas homeostatic devices prevail for short-term control, and may overide the long-term regulation to preserve vital functions. Bauman et al (18) suggested that prolactin might alter the capacity of muscle for net protein accretion as well as alter the metabolism of other tissues (such as adipose tissue) to allow a greater partitioning of nutrients to support muscle growth. They suggested that prolactin either directly or indirectly decreases LPL activity in adipose tissue, decreases de novo fatty acid synthesis in liver and adipose tissue and increases lipolysis in adipose tissue. The results of their first study in growing lambs did not demonstrate a clear role for prolactin in the regulation of growth (18).

According to Weekes and Godden (159) an anabolic role for prolactin was suggested by Forbes et al (1979) in growing lambs and by Beeby and Swan (1979) in steers, although definitive evidence is lacking. They further stated that prolactin levels are increased after feeding in cattle.

2.4.3.9 Prostaglandins - Vernon (156) cited only one in vivo study of the effect of prostaglandin on lipolysis; infusion of PGE_1 into fasted sheep reduced the plasma FFA concentration. The effect was apparently not mediated by insulin or glucagon.

Two in vitro studies using subcutaneous adipose tissue biopsied from Holstein steers produced conflicting results. Di Marco et al (44, 45) found that PGE_2 did not affect the basal rate of lipolysis, but in one study it reduced adrenalin stimulated lipolysis (44). In the other study (45) this effect was not found.

The effects of prostaglandins on lipid metabolism in other species were reviewed by Curtis-Prior (40). Briefly prostaglandins of the E type have been shown to reduce lipolysis stimulated by various lipolytic hormones. It was proposed that prostaglandins are involved in the maintenance of adipose tissue homeostasis by way of a physiological negative feed-back control mechanism (40).

2.4.3.10 Thyroid hormones - apparently the effect of thyroid hormone administration on lipolysis in ruminant adipose tissue has not been directly studied. Kirton and Barton (91) studied the components of live weight loss in Romney ewes subjected to thyroxine treatment and a low plane of nutrition. They reported significant liveweight losses due to both treatments but that neither treatment reduced the weight of chemical fat or dissectible fatty tissue. A low plane of nutrition produced its main weight reducing effects by lowering the protein and water content of carcass muscle in contrast to the dehydration of the muscle in animals treated with thyroxine (91). According to Kirton and Barton (91) such results were not in agreement with the literature which indicated that fatty tissue is the first to be removed. Unfortunately they did not report plasma FFA or glycerol levels.

Further indirect evidence for a lack of primary lipolytic action by thyroid hormones in sheep was provided by Luthman and Jonson (102) who found no change in plasma FFA levels in response to TSH.

Lister (100) suggested that there was an association between thyroid activity and his Fat Partition Index (FPI) in sheep. Apparently thyroid activity is higher in breeds which are lower on the FPI i.e. have lower proportions of subcutaneous fat in the carcass in relation to the sum of the weights of intermuscular, kidney knob and channel fat (100).

Nunez et al (116) reported that responses to several lipolytic hormones were completely abolished in fat tissue from thyroidectomised animals. Hales et al (71) stated that the effects of acute lipolytic hormones are modulated in the long term by thyroid hormone and corticosteroids. Fain and Shepherd (55) indicated that thyroid hormones modulated the sensitivity of adipose tissue to catecholamines released at nerve endings.

Weekes and Godden (159) reported that thyroxine (T_4) levels decreased after feeding but that this may have been part of a natural circadian rhythm. They also stated that generation of triiodothyronine (T_3) from T_4 was reduced by starvation in the rat and that the majority

of circulating T_3 in sheep is of extrathyroidal origin. Energy restriction in steers was associated with reduced levels of T_3, T_4, insulin, glucose and α-amino nitrogen while GH and FFA were elevated (159). Refeeding resulted in a greatly increased growth rate while T_3 levels initially fell further and insulin and T_4 returned to control values.

Holdaway et al (81) observed that serum T_3 levels in humans were low during fasting and starvation and rose during weight gain. T_4 levels were unchanged during weight loss leading to a fall in the T_3/T_4 ratio while reverse $T_3(rT_3)$ (which is metabolically inactive) levels increased. Holdaway et al (81) postulated that mechanisms controlling thyroid hormone production and the $T_4/T_3/rT_3$ conversions could provide a sensitive means for weight regulation in humans perhaps even on a day to day basis.

2.4.4 Non Hormonal Factors Affecting Lipolysis

2.4.4.1 Glucose - glucose administration to ruminants decreases plasma FFA levels (20) and FFA entry rates (160). Glucose precursors such as propionate and glycerol can also have a similar effect (156). According to Prior and Smith (127) glucose and lactate are much greater stimuli than insulin for in vitro lipogenesis. Insulin is needed for the suppression of lipolysis by glucose (156). Glucose usually inhibits fat mobilisation by stimulating re-esterification, but Metz and Van Den Bergh (111) found that this did not occur in adipose tissue slices from dairy cows in early lactation. They attributed this to a complete loss of re-esterifying activity by the adipose tissue. In vivo however, if ketotic cows are given glucose intravenously the FFA concentration of blood is lowered. Accordingly Metz and Van Den Bergh assumed that the effect of glucose in vivo is not a direct effect on fat mobilisation in the adipose tissue, but may affect other factors regulating plasma FFA concentration. It is interesting to note that in their experimental conditions (in vitro), insulin had no effect on fat mobilisation from bovine adipose tissue in the absence and in the presence of glucose. They could not explain this total lack of effect of insulin (111).

2.4.4.2 β-hydroxybutyrate and butyrate - both β-hydroxybutyrate and butyrate have been shown to reduce release of FFA and glycerol from bovine adipose tissue when supplied in high concentrations. Vernon (156) suggested that the inhibition of lipolysis by β-hydroxybutyrate is probably of physiological significance, but only when the concentration is raised to the levels found during ketosis. Metz and Van Den Bergh (111) stated that β-hydroxybutyrate inhibits fat mobilization in dairy cows and protects the animal against further intoxication by ketone bodies. Conversely Yang and Baldwin (162) found that β-hydroxybutyrate increased the rate of fatty acid release stimulated by adrenalin in isolated cow adipocytes. Chase et al (35) suggested the β-hydroxybutyrate is involved in the control of feeding in ruminants.

2.4.4.3 Long-chain fatty acids and albumin - FFA are transported in plasma bound to albumin (111, 156). It has been shown by Metz and co-workers that an increase in the molar ratio of FFA to serum albumin leads to increased levels of tissue-associated fatty acids (TAFA), resulting in an inhibition of lipolysis (111, 156). Vernon (156) summarized thus; "FFA themselves have the potential for limiting the rate of lipolysis, but this would not appear to be important except when plasma FFA concentrations exceed about 1mM (possibly during fasting or peak lactation, or under pathological conditions). Furthermore, the rate of blood flow through adipose tissue may influence the rate of FFA release and hence lipolysis, by altering the availability of albumin" (156).

2.4.4.4 Calcium - limited evidence suggests that calcium may modulate the rate of lipolysis via an increase in intracellular calcium ion concentration. In sheep hypocalcaemia is associated with increased plasma FFA levels and the infusion of calcium borogluconate (a common treatment on N.Z. farms) into normal or hypoglycaemic animals reduced the plasma FFA levels (156). The latter effect is probably confounded by the effect of the borogluconate which may stimulate esterification.

2.4.4.5 Other agents - Vernon (156) briefly reviewed the effects of various other compounds and drugs including nicotine, carnitine and heparin. None were concluded to have physiologically significant effects in the normal regulation of lipolysis.

2.4.5 Evidence for Genetic Differences in Control of Lipolysis Between Animals

Broad (28) reviewed the effect of genetic factors on fatness in ruminants and described the problems in deciding what components (of fatness) to measure and how they should be quantitated. Bowman (26) suggested that there was considerable genetic variation in fat percentage in sheep and Broad (28) reviewed evidence for breed differences in fatness in sheep. Of particular interest to this study, he noted that Southdowns were smaller and fatter than Romneys and that on N.Z. hill country Romneys were leaner than Merinos, Corriedales, Perendales, and Border Leicester and Romney crossbreds. He further stated that there is little evidence to suggest that the efficiency of energy utilization is responsible for the differences in fatness between breeds (28).

Broad (28) also briefly reviewed the effect of selection on fatness and although breeding programmes designed to reduce the fatness of ruminants had not begun it seemed feasible that fatness could be reduced by such a programme. Apparently fat deposition among domestic animals treated alike is 40-60% heritable (28). As early maturing animals tend to produce fat at a lower body weight he suggested that larger, later maturing animals should be selected. However since the genetic variation in mature body size of sheep is relatively small, he warned that gains achieved by such a programme would not be spectacular (28).

Evidence for genetic differences in fat metabolism in sheep is scarce. Emery (52) showed that triglyceride synthesis is greater in adipose tissue of sheep with a greater propensity to fatten by virtue of genetic background or sex. In particular lean types (Suffolk breeding) sheep showed less glyceride synthesis in subcutaneous adipose tissue

than did fat types (Southdown breeding). Similarly ewes synthesised more glyceride in subcutaneous adipose tissue than did wethers and rams (52). Sidhu et al (141) studied the effects of age, breed and sex on activity of HSL in homogenates of lamb subcutaneous tissue. Basal lipolysis increased with age and apparently also with fatness. Southdown sired lambs showed higher basal lipolytic activity than Suffolk sired lambs and there was an age x breed interaction due to a high level of HSL activity in 32 week Southdown sired lambs. Lipolytic activity did not differ significantly with sex although ewes and wethers tended towards higher lipolytic activity than rams. Furthermore they found that potential (cAMP stimulated) lipolysis did not differ significantly between breeds and since basal activity did differ they suggested that this could indicate the presence of a greater proportion of the active form of HSL in lambs sired by Southdown rams than those sired by Suffolk rams. They concluded that this could have been due to faster deactivation of HSL during storage of triglyceride in the Suffolk group. Since increased lipolysis was apparently related to increased lipid deposition in adipose tissue, Sidhu et al (141) postulated that factors affecting deposition of fat must have compensated for the increase in lipolysis.

It has been shown that some sheep do not readily lose fatty tissue during submaintenance feeding (4, 28, 79, 91) and in at least one case, that surplus fat cannot be removed from ewes in a relatively short period of time without endangering their lives (91). However, other reports have indicated relative ease of fat loss in sheep (23, 76). Also feed restriction followed by refeeding has been reported to have a very variable effect on fatness in sheep (28).

A growing body of evidence for genetically mediated differences in metabolism has been provided by work with cattle. High-yielding dairy cows were found to have significantly higher levels of plasma GH (21, 72). There was also a positive relationship in the same cows between changes in circulating GH and changes in milk yield (72). However, Beeby and Swan (19) reported that hormone concentrations (GH, insulin and prolactin) were not related to maturity type in a comparison of 10 breeds of growing steers. They found that GH concentrations declined and insulin concentrations increased as the steers matured. This

evidence is suspect however since blood samples were collected at approximately 3 month intervals. Spencer (147) indicated that age-related fatness was associated with higher insulin-secreting ability in both Herefords and Friesians (69) but noted that the "fatter" Herefords exhibited less insulin-secreting ability than the Friesians. Bines and Hart (21) found that plasma GH levels were higher and insulin levels lower in Friesians than in Hereford-cross heifers during lactation. During the dry period, when the Friesians were gaining weight, their insulin levels rose significantly and were higher than those of the beef heifers, though not significantly so. Plasma levels of FFA's and β-hydroxybutyric acid were significantly higher in the dairy type heifers during lactation (21).

Flux et al (57) reported that plasma insulin concentrations rose more rapidly and were sustained at higher levels longer in cows of high genetic merit fed restricted rations than in other cows. The high genetic merit cows had higher mean levels of plasma glucose than cows of average genetic merit when both groups received similar restricted intakes. They concluded that this was evidence that selection for improved milkfat production has produced cows with measurable differences in endocrine control of metabolism.

Land et al (94) found that dairy calves release less insulin than do beef-type calves after feeding. They also reported that Friesian calves sired by bulls of high genetic merit mobilised fat reserves more effectively and produced a higher plasma concentration of FFA's on fasting than did calves from low genetic merit bulls. In another study of Friesian calves of high or low genetic merit, Tilakaratne et al (150) found significant differences in levels of 3 plasma metabolites between the high and low groups. During fasting FFA concentrations increased more rapidly to higher levels in the high genetic merit group, indicative of greater lipolysis. Blood urea was higher while fed and during fasting in the low group while total blood protein was higher during both treatments. Tilakaratne et al (150) postulated that the high group possessed a fat-based energy economy whereas the low group obtained a relatively greater proportion of their energy from protein catabolism.

Kiddy (89) reviewed evidence of the existence of 2 different types of BGH. Studies of a few individual cattle of different breeds revealed differences between breeds and within Holsteins in the type of BGH. However he reported that such variation was not found in ovine GH or prolactin. Indirect evidence of the expression of genetic factors involved in fat metabolism was provided by Hafs et al (70) who reported that in their study of GH in bulls, none of the measures of GH status were closely related to measures of body growth. However in a study of carcass quality in Holstein heifers they found that blood levels of GH were significantly ($r = -0.37$) related to growth but not to any measures of carcass composition. They warned that interpretation of that result was difficult since pituitary GH concentration was correlated with lean carcass characteristics. They suggested that animals with high pituitary GH tended to be more lean, but that GH had more influence on lipolysis than on protein anabolism because animals with high pituitary GH tended to grow more slowly.

Stronger evidence of genetic differences in fat metabolism has been found in pigs. Gregory and Lister (68) reported that lean Pietrain pigs had a higher lipolytic response to noradrenaline than fatter genotypes. They also reported an enhanced release of noradrenaline which combined with enhanced lipolytic sensitivity to noradrenaline could have led to greater fat mobilization and hence a leaner body. Spencer (147) stated that the greater insulin-secreting ability of large white pigs was partly responsible for their greater fat deposition.

Shire (140) extensively reviewed genetic variation in endocrine systems with reference to many species including mice, rats and humans in which most studies have been carried out. He presented evidence for genetic variation in virtually every component of the endocrine system and concluded (among many other conclusions) that such genetic variation can underlie variation in endocrine systems and result in variant phenotypes.

2.5 Summary of Review

The degree of fatness depends upon the relative extent of lipogenesis and lipolysis. The main factors controlling these processes appear to be the sympathetic nervous system and catecholamines, GH and insulin. The prime initiator of lipolysis is probably sympathetic release of noradrenaline at nerve endings in adipose tissue. This activity is modulated by the presence of other hormones. GH assists lipolysis and may be considered to be catabolic in adipose tissue, but is anabolic in its defence of tissue protein. Insulin is primarily anabolic and to this end modifies the cellular environment to assist lipogenesis. To this extent it can be considered antilipolytic but no mechanism for a direct antilpolytic action of insulin has been elucidated. Corticosteroids and thyroid hormones are permissive and essential to the above actions in adipocytes. ACTH and TSH are apparently not involved in ovine lipid metabolism, while prostaglandins may be involved in feedback control mechanisms. The role of prolactin is unclear. There is very little direct evidence for genetic differences in fat metabolism in sheep. Triglyceride synthesis was greater in sheep with a greater propensity to fatten but basal lipolysis also apparently increased with fatness. It has been postulated that sheep of fatter genotype possess a greater proportion of the active form of LPL or that it is inactivated less quickly. Variation has been reported in the ease with which sheep mobilize surplus fat.

Some evidence from cattle has indicated that lean animals have lower insulin levels and higher GH levels than fat ones, while other evidence suggested that fatter animals have higher insulin levels. Some studies have failed to show relationships between either hormone and fatness. These differences doubtlessly result from the different physiological states (e.g. lactation, growth, sex, age) of the animals studied. Genetic differences in ability to mobilize fat were reported and it has been suggested that some animals possess a fat-based energy economy whereas others utilize relatively more protein.

Work with pigs has indicated that higher sympathetic activity was related to genetically leaner bodies, and that greater insulin-secreting ability was partly responsible for greater fat deposition in one genotype.

Figure 3.1 The first group of ewes; Romneys and Southdowns of different phenotypes.

The Romneys are on the left and the Southdowns on the right.
The short,fat ewes are on the outside and the long,lean ones in the middle.
Identification numbers are visible on the feed bins.

CHAPTER THREE

MATERIALS, METHODS AND DEVELOPMENT OF METHODS

3.1 THE SHEEP

A total of 9 ewes were used in these trials. They should be considered as two distinct groups for reasons which will become evident in the following descriptions.

The first group of 4 ewes consisted of 2 Romney ewes and 2 Southdowns. They were selected from the Massey-DSIR research flock on the basis of previous records of backfat thickness and body length with the objective of obtaining one long lean and one short fat ewe of each breed. Soon after obtaining them the sheep were weighed and their backfat thicknesses measured ultrasonically (method described later in this chapter). These measurements are presented in Table 3.1. Length and height were not recorded but the differences were visibly apparent particularly between the Southdowns.

Photographs of the first group of ewes are presented in Figure 3.1.

TABLE 3.1: INITIAL PHENOTYPIC CHARACTERISTICS OF THE FIRST GROUP OF EWES

Breed	Ewe No.	Apparent Phenotype	Backfat Thickness	Liveweight
Southdown	56	short/fat	19 mm	53 kg
Southdown	73	long/lean	13 mm	60 kg
Romney	313	short/fat	12 mm	53 kg
Romney	323	long/lean	10 mm	67 kg

It was thought that these and previous data possibly indicated the existence of genetic differences between the 2 ewes of each breed with respect to adipocity.

Figure 3.2 The second group of ewes; cull Romneys of similar phenotype and unknown genetic background.

They have just been cannulated.
The shorn patches on their backs mark the site of ultrasonic back-fat thickness measurements.

The second group of 5 ewes were all Romneys. They were obtained from the Massey sheep farms at 5 years of age having been culled because they were not pregnant. Four of the ewes (ewes 57, 116, 142 and 186) were obtained in August 1977 and were selected from a group of only 6 available cull ewes. The other 2 were rejected; one due to ill health, the other because it would not eat the rations provided. The fifth ewe used (ewe 86) was another non pregnant 5 year cull ewe obtained in August 1978 to replace Ewe 186 which became pregnant in that year.

Two weeks after being housed indoors the second group of ewes (except Ewe 86) were weighed and their backfat thicknesses measured. These data are presented in Table 3.2 and are considered typical values for that class of sheep (5yr cull ewes) at that time of year. There was no reason to suspect that there might be any marked differences in the metabolism of these ewes.

TABLE 3.2: INITIAL CHARACTERISTICS OF THE SECOND GROUP OF EWES (ROMNEYS)

Ewe No.	Backfat Thickness	Liveweight
57	4 mm	48 kg
116	4 mm	50 kg
142	3 mm	40 kg
186	4 mm	44 kg

A photograph of the first 4 of the second group of ewes is presented in Figure 3.2.

All ewes were penned individually in metabolism crates (see photos) and housed in the Animal Physiology Unit (APU) except during the longer intervals between experiments when they were grazed outdoors.

3.2 CANNULATION

Ewes were cannulated in the jugular vein using a variety of tubing materials with varying degrees of success. Initially less expensive cannulas, P.V.C. and silastic tubing were used for reasons of economy. Because cannulas often blocked, larger diameter tubing was tried, necessitating the manufacture of 10 gauge hypodermic needles from surgical quality stainless steel tubing. This did not solve the problem however and it was eventually found that the best results were obtained with more expensive cannulas ready made for medical use with humans. Those preferred were Ezycath and Bardik cannulas.

The following generalised description of the method of cannulation was modified slightly according to problems encountered. Two people were needed for a satisfactory operation unless a general anaesthetic was used.

Requirements:

- Wool clippers
- Portable light source (if necessary)
- Cannulae and plugs
- Heparinised saline
- Hypodermic needles (various gauges)
- Alcohol 70.0%
- Local anaesthetic (xylocaine) or
- General anaesthetic (Rompun or Nembutal)
- Antibiotic powder or spray
- Broad spectrum antibiotic
- Antiseptic solution
- Surgical swabs
- Adhesive tape (sleek)
- Sterilizer and surgical equipment including;
  - Suture material
  - Surgical needles
  - Needle holder
  - Scissors
  - Forceps
  - Drapes

Method:

Cannulae were prepared and sterilized if necessary and all parts including plugs were washed in 70.0% alcohol. All surgical equipment was sterilized and placed within reach, usually on a stainless steel trolley.

The wool was clipped away from the neck of the ewe over the jugular veins and if necessary the area was shaved in order to see the vein as clearly as possible. In very fat sheep the vein was harder to see. A good light source was found to be important and a strong portable light was helpful.

The area over the jugular vein was washed with alcohol. (It dries faster than water - this is important when using adhesive tape). 1.5cm^3 of local anaesthetic (Xylocaine) was injected at the desired site. The preferred site was fairly high up the neck where the jugular is closer to the surface. When general anaesthesia was required either Rompun or Nembutal were used. Doses were calculated according to the makers instructions. When administered to a 50kg ewe 1.5cm^3 of Rompun (2% solution) induced light surgical anaesthesia for about half an hour. Disadvantages of Rompun included a variable time of onset of action, markedly increased salivation and a long recovery period. Ewes were not intubated but their heads were lowered or hung over the end of a table to avoid choking. Due to the relatively long action of Rompun, doses were kept to a minimum and cannulation was carried out 1 or 2 days prior to trials. Eventually Nembutal (Pentobarbitone sodium) became the preferred anaesthetic. 17cm^3 of Nembutal when injected rapidly i.v. into a 50kg ewe gave almost exactly 30 mins of deep surgical anaesthesia followed by a relatively rapid recovery. Initially about 90% of the calculated dose was injected very rapidly; the remainder was administered as necessary to obtain or maintain the desired effect. Rapid administration resulted in almost instantaneous unconsciousness, about 30 minutes of surgical anaesthesia and a rapid recovery. Slower administration resulted in a slightly slower onset and apparently a slightly longer period of anaesthesia and slower recovery. While the safety margin may be smaller with Nembutal than with Rompun, the effect of the former was

preferred although more care was needed in monitoring the state of the animal.

When local anaesthetic only was used the sheep was restrained either standing or "seated" on its backside with the head held slightly to one side by the assistant. The "seated" position was preferred and it was found to be best if the sheep's chin was only raised marginally. When using the specially made hypodermic needles it was found necessary to cover the "blunt" end with a folded gauze swab to protect ones fingers as considerable pressure was needed to penetrate the skin. A special stainless steel aid was made for this purpose but its use was abandoned as it eliminated the sensitivity or "feel" which aided the precise location of the needle in the vein. With the left thumb (right-handed operator) firm pressure was applied to the jugular below the entry site to occlude the vein. With the right hand the needle was inserted, keeping the bevelled edge away from the sheep (i.e. towards the operator). This aided penetration when the vein was deep. The needle was inclined at about 70° to the skin and inserted in the direction of blood flow (towards the heart) with a short, sharp push.

If the jugular was not found with the first push, the needle was withdrawn as far as the skin. The vein was located by tapping or feeling with the right hand all the while maintaining pressure with the left. With needles of 16-18 gauge or larger, the vein could often be "felt" with the tip of the needle, which was aimed at the centre of the vein to avoid the needle rolling or sliding off the side. (The vein was found to be surprisingly mobile).

When blood was obtained (in large quantities) the pressure applied by the left hand was released and ensuring that the needle did not move, the cannula was inserted about 15-20cm. After testing the cannula was filled with about $2cm^3$ of heparinized saline and the plug inserted. Initially the cannula was secured by a mattress suture but this was found to be unnecessary. A piece of adhesive tape about $2.5cm^2$, folded around the cannula and sutured to the skin just above the point of entry was found to provide adequate security. This was repeated at one or more sites as the cannula was looped over the neck and secured to the back of the neck and along the mid line of the

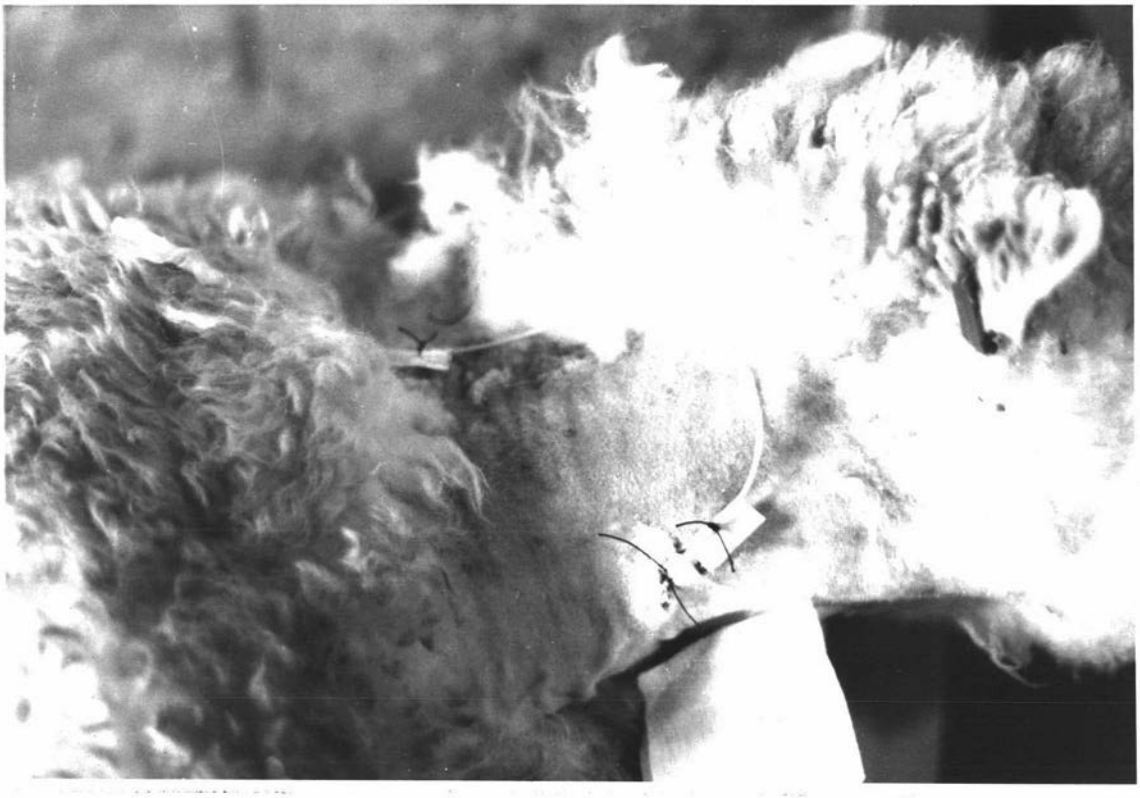

Figure 3.3 Cannulated Romney ewe.

Note adhesive tape and sutures.

back as far as the length of the cannula allowed. When sufficient wool was present the cannula was tied under the wool, otherwise adhesive tape and sutures were used. A little puff of antibiotic powder or spray was applied at each wound after cleaning with antiseptic solution and drying. Penicillin (Propen L.A.) (about $4cm^3$) was administered if incisions had to be made to locate the jugular, but otherwise it was only used if signs of infection developed. An example of a completed cannulation can be seen in Figure 3.3.

3.3 HEALTH, FEEDING AND CARE OF SHEEP

The health of the animals was monitored by close attention to behaviour. Observation of eating and drinking behaviour, faeces volume and texture, urine volume and colour as well as body temperature was usually adequate in early detection of ill health. Body temperature was usually monitored by touch, especially by feeling the ears, but rectal temperatures were taken at the slightest sign of problems. The best indicator of ill health was found to be aberations in behaviour, such as inactivity, drooping head and ears and decreased eating. A small degree of scouring occured during changes in diet from pasture to hay and sheepnuts, but this was corrected by withdrawing the nuts, providing some fresh grass and/or increasing the hay ration.

The ration, as mentioned above, consisted of good quality meadow hay (ryegrass and white clover) and lucerne based sheepnuts (Farm Products, Palmerston North). Unfortunately the suppliers would not reveal the composition or analysis and this was never determined. The author was unaware of the potential mineral imbalances associated with lucerne. In a memorandum to Dr F.R. Cockrem in 1966, I.J. Cunningham (39) supplied an analysis of a Lucerne meal containing 8.9 p.p.m. Cu, 0.1 p.p.m. Mo and 0.06% SO_4. He stated that appropriate additions of Sodium molybdate and sodium sulphate would give a reasonable likelihood that a diet containing such a (high) copper level could be fed without the occurrence of chronic copper poisoning. He also said that it was desirable to have about 2 p.p.m. Mo in the diet and about 0.35% SO_4. When copper poisoning was diagnosed in Ewe 56 (as described

in Chapter 4) the proportion of sheepnuts in the diet was decreased and a food additive was formulated. The additive was based on a formula supplied by Dr A.W.F. Davey (Dairy Husbandry Dept., Massey University) (Personal Communication 1977) and Prof. Cunninghams recommendations.

The formula is detailed in Table 3.3.

TABLE 3.3: COMPOSITION OF FEED ADDITIVE

Bone flour	250g
NaCl	150g
Na_2SO_4	520g
Na_2MoO_4	4g

About one heaped teaspoon of this mixture was sprinkled on the ration of each sheep daily for several weeks.

This however proved unpalatable to the sheep and was discontinued since it was considered that the decreased proportion of sheepnuts in the ration probably minimized the risk of copper poisoning sufficiently.

The calculation of the sheeps' feed requirements was based upon the method of Coop (38) with conversion of values to the metabolizable energy system (24). 1kg DOM was assumed to be equivalent to 15.5 MJME (A.W.F. Davey personal communication 1977). The calculated estimate of the daily maintenance energy requirement of the sheep was 0.37 $MJ.ME.kg^{-0.75}$. This value was used to calculate the maintenance energy requirement of each sheep and hence their maintenance requirements in the form of hay and sheep nuts.

These values are presented in Table 3.4.

TABLE 3.4: MAINTENANCE REQUIREMENTS OF THE EXPERIMENTAL EWES

Ewe No	Live Weight (kg)	Maintenance energy requirements (MJ.ME. d^{-1})	Maintenance Requirements in the form of Hay (Kg d^{-1})	Sheepnuts (Kg d^{-1})
56	53.4	7.31	0.81	0.66
73	60.0	7.98	0.88	0.72
313	53.0	7.27	0.81	0.66
323	67.5	8.69	0.96	0.79
116	50.3	6.99	0.78	0.64
142	40.6	5.95	0.66	0.54
186	44.1	6.33	0.70	0.58
57	47.8	6.73	0.75	0.61

The feed requirements were calculated assuming the ME concentration in hay to be 9 MJ.ME. Kg^{-1} and that of the nuts 11 MJ.ME.Kg^{-1}. The GE of the nuts was (later) determined to be about 18.8 MJ GE Kg^{-1} which supported the ME value used. Now an animals energy requirement (MJ.ME d^{-1}) is lower for feeds of higher ME concentration (because the utilization of ME is more efficient).

The addition of nuts increases the efficiency of utilization of ME in the hay, and conversely the hay reduces the efficiency of utilization of ME in the nuts. For this reason the estimate of the ME concentration in the hay was about the highest value one would expect while that for the nuts is about the minimum. This should have tended to correct for the alteration of ME concentration by mixing hay and nuts in the ration. However, since no determinations were made of the ME concentrations in the hay and nuts, the precision of the calculated requirements was not high. The only really important consideration was that the ewes should all be fed the same amount of the same ration on a metabolic bodyweight basis. They were fed either 1.5 or 1.25 times their initial calculated maintenance requirements with various proportions of nuts and hay ranging from 0.25xM (maintenance) as hay and 1xM as nuts to 0.25xM nuts and 1xM as hay. The relevant details are described in each experiment in chapter 4.

3.4 BLOOD SAMPLING

3.4.1 Requirements

Dry, labelled centrifuge tubes (1 per ewe)
Na EDTA solution (9mg cm^{-3} for 9cm^3 sample)
100 µl micropipette
3 x 10cm^3 hypodermic syringes
Sterile heparinized physiological saline (200 iu heparin cm^{-3})

3.4.2 Procedure

Just prior to the scheduled sampling time 100µl of the EDTA solution was placed in each centrifuge tube using the micropipette. Then using a syringe the contents of the cannula were withdrawn until pure blood was entering the syringe. Using a second syringe the required sample was withdrawn and placed immediately in the appropriate labelled centrifuge tube. Using a third syringe (prefilled) the cannula was filled with the appropriate volume of heparinized saline (usually about 2cm^3) to maintain patency of the cannula. At all times care was taken to avoid air bubbles entering the circulation. All going well it was possible to sample the 4 sheep in a little less than 3 minutes. All samples were mixed by inverting (capped tubes) and placed immediately in a bench centrifuge.

If a sample could not immediately be withdrawn (as happened on many occasions) an initial attempt was made to clear the cannula by infusing 1-2cm^3 of heparinized saline and/or sliding the cannula up and down in the jugular (as far as allowed by the securing arrangements). If this failed the remainder of the sheep were sampled and then further attempts were made to clear the blocked cannula. In some cases a fine monofilament nylon line or fine flexible stainless steel wire was run through the cannula in an effort to clear it. If this was unsuccessful a fine diameter polythene tube was passed through the cannula into the jugular (if possible) to collect the sample). When cannulae reached this state they very seldom remained patent for long and none became properly functional again. Many samples were missed through such problems and in some cases samples were collected by

venepuncture. The objective was to take samples as quickly as possible with the minimum of disturbance to the sheep and at the specified time. If the sample was taken late, the time was noted.

Blood samples were not kept on ice between collection and centrifugation. As the centrifuge was not refrigerated it was thought that there was little to be gained from the added inconvenience of using ice. In addition, most experiments were carried out in the winter and spring when ambient temperatures were low. In any case, samples were processed rapidly and placed in a freezer as quickly as possible (usually 15-20 minutes). In retrospect, and considering the doubts expressed in chapter 4 about the condition of some samples, it would have been preferable to chill the samples immediately after collection.

Samples were centrifuged at 2000 r.p.m. for at least 10 minutes and the plasma was removed using a hypodermic syringe and needle. Disposable pasteur pipettes were tried but it was found that more plasma could be removed without disturbing the blood cells when using the syringe. Each plasma sample was aliquoted into 3 vials. $0.5cm^3$ was placed into an autoanalyzer vial (for glucose assay) and the remainder (about $5cm^3$) was divided between two glass vials (one for hormone assay and the other for plasma metabolites). Adhesive tape was applied to all vials and this was labelled using pencil or indelible pen. In addition the sample number was written on the top of the cap to aid identification of vials when packed in boxes. Samples were immediately placed in a freezer room and later packed in boxes and stored in a domestic freezer.

Following each sample, all tubes, syringes etc were washed in water and 70% alcohol and dried in an incubator. Syringes were replaced at intervals, especially those used for collecting the sample and removing the plasma. Large variation was encountered in the quality of syringes. Brunswick brand were preferred as they had the most effective seal, lasted longest, and the calibrations did not wash off so easily. From time to time the cannula plugs were washed in 70% alcohol.

3.5 MEASURING BACK-FAT DEPTH

The degree of fatness of the ewes was assessed at intervals by estimating the thickness of subcutaneous fat on the back of the sheep. This was done using a prototype ultrasonic fat depth sounder under development by the DSIR's Applied Biochemistry Division at Palmerston North and the Industrial Development Division in Auckland. The equipment at the time consisted of a transducer wired to a modified Hewlett Packard Ultrasonic Echoencephalograph as described by Gooden et al (63). The principles of the device were also adequately reported by Rennie (133).

Measurements of the fat thickness were made at 2 sites, 50mm either side of the midline (spine) over the twelfth rib. Wool at the site was clipped as close as possible to the skin using Oster clippers. The position of the twelfth rib was identified by feeling the spinal processes and the sites 50mm either side of the midline were marked with indelible ink. A small amount of petroleum jelly was applied to the face of the transducer to provide satisfactory acoustic coupling with the skin. The transducer was held lightly but firmly on the skin perpendicular to the skin surface, and the thickness of the skin and fat read (as indicated by peaks on a line) from a calibrated scale on the oscilloscope screen. An experienced operator, Dr Roger Purchas (Sheep Husbandry Dept. Massey University) performed this operation on all occasions as it was believed that live/carcass relationships differed between operators and that better repeatability would be obtained by experienced operators. Purchas and Beach (129) confirmed the former assumption but found that experience did not significantly affect the repeatability of measurements.

The 2 records of fat plus skin depth were averaged and the estimate of skin thickness (usually 3mm) was subtracted to obtain an estimate of the back-fat thickness over the 2 sites. Some problems were experienced in deciding which peak on the graph corresponded with which tissue interface and doubts were held about the accuracy of the measurements, particularly in very fat ewes. It was felt that an additional interface may be present in the very fat sheep as 2 peaks were sometimes detected in the expected zone. The deeper of

these was thought to represent the muscle/fat interface as it was detected with lower amplification. The intermediate peak may have represented an interface within the subcutaneous fat; layers within the adipose tissue. It was not practical to slaughter the ewes or to perform surgery to confirm the estimates, however the death of Ewe 56 provided an opportunity to confirm by dissection that fat depths were indeed in the range of those recorded (about 20mm). Examination of the tissue, both fresh and frozen, did not confirm or refute the hypothesis regarding an interface in the fat. (Unfortunately this was not subjected to expert scrutiny). However layering of the subcutaneous fat has been observed elsewhere in fat sheep (R. Purchas, personal communication, 1977).

It was initially hoped that the ultrasonic fat depth measurements would provide evidence of changes in adipocity in response to lipolytic stimuli in addition to providing an index of fatness of the sheep. No differences were detected, but this may have been due to either a lack of sensitivity or to the fact that no differences existed.

3.6 PLASMA GLUCOSE ASSAY

The method used for plasma glucose determinations was an automated method described by Rosevear et al (136), using glucose oxidase, peroxidase and o-dianisidine dihydrochloride. The Auto Analyzer incorporated a dialyzer through which the glucose from the plasma sample passed into a recipient (sodium sulphate) solution where it was combined with the enzyme-dye solution and after incubation in a water bath, estimated colourimetrically. The absorbance was recorded graphically on an automatic recorder. Glucose standard solutions of 2.0, 2.5, 3.0, 3.5 and 4.0mM were included in each assay. The absorbance of all standards used in each assay were used to construct a standard curve from which the glucose concentrations of the unknown plasma samples were estimated.

The glucose determinations were not made by the author but were performed by courtesy of Dr R.M. Greenway (Chemistry, Biochemistry, Biophysics Department, Massey University) in his laboratory.

3.7 PLASMA FREE FATTY ACID ASSAY

Plasma FFA concentrations were determined by a slightly modified version of the method described by Dole (46).

3.7.1 Reagents

Extraction mixture: Lipids were extracted from the plasma using a mixture consisting of 40 parts propan-2-ol, 10 parts n-heptane and 1 part 1N H_2SO_4.

Titration mixture: the end point in the titration was determined using the indicator thymol blue (0.01%) and 90% ethanol in water, made by dilution of a stock 0.1% thymol blue solution in water with 9 parts of ethanol.

Alkali: the FFA's were titrated using approximately 0.18* N NaOH made fresh daily by 1/100 dilution of saturated NaOH with CO_2 free distilled water. (Both the saturated NaOH stock and the diluted solution were protected from CO_2 during storage by a soda lime column).

Standards: the fresh alkali was calibrated each day by titration of standards of recrystallized palmitic acid in heptane.

All solvents were double redistilled.

3.7.2 Method

As soon as samples were thawed the lipids were extracted from $1cm^3$ of plasma by adding $5cm^3$ of extraction mixture, shaking vigorously in a glass stoppered tube and standing for 10 minutes. The contents were then divided into 2 phases by the addition and mixing of $2cm^3$ of heptane and $3cm^3$ of distilled water. The phases separated rapidly forming a sharp interface. A $2cm^3$ aliquot was transferred to a glass test tube containing $1cm^3$ of titration mixture. Dole specified a $3cm^3$ aliquot but as it was found that the upper phase measured almost exactly $3cm^3$ it was impossible to remove this amount satisfactorily without risking removal of some of the lower phase. Accordingly a $2cm^3$ aliquot was used and the appropriate correction factor applied to the titre. The aliquot was titrated with the alkali which was delivered from a microsyringe through a fine calibre polythene tube

* An apparent error was noted in the reference by Dole (1956) (46) in which he quotes 0.018N NaOH. This does not agree with his method of preparation of the solution.

and ultimately passing through the barrel of a 21 gauge hypodermic needle. This last item was employed to minimize the size of alkali droplets exiting the delivery tube. Carbon dioxide-free Nitrogen was delivered to the bottom of the tube through a pasteur pipette to expel CO_2 from the sample and keep it mixed during titration. The gas stream was interrupted at intervals to check the indicator colour. Good lighting was essential to determine the green-yellow endpoint as the colour changed very rapidly with the addition of minimal alkali once the endpoint was reached. The titratable acidity of the system due to non-plasma components was monitored by the inclusion of blank tubes containing $1cm^3$ of titration mixture and $2cm^3$ of heptane. The titre of all other tubes in each run was corrected by subtracting the average value of the titres for the blank tubes. Standard solutions of 0.1, 0.2, 0.3, 0.4 and 0.5mM Palmitic acid were included in the assay. The corrected titres of all standards were used to construct a standard curve from which the FFA concentration of the plasma samples was estimated.

This assay was found to be sensitive in the extreme to a multitude of factors which could affect the value of the titre obtained. The main identifiable factors involved were the cleanliness of glassware, the purity and pH of reagents, the time elapsed during each stage of the assay and operator skill.

The cleanliness of the glassware (especially the titration tubes) was the major source of variation. Test tubes which had not been previously used in the assay (both new and used tubes) initially produced high and variable titres when used as blanks compared to those which had been in use for some time. Accordingly 2 extra identified tubes (containing blank solution) were included in each run in order to "break them in" so that they could be used to replace damaged tubes without introducing extra variation into the assay. (The titres obtained from tubes being "broken in" were not included in the calculation of the average value of the blanks). The technique of cleaning the glassware was found to be critical. Tubes were soaked in Decon 75 (Selby Wilton Scientific Ltd) (chosen for its neutral pH) for up to 20 mins then scrubbed twice in the same solution after

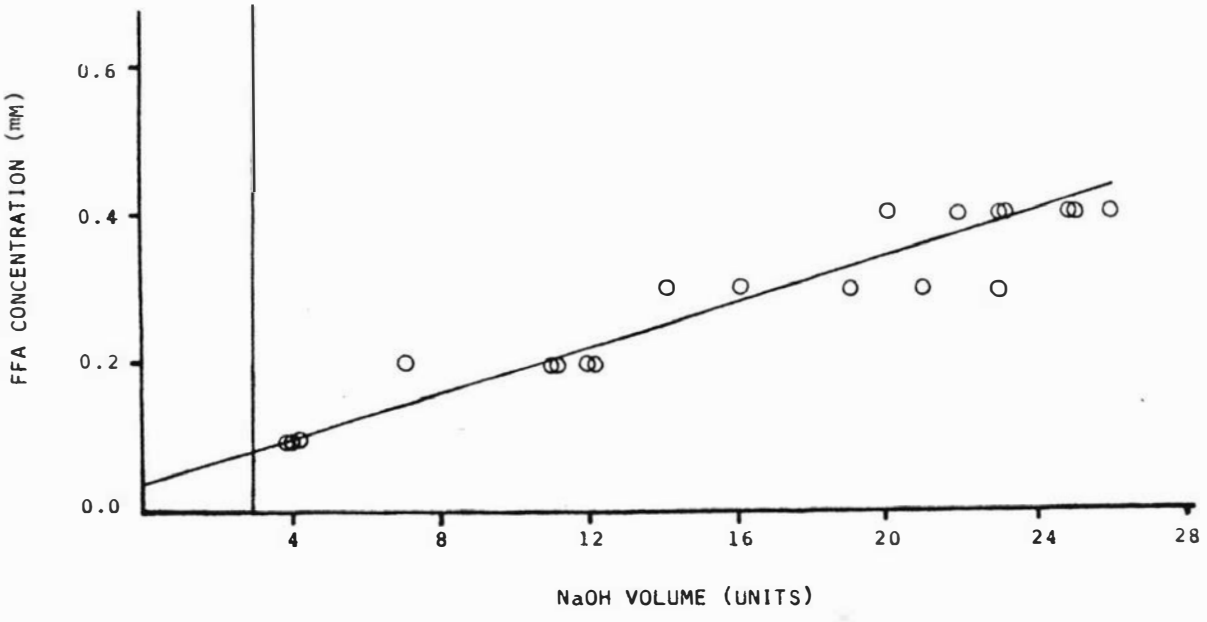

Figure 3.4 FFA standard curve ; Regression of FFA concentration on titre of NaOH.

$Y = 0.0156X + 0.035$ $r = 0.94$

which they were rinsed in tap water then distilled water a total of 7 times. It was possible to reduce the number of rinses to 3 or 4 when the assay had been operating successfully for a number of days and when the routine was fairly constant. Changes in routine were believed to have been responsible for some of the major variations in the titratable acidity of the system (blanks) e.g. soaking tubes in Decon solution over lunch hour or overnight often produced unsatisfactory results as did inadequate washing of tubes or changes in the routine of washing the glassware used to prepare the alkali. Extended extraction times or delays between extraction and titration caused increased titres also.

Changes in the titratable acidity of the system were also due to changes in the acidity of reagents. The alkali was protected from CO_2 by soda lime but the other reagents were not. Dole warned that the titratable acidity of ethanol slowly increases over several days (46) but this effect was not clearly identified. Rather, on 2 occasions, large changes in acidity occured very suddenly (within hours). The source of the acid was not specifically identified but it appeared to be in the heptane. Certainly it was in the titration mix. All solvents were double redistilled and the acidity of the system declined over several days. Eventually, after major efforts to trace the source of the acid and to resolve the problem by cleaning of glassware and purification of reagents it was decided to follow Doles hint (46) and counter it by addition of NaOH. This was successful (but cleanliness was still essential).

The precision of the assay was monitored by the repeatability of standards and blanks. At least 2 standards and 4 blanks were included with each batch of 8 samples. A typical standard curve is shown in Figure 3.4. Analysis of variance showed that the differences between palmitate standard concentrations were significant ($p < 0.0001$). The standard error of the mean titre for any FFA standard concentration was 1.24 units of NaOH.

3.8 RADIOIMMUNOASSAYS

GH and insulin were assayed using specific double antibody radioimmunoassays. The hormones used in the assays were; Growth hormone: Bovine GH NIH GH B8 Kindly donated by Professor A.E. Wilhelmi (National Institute of Health, U.S.A.).
Ovine GH Ext. IIB S(A) Kindly donated by Dr Carolyn Redekopp (Princess Margaret Hospital, Christchurch)
Insulin: Bovine pancreatic insulin, Sigma Chemical Co
No 1-5500 lot 121 C-1350 (26.4 iu mg^{-1})

3.8.1 Growth Hormone R.I.A.

The GH RIA was basically the same as the method of Hart, Flux, Andrews and McNeilly (73).

1) HORMONES

Bovine GH
In order to remove contaminating hormones the BGH was purified by ion-exchange chromatography as described by Hart et al (73). It was used for radioactive tracers, for standards in the RIA's and for raising antibodies.

Ovine GH
The OGH was used only for standards in the RIA's

2) IODINATION

The BGH was iodinated using the Chloramine T method described by Greenwood, Hunter and Glover (66) except that borate buffer was used instead of phosphate buffer for the initial steps and the BGH was dissolved in 2M urea at room temperature 55 mins before iodination (73). The iodinated BGH was separated from the salts on a Sephadex G50 column and batches to be used more than 2 weeks after iodination were further purified on a Sephadex G100 column.

3) ANTISERA

Antiserum to BGH was raised in guinea-pigs by injection of BGH conjugated with guinea-pig serum albumin in Freunds complete adjuvant (Ist antibody. GP_1 (March 1974) and antiserum to guinea-pig γ-globulin by injection of guinea-pig γ-globulin into a Romney wether (2nd antibody, Phil (Dec 1975)). Both antisera were kindly provided by Professor D.S. Flux (Dairy Husbandry Dept., Massey University) and had been proven effective in previous assays.

He also tested the antiserum to BGH for cross reactivity with TSH, FSH, LH, ACTH and Prolactin (which were kindly donated by Dr K.R. Lapwood, Department of Physiology and Anatomy, Massey University). Prof. Flux showed evidence of some slight non-specific reactivity with these hormones in the BGH assay but all regressions of binding on dose of hormones were not significant. The greatest depression was caused by ACTH at 1000 ng cm^{-3} and was equivalent to that caused by about 4 ng cm^{-3} of BGH (D.S. Flux, unpublished results).

4) RADIOIMMUNOASSAY METHOD

The final form of the assay after testing and modification, was as follows:

The diluent buffer (assay buffer) was 2% Human plasma in 0.01 M phosphate buffered saline (PBS) pH 7.5 containing 0.01 M EDTA. The human plasma was filtered before use by passing through a millipore filter to remove large particles. The reagents were added to $3cm^3$ plastic LP3 tubes in the following order: 100µl of assay buffer containing antiserum to GH (diluted 1:20,000) and 1 µg guinea-pig γ-globulin; 100µl of a solution of standard GH or 100µl of sample or 50µl of sample plus 50µl of assay buffer. The reagents were mixed on a vortex mixer and incubated at room temperature for 24 hours, after which 50µl of assay buffer containing ^{125}I-GH with a radioactivity of 5000 ± 100 counts per minute (cpm) was added. The contents were again mixed and incubation continued. After a further 24 hours, 50µl of antiserum to guinea-

TABLE 3.5: DESIGN AND RESULTS (CPM) OF FACTORIAL EXPERIMENTS TO DETERMINE OPTIMAL RIA METHOD

INCUBATION TIME*	2ND ANTIBODY DILUTION	No Standard BGH					1ng Standard BGH				
		FIRST ANTIBODY DILUTION									
		1:12000	1:14000	1:15000	1:16000	1:20000	1:12000	1:14000	1:15000	1:16000	1:20000
72 hours	1:30	1227	1338	1334	1304	1346	785	672	706	656	604
	1:36	1367	1306	1969	1298	1330	740	679	689	635	626
	1:40	2415	2328	2218	2142	2111	757	776	673	685	638
	1:45	1504	1573	1745	1365	1344	780	775	749	761	662
105 hours	1:30	1063	857	1015	796	591	466	497	426	483	483
	1:36	713	911	1007	842	877	543	526	530	532	453
	1:40	617	687	670	542	562	415	566	424	447	452
	1:45	774	1174	748	865	632	469	476	410	388	406

N.B. Each datum is the mean of 2 (duplicate) determinations

* Incubation time following the addition of 2nd antibody

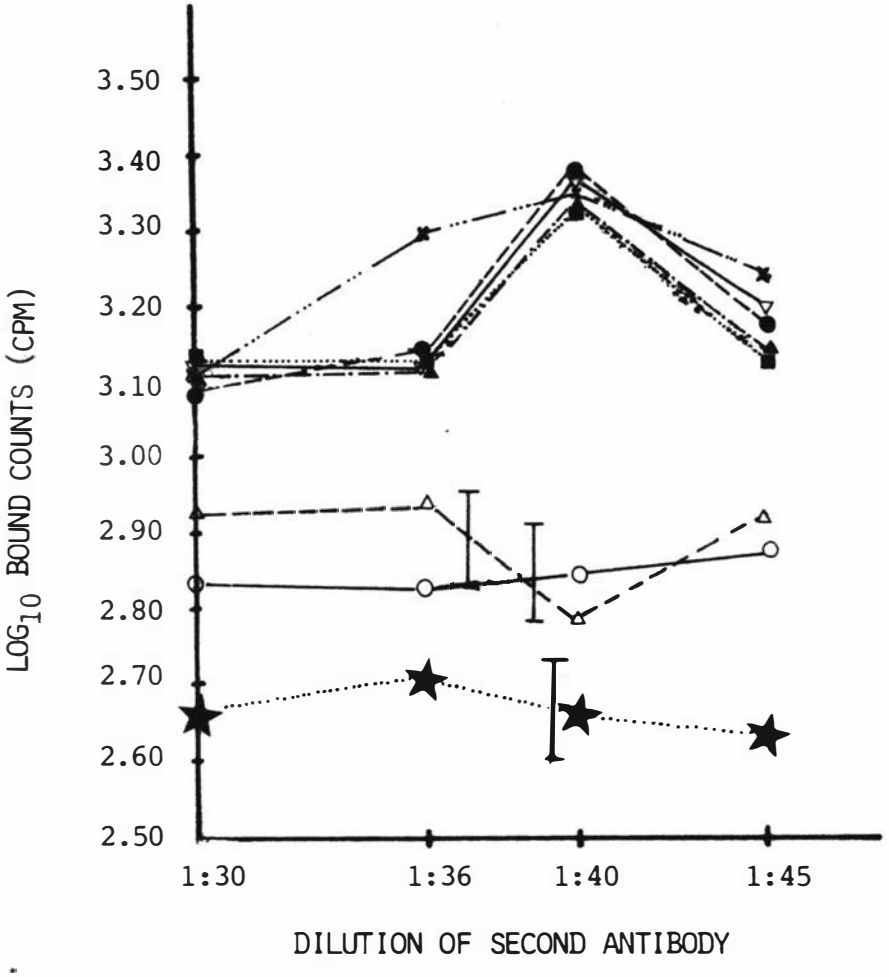

Figure 3.5 Binding of BGH using 5 dilutions of 1st antibody, 4 dilutions of 2nd antibody, 2 levels of BGH and 2 incubation times.

1:12000 dilution of 1st antibody,0ng BGH,72 hours
1:14000 " " "
1:15000 " " "
1:16000 " " "
1:20000 " " "

Mean of 5 levels of 1st antibody,0ng BGH,105 hours
" " " 1ng BGH, 72 hours
" " " 1ng BGH,105 hours

pig- γ-globulin (diluted 1:40) in assay buffer was mixed in and incubation continued at 4°C. After a further 48 hours the reaction tubes were centrifuged at 2000 rpm for 60 min and the supernatants (which contained free ^{125}I-GH) were removed by aspiration and discarded. The radioactivity remaining in the precipitate, (bound ^{125}I-GH) was determined in a gamma counter (NE. 1600). All assays were performed in duplicate and with 2 levels of sample (i.e. 2 x 50µl and 2 x 100µl).

5) DEVELOPMENT OF THE GH RIA

In order to determine the optimum dilutions of antisera and incubation time a factorial experiment was carried out incorporating 5 levels of 1st antibody, 4 levels of 2nd antibody and 2 incubation times. The design and results of this experiment can be seen in Table 3.5 and Figure 3.5. Two levels of standard BGH were used; 0 and 1ng cm^{-3} . Since radioactive counts (cpm) are not normally distributed the data in Table 3.5 were transformed to logarithims for analysis of variance and graphing. Over all the data there was no significant interaction between levels of 1st and 2nd antibodies ($P = 0.88$) and no significant difference between 2nd antibody levels ($P = 0.65$) but as can be seen in Figure 3.5 there was an apparent advantage in using a 1:40 dilution of 2nd antibody during the 72 hour incubation when no GH was present. The binding resulting from the 2 incubation times was significantly different ($P < 0.001$) with bound counts for the 105 hour incubation lying between a half and a quarter of the magnitude of the 72 hour values. There was also a significant interaction between 2nd antibody levels and incubation times ($P < 0.001$).

Accordingly the 72 hour incubation with 1:40 dilution of 2nd antibody was selected as the optimal combination for further assays as it apparently resulted in the lowest levels of non-specific binding. Although there was evidence of some difference between binding achieved with different 1st antibody levels ($P = 0.03$) this was considered too small to be important (see Figure 3.5) at these levels of BGH, so the middle value of 1:15,000

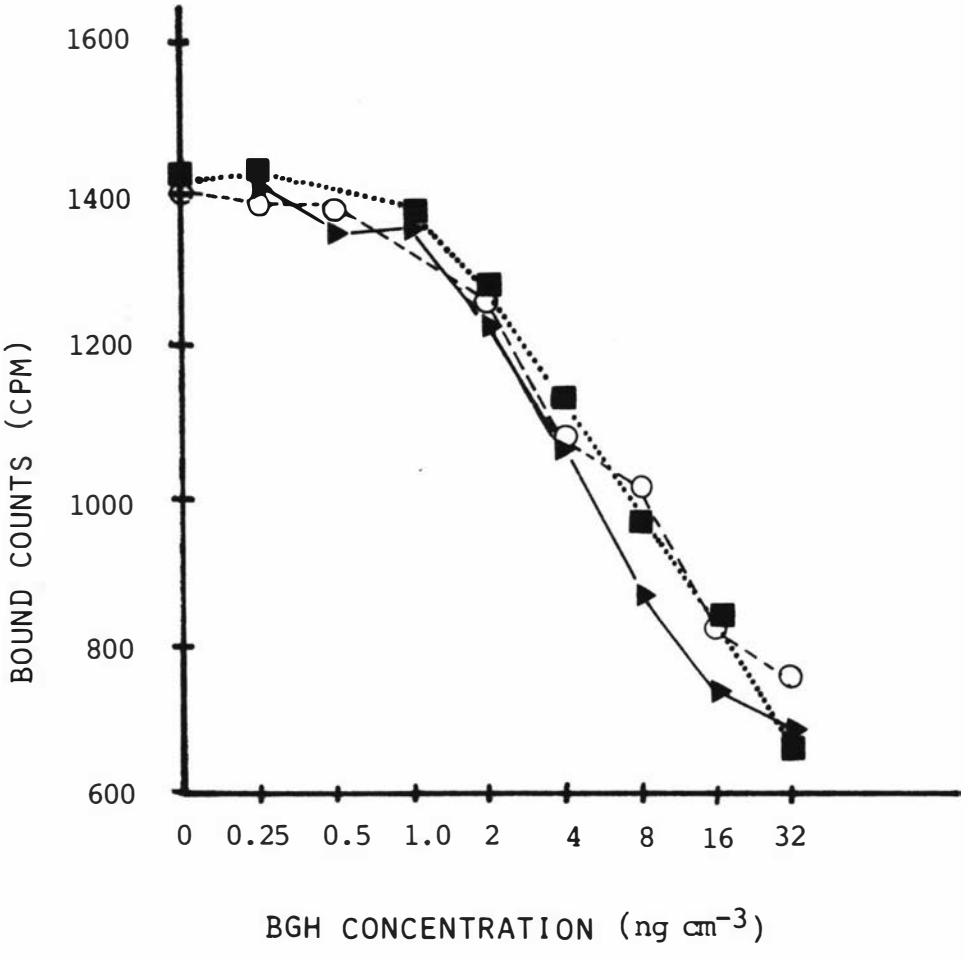

Figure 3.6 BGH dose response curves with 3 levels of 2nd antibody after 96 hour incubation

1:35
1:40 dilution of 2nd antibody
1:45

was selected for following work. It is worthwhile noting that there was a significant difference ($P < 0.001$) between the binding recorded with the 2 levels of BGH indicating that the assay could distinguish between 0 and 1 ng cm^{-3}.

A summary of the analysis of variance results can be seen in Appendix 3.8.1a.

It should be noted that in the early stages of development of the RIA 100µl of human plasma (HP) was added to all tubes. This was due to a misunderstanding and was later amended to 100µl added to tubes containing standard solutions only in order to equalise the protein concentration in those tubes with the protein levels in the tubes containing samples, because it was thought that protein concentration influenced the degree of binding achieved.

The next step taken was to determine dose response curves using a first antibody dilution of 1:15,000, 2nd antibody at 3 levels (1:35, 1:40 and 1.45) and 2 incubation times. It was intended to incubate for 72 and 96 hours following addition of 2nd antibody but an error in recording resulted in incubation times of 96 and 120 hours. BGH standard solutions were 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ng cm^{-3}. In addition a sample of calf plasma was included at 3 dilutions. The dose response curves for the 96 hour incubation can be seen in Figure 3.6. The curves for the 120 hour incubation were similar but exhibited considerably more variation, and it was assumed that proteolysis had taken place during the longer incubation.

The bound counts for the calf plasma samples are listed in Table 3.6.

TABLE 3.6: BOUND COUNTS (CPM) OF 3 DILUTIONS OF CALF PLASMA WITH 3 LEVELS OF SECOND ANTIBODY AFTER 96 HOUR INCUBATION

CALF PLASMA (µl)	HUMAN PLASMA (µl)	2ND ANTIBODY DILUTION 1:35	1:40	1:45
100	0	1058	1407	926
50	50	1533	2152	1041
25	75	2653	2820	1154

N.B. Data are the mean of duplicate samples

While all 3 dose response curves in Figure 3.6 appear satisfactory and are very similar the calf plasma samples exhibited different binding characteristics with different dilutions of 2nd antibody. The 1:40 dilution of 2nd antibody resulted in higher binding than the 1:35 dilution which was in turn higher than the binding of the 1:45 antibody dilution at each level of calf plasma. What was more significant however was the fact that the bound counts of 5 of the 6 dilutions (50 and 25µl) of calf plasma were considerably higher than the dose response curves.

To investigate this anomoly another similar factorial experiment was carried out incorporating additional replicates of the 0, 0.25 and 0.5 ng cm^{-3} BGH standards to confirm the binding levels in the upper regions of the dose response curves. In addition ewe and lamb plasma samples were included at levels of 50,100 and 200 µl in order to see whether their binding corresponded with levels on the dose response curves (standard curves). Unfortunately the assay did not work satisfactorily, probably due to the omission of EDTA from the assay buffer.

Because the first does response curves (those in Figure 3.6) exhibited binding somewhat lower than that of the unknown samples it was considered wise to check the accuracy of the standard BGH solutions by comparison with other BGH standards. Accordingly standard BGH solutions prepared by Professor D.S. Flux were included in the next experiment; in addition to the authors own standard solutions. Since the BGH

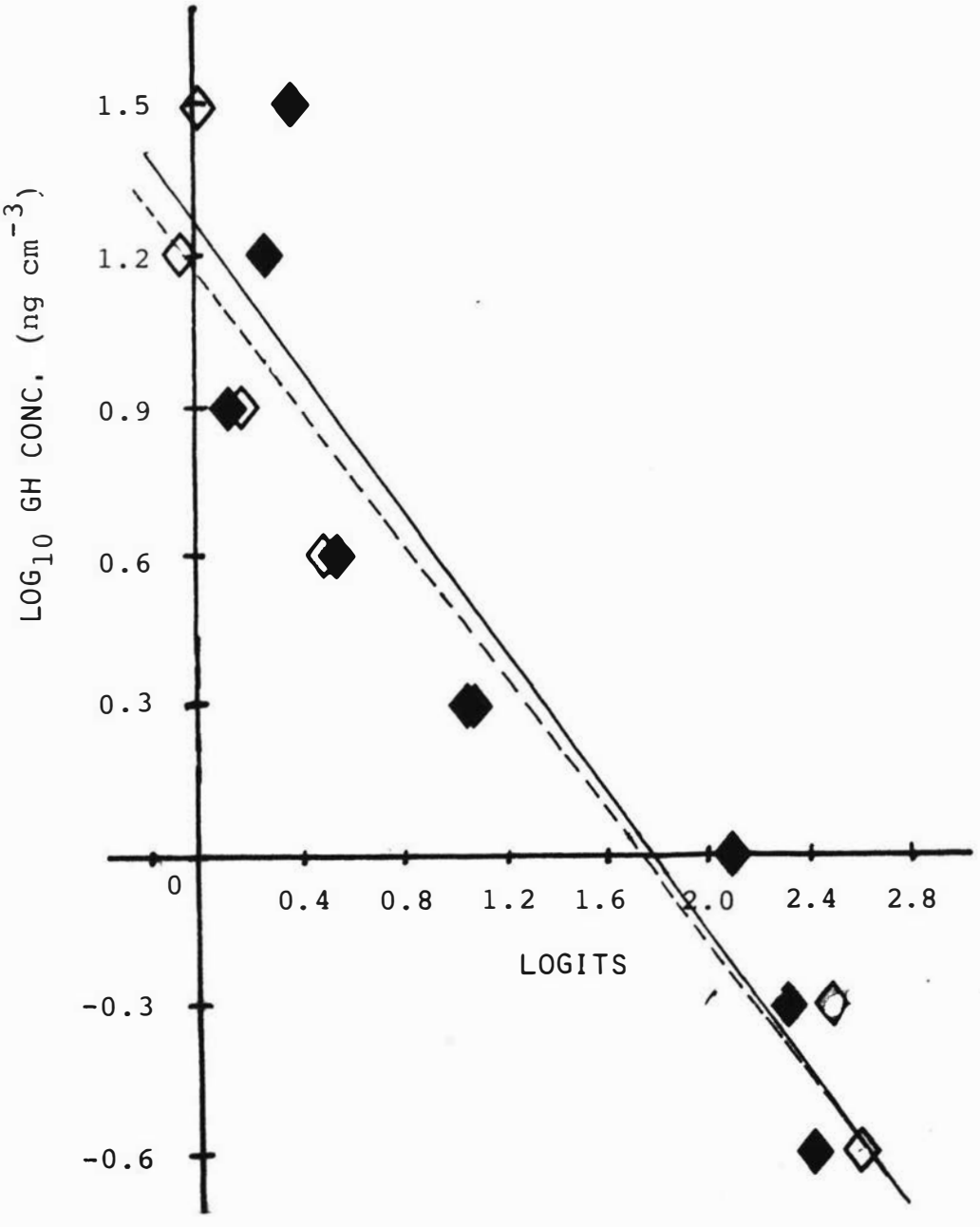

Figure 3.7 Standard curves of authors BGH standards compared to Prof. D.S. Flux's BGH standards.

Curves are the regression of Log_{10} GH concentration on logits.

Prof. Flux's standard curve

authors standard curve

standards seemed little affected by differences in dilution of 2nd antibody (see Figure 3.6) it was considered unnecessary to include standard curves with different 2nd antibody dilutions, so a 1:40 dilution was used for the standards.

However the unknown calf plasma samples (see Table 3.6) were apparently affected by 2nd antibody dilution. Hence the plasma samples in this trial were assayed using 3 levels of 2nd antibody once more i.e. 1:35, 1:40, 1:45. The unknown plasma samples used were; a bulk ewe plasma sample at 4 dilutions (100, 50, 25 and 12.5 µl made up to 100 µl with human plasma), calf plasma, known to contain high GH levels at 3 dilutions (100, 50 and 25 µl made up to 100 µl with human plasma) and lamb plasma at the same dilutions as for calf plasma.

Since the bound radioactivity (cpm) in the reaction tubes was lower than expected, tubes were counted for 2 minutes in order to improve accuracy. The standard curves obtained are shown in Figure 3.7. Analysis of covariance showed that the curves did not differ significantly in either slope or elevation ($P = 0.71$ in both cases). The analysis of covariance can be seen in Appendix 3.8.1b. For the purpose of analysis, data were transformed to logits using the formula;

$$\text{Logit} = \frac{B}{T-B} \log_e$$

where B = Bound counts

T = Bound counts with 0ng cm^{-3} BGH

in order to obtain values normally distributed about the mean. Another means which often achieves this is to simply take square roots of the data. This was carried out on the unknown data prior to analysis of variance.

TABLE 3.7: ANALYSIS OF VARIANCE OF BINDING OF SHEEP PLASMA AT 4 LEVELS WITH 2 DILUTIONS OF SECOND ANTIBODY

Source of Variance	d.f.	M.S.	P
TOTAL	12		
Between dilutions of 2nd antibody	2	3.3316	p = 0.13
Between levels of sheep plasma	3	4.7235	p = 0.06
Residual (error)	7	1.1920	

As indicated in Table 3.7 there was no significant difference between the results achieved with different levels of second antibody (p = 0.13) indicating a degree of safety in using dilutions around 1:40. A significant difference (p = 0.06) was found between results obtained with different levels of sheep plasma indicating that the assay was capable of detecting such differences. Analysis of variance of the calf plasma gave similar results.

Most of the bound counts for the unknowns fell within the range of the standard curves except for the highest levels (100 µl) of calf and lamb plasma. Estimates made using the fitted regression lines indicated that the calf and lamb plasma had GH concentrations in the range 20-30 ng cm^{-3} while the bulk ewe plasma had levels around 2-3 ng cm^{-3}.

It seemed then that the assay was working satisfactorily, that the 1:40 dilution of 2nd antibody was safe and that the BGH standards were sufficiently accurate. What is more, the levels of GH in the ewe plasma corresponded with the central, straightest part of the standard curves. With this apparent success it was only logical to begin the assays of the unknown experimental ewe plasma samples.

Two assays were carried out to determine the GH levels in samples from Experiments II, III and IV; but unfortunately few of the counts for the unknowns fell within the range of the standard curve. Most counts were higher than the highest counts on the standard curve. It was thought that ovine GH (OGH) might exhibit different binding characteristics to BGH so it was decided to include OGH in future assays. It

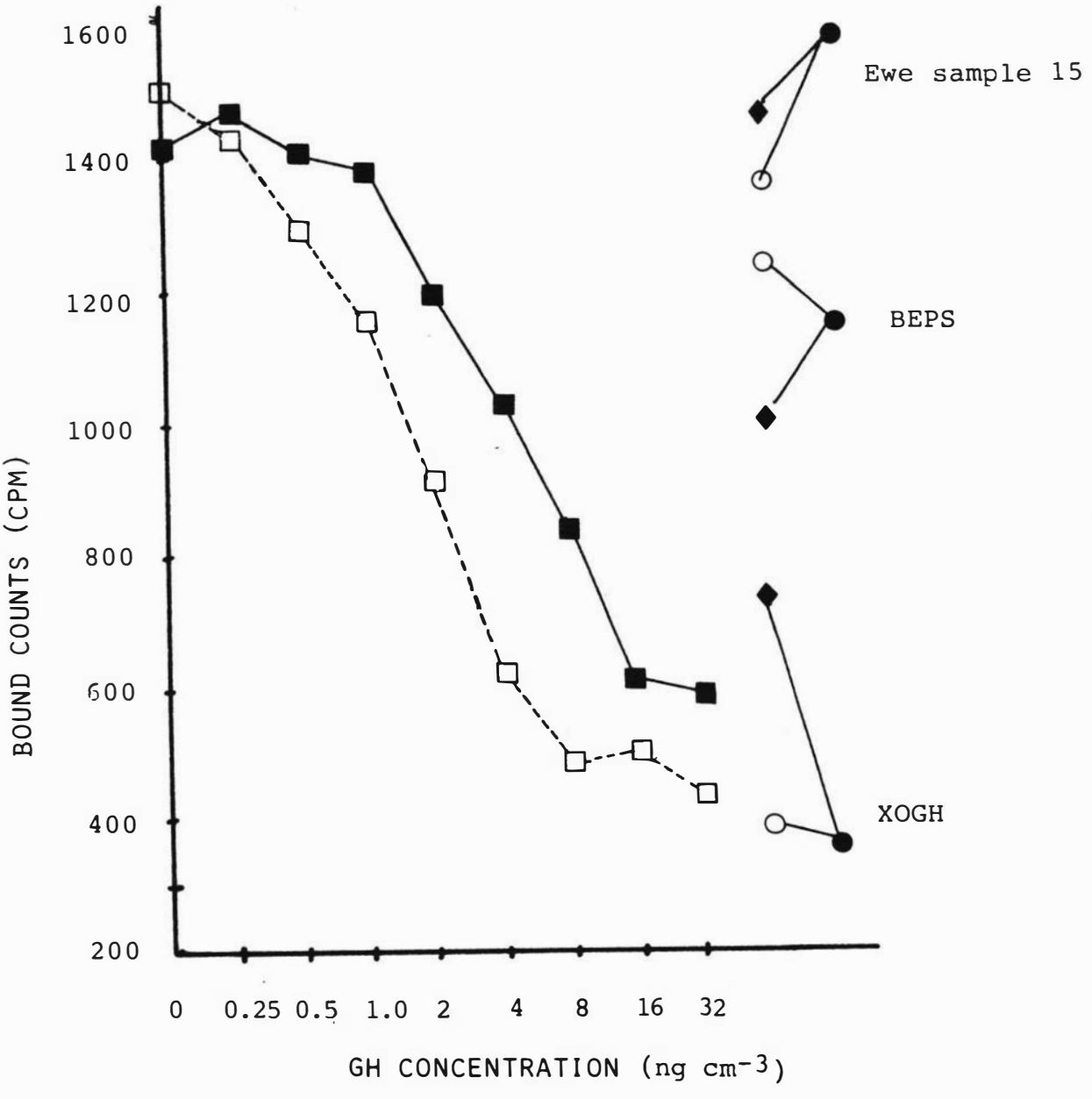

Figure 3.8 BGH and OGH standard curves at 1st antibody dilution of 1:20,000 and unknowns with or without HP in the 50ul sample.

BGH standards
OGH standards
100ul unknown sample
50ul-HP unknown sample
50ul+HP unknown sample

was also possible that something was depressing the BGH standards relative to the unknowns. Professor D.S. Flux had shown (using the same procedure and the same antisera in the same lab) that increasing levels of HP (human plasma) added to the reaction tubes progressively depressed the BGH standard curve, especially at lower levels of BGH. (D.S. Flux, personal communication 1980). In addition he found that removal of low molecular weight components (<20,000) from HP resulted in less depression of the standards i.e. 100µl of "Big HP" (low mwt components removed) did not depress the standards significantly more than did 25µl of "Big HP" or more than 25µl of untreated HP.

Another assay was carried out including BGH and OGH standards with plasma samples from Experiment IV. All HP used in the assay was "Big HP" prepared by Prof. Flux by passing HP through a "Diaflow filter". The BGH and OGH standard curves obtained were very similar and were satisfactory over the range 1-32 ng cm^{-3}. However the unknown plasma samples correspond with the poor part of the curves below 1 ng cm^{-3}. The bulk ewe plasma standard (BEPS) only, corresponded with the useable part of the curve showing a concentration of 2-3 ng GH cm^{-3}. It was concluded that the unknown plasma samples must indeed contain very low levels of GH and that the sensitivity of the assay below 1 ng cm^{-3} needed to be much improved by increasing the first antibody dilution.

A factorial experiment was carried out incorporating three levels of first antibody (1:16,000, 1:20,000, 1: 24,000) in an effort to improve the sensitivity of the assay. Both BGH and OGH standards were included at each level of first antibody, along with BEPS, samples from Experiment IV and an unknown OGH standard (XOGH) which had been weighed out inaccurately. The 50 µl levels of all unknowns were made up to 100 µl with Diaflow filtered HP as usual (50 + HP) and an additional 50 µl was made up to 100 µl with assay buffer (50-HP) to see whether HP was depressing binding and therefore affecting parallelism. The results of the experiment for the 1:20,000 dilution of first antibody are shown in Figure 3.8. Increasing the first antibody dilution to 1:20,000 achieved a marked improvement in the standard curves below 1 ng cm^{-3} compared to the 1:16,000 dilution.

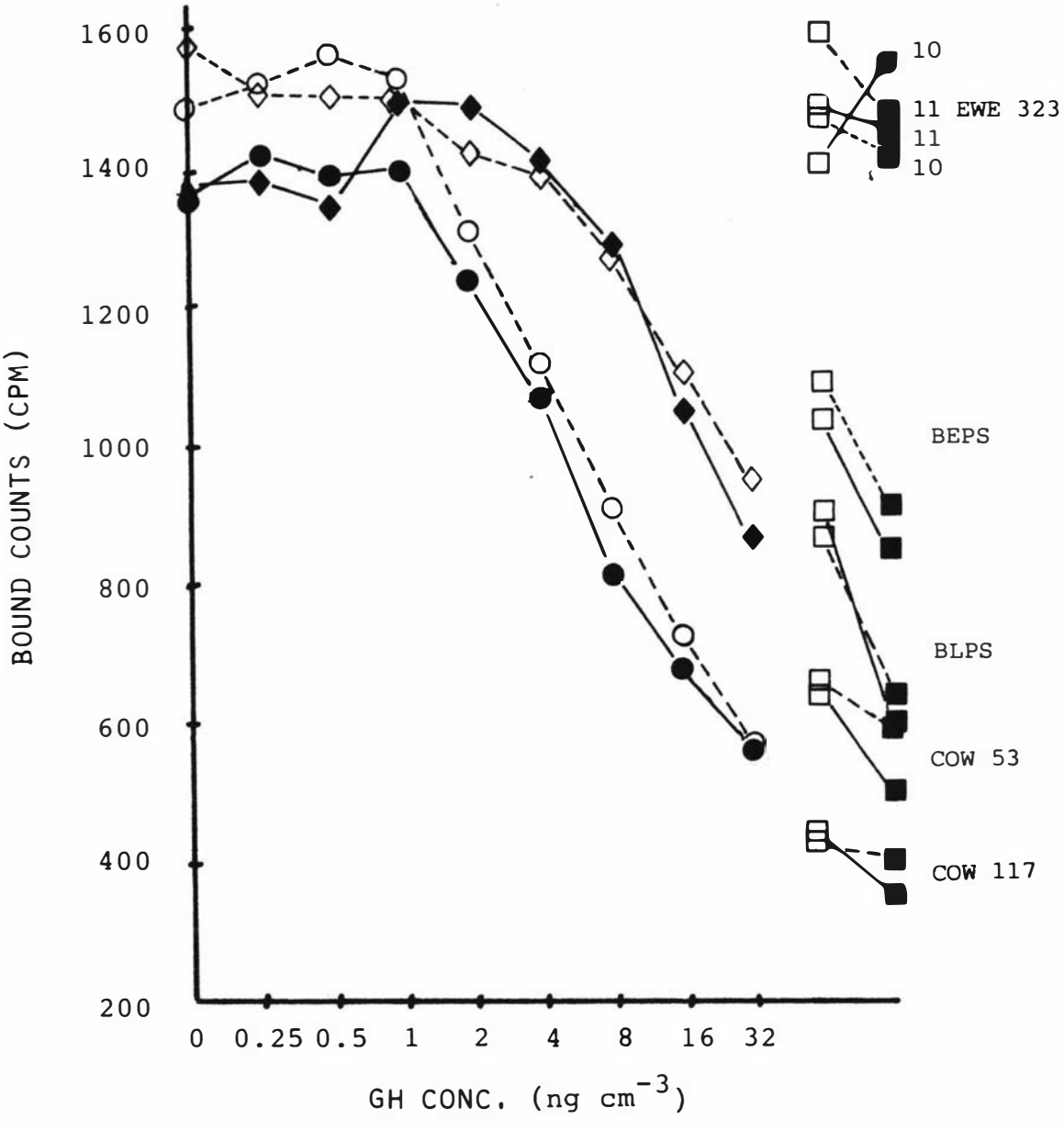

Figure 3.9 BGH and OGH standard curves with and without HP and some unknowns at 2 levels with and without HP.

- BGH + HP
- BGH - HP
- OGH + HP
- OGH - HP
- 50ul unknown sample
- 100ul unknown sample
- unknown sample with HP
- unknown sample without HP

The standard curves for the 1:24,000 dilution were not better than those for the 1:20,000 dilution. To improve sensitivity it was necessary to accept a considerable loss of precision at GH levels above 8 ng cm^{-3} i.e. it was not possible to distinguish between levels greater than 8 ng cm^{-3} with any degree of accuracy. However this was considered unimportant since the unknowns corresponded with the other (low) end of the GH concentration scale. It can be seen in Figure 3.8 that HP was exerting a marked effect on parallelism. HP depressed BEPS, raised the unknown sheep plasma samples and raised the XOGH binding. Thus it was apparent that something in the HP was interacting with different samples in a different way, but no explanation could be offered for this. Since the validity of an assay depends upon all samples reacting in the same manner it was important that this effect be eliminated. It was decided to include some standards with no HP (except that in the assay buffer) in the next assay. It was known that the presence of some plasma protein in the reaction tubes was essential for the action of the assay, but a suitable level and source of protein for OGH assays had evidently not been found.

During the incubation of the experiment just described, the author was injured and as a result the development of the GH RIA ceased for 9 months. The next assay was therefore composed of totally new reagents in all cases except for the antisera. The assay was another factorial including both BGH and OGH standards, BEPS, bulk lamb plasma sample (BLPS), unknown sheep plasma samples from Experiment IV and several cow plasma samples. The cow plasma samples were known to contain high BGH levels and the BLPS was assumed to have high OGH levels. It was thus expected to achieve a range of results over the whole range of the standard curves. All samples were duplicated; one set contained no HP except that in the assay buffer (2%) while the other set contained 50µl HP in the standards and an additional 50µl of assay buffer in all unknowns. (The 50µl level unknowns thus still had 50µl more assay buffer than the 100µl level unknowns). The results as shown in Figure 3.9 indicated that HP was depressing the standards, especially at lower GH levels (<2 ng cm^{-3}). The first antibody dilution of 1:20,000 increased the ability to distinguish between such low levels of GH and it was clear that there was a considerable difference between

standards with HP and those without HP below 2 ng cm^{-3} GH. The unknown ewe plasma samples (Ewe 323) corresponded with the zone of depression between the standards with HP and those without. Eleven of the twelve estimates of the unknown ewe plasma samples were elevated by the addition of HP (as 2% HP assay buffer) so that the net result of reducing HP in both standards and unknowns was to raise the standards and depress the unknowns so that they coincided to a much better degree. However the standard curves were not sufficiently linear below 2 ng cm^{-3} and parallelism was not good amongst the unknown ewe plasma samples so accurate determinations of the unknowns was still not possible.

The BEPS, BLPS and cow plasma samples were also affected by HP but in those cases additional HP depressed binding. Parallelism however was good and using the BGH standard curve BEPS was estimated to contain about 8 ng GH cm^{-3} while BLPS contained about 28 ng GH cm^{-3}. The cows had BGH concentrations greater than the highest BGH standard (32 ng cm^{-3}).

As a result of this experiment it was decided to omit HP from future assays except for the 2% HP in the assay buffer. Furthermore, the successful binding achieved with BEPS, BLPS and cow plasma indicated that in all probability the assay was working satisfactorily over the range 2-32 ng cm^{-3} and that the levels of GH in the unknown ewe plasma samples were truely less than 2ng cm^{-3}. The BEPS, BLPS and cow plasma were relatively fresh samples compared to the unknown plasma samples of Experiment I, II, III and IV used in assays to date and it was considered that the older samples may have deteriorated for some reason. With these thoughts in mind it was decided to embark on full scale analysis of the experimental unknown ewe plasma samples beginning with those most recently collected, from Experiment IX. Should these unknowns have exhibited binding corresponding with GH levels less than 2ng cm^{-3} it would have to be accepted that the GH levels were lower than the sensitivity of the assay.

Experiment IX involved BGH infusions into ewes and it was expected that high GH levels would be detected. Standards up to 128 ng cm^{-3} were included in the assay which did indeed (as shown in the results

in Chapter 4) detect high GH levels in the unknowns. Many determinations were in fact in excess of the highest standard so that it was necessary in following assays to improve the precision of the assay at high GH levels by returning the first antibody dilution to 1:15,000. The assay was deemed to be working satisfactorily and the majority of problems experienced were attributed to genuinely low levels of GH in the unknown ewe plasma samples from Experiments I, II, III and IV which may have deteriorated in storage.

3.8.2 Insulin RIA

The insulin RIA was basically the same as the method for GH described in section 2.8.1. The insulin RIA has also been described by Bohn (25).

1) HORMONE

Bovine insulin only was used as no ovine insulin was immediately available. In any case the difference in the primary structure of ovine and bovine insulin is only one amino acid residue at the A-9 position in the disulphide ring, the structure of which is not believed to be crucial to the activity of the parent molecule (66).

The insulin used is specified in Section 3.8. It was used for radioactive tracers, for standards in the RIA's and for raising antibodies. The high biological activity of the bovine insulin used (26.4 iu mg^{-1}) indicates that it was quite pure and no attempts were made at purification. It was compared in an assay with monocomponant insulin (Novo actrapid human insulin) by Professor D.S. Flux who found that the regression of dose on logit counts for the two sources did not differ significantly. In both cases the correlation was -0.99 (D.S. Flux, personal communication 1982).

2) IODINATION

The insulin was iodinated by Professor D.S. Flux using the chloramine-T method which was substantially that used at Princess

Margaret Hospital (Christchurch) (Personal communication, Dr R.A. Donald to Professor D.S. Flux).

3) ANTISERA

Antisera to bovine insulin was raised in guinea-pigs by injection of polymerised bovine insulin in Freunds complete adjuvant. (1st antibody GP_7). It was raised by Dr G.M. Bohn who described the method in her thesis (25). The antiserum was tested by Professor D.S. Flux who found no significant cross reactivity with glucagon.

4) RADIOIMMUNOASSAY METHOD

The method used was essentially unaltered for both assays performed. It was as follows.

The assay buffer was identical to that used for GH RIA described in Section 3.8.1. Fresh HP was used in light of the effects of HP encountered in the GH RIA and the experience of Professor D.S. Flux with the insulin RIA. The reagents were added to $3cm^3$ plastic LP3 tubes in the following order: 100µl of assay buffer containing GP_7 antiserum to bovine insulin (diluted 1:25,000) and 1µg of guinea-pig γ-globulin; 100µl of a solution of standard insulin or 100µl of sample plasma or 50µl of sample plasma plus 50µl of assay buffer. The remainder of the procedure was identical to that described for the GH RIA in Section 3.8.1.

3.8.3 RIA Data Transformation

The estimates of bound ^{125}I-GH obtained from the gamma counter printout were expressed in counts per minute (CPM). In order to facilitate handling of the large numbers of figures it was necessary to transform the data so obtained into a form suitable for computer calculation. This involved obtaining the line of best fit of the standard hormone response curve in terms of a mathematical function and the use of that function in the automatic (or manual) calculation of unknown hormone concentrations.

Two methods of such data transformation were used. The first was a programme developed by Professor R.E. Munford (Department of Physiology and Anatomy, Massey University) based on Burger, Lee and Rennie (31). The second was a programme written by the author based on the log-logit transformation as explained by Professor D.S. Flux (personal communication). Both programmes were written in Basic and used on a SORD M222 microcomputer. In the following sections both programmes will be described and compared.

Munford Programme:

The Munford Programme (designated RIAPRT) was based upon the method of Burger et al (31) and a similar version for use on an IBM 1620 computer has been described by Bohn (25). The untransformed standard curve data were represented by the equation;

$$Y = \frac{A}{C + X^E} + e$$

Where Y was the amount of radioactive hormone bound by first antibody, and X was the amount of non-radioactive hormone present in the tube. A, C and E were constants specific for each assay and e was the random error. A, C and E were determined by an iterative technique and the estimate of the amount of hormone present in each sample ($\hat{X}$) was calculated from the equation;

$$\hat{X} = (\frac{A}{Y} - C)^{\frac{1}{E}}$$

The printout provided estimates of hormone present in each of the 50 and 100µl samples.

Logits Programme:

The bound counts for each dose of standard hormone were averaged and the resulting values transformed using the formula;

$$\text{Logit} = \frac{B}{T-B} \log_e$$

as described in Section 3.8.1. If any negative logits were obtained all values were coded by the addition of an integer so that all values were positive. The hormone standard doses (except for the zero dose) were converted to $\log_{10}$. Next the regression of $\log_{10}$ standard hormone concentration on logit was calculated. This was repeated several times using different estimates of T until the line of best fit was obtained as indicated by the highest correlation coefficient (r). A correlation coefficient of 0.95 or better was considered satisfactory i.e. 90% (r^2 = 0.90) of the variance was accounted for by the linear regression. The steps thus far were performed on a programmable calculator (Texas Ti 59). Examples of the standard curves obtained can be seen in Figure 3.7.

Following this the LOGITS programme on the microcomputer was used to obtain estimates of the unknown hormone concentrations from the regression equation of the standard curve. The estimates of the unknown hormone concentration in each ($\hat{Y}$) were calculated from the equation;

$$\log_{10} \hat{Y} = b\left(\frac{B}{T-B} \log_e + C\right) + Yo$$

where b is the regression coefficient (slope), Yo is the Y intercept of the regression line (when X = 0), C is the integer code and $\frac{B}{T-B}\log_e$ is the logit (as previously defined) derived from the bound counts of the unknown plasma sample.

The estimates of unknown hormone concentration in the 50 µl samples were doubled to obtain a correct estimate of the hormone concentration in the plasma sample (since the standard hormone doses were in aliquots of 100 µl). A weighted average of the unknown hormone concentration ($\hat{W}$) in the 50 and 100µl samples was calculated by the equation;

$$\hat{W} = \hat{Y}_{50} + 2(\hat{Y}_{100})$$

where $\hat{Y}_{50}$ and $\hat{Y}_{100}$ are the estimates of the hormone concentration in the plasma sample obtained from the 50 + 100µl samples respectively.

Since the estimate from the 100µl sample (Y_{100}) is usually more accurate (due to being twice as large as the 50µl sample) this value was allocated twice the weight.

The final estimate of unknown hormone concentration, derived from duplicates of 50 and 100µl samples (4 tubes in total) was printed out in units of either $ng.cm^{-3}$ or $pg.cm^{-3}$ and in log_{10} terms.

If the bound counts for either the 50 or 100µl samples were rejected as erroneous by the operator the value printed out was derived from the (unweighted) estimate of the other sample.

A copy of the logits programme is presented in Appendix 3.8.3.

Comparison of Data Transformation Methods:

While Burger et al (31) have discussed the relative merits of these 2 basic methods it was necessary to compare the success and ease of application of each in the current context in order to choose which method to use to provide results for this research. While the Burger method is arguably a better formula the Munford programme (RIAPRT) entailed more data entry into the microcomputer and provided output which required further calculation. In particular RIAPRT provided independent estimates of the hormone concentration in the 50 and 100µl samples from which the weighted average had to be determined by further calculation, while LOGITS provided both independent estimates and a weighted average without any further operations. On the other hand RIAPRT calculated the coefficients of the standard curve equation, while LOGITS required previous calculation of the coefficients of the standard regression. The major benefits of each programme were lacking in the other. Even though the author wrote another programme (WEIGHT) to calculate weighted averages from the output of RIAPRT it was still necessary to re-enter data for all the unknowns. On balance, the calculation of the regression equation for LOGITS was considered to be considerably less onerous than the re entry of the unknown data for WEIGHT. Since the ease of application favoured LOGITS it was decided to use it as long as the results obtained did not differ

greatly from those obtained by RIAPRT and WEIGHT i.e. so long as the interpretation of results and the conclusions obtained were not altered by the choice of programme.

TABLE 3.8: COMPARISON OF RESULTS FROM TWO METHODS OF DATA MANIPULATION: ESTIMATES OF GH LEVELS IN PLASMA OF EWE 57 OBTAINED FROM LOG-LOGIT TRANSFORMATION AND FROM BURGER TRANSFORMATION USING THE SAME SET OF BGH STANDARDS (Expt VII)

Sample Number	TRANSFORMATION	
	RIAPRT & WEIGHT (ng BGH cm^{-3})	LOGITS (ng BGH cm^{-3})
1	6.64	6.02
2	0.43	0.52
3	1.55	1.57
4	55.78	49.30
5	56.52	49.56
6	42.40	37.19
7	0.18	0.24
8	95.18	83.34
9	133.51	118.34
10	58.71	51.71
	450.90	397.79

Table 3.8 compares the estimates of GH concentration in several unknown samples derived using the 2 methods and the same set of BGH standards. The difference between estimates of each sample is small compared to the differences between samples. In this example RIAPRT and WEIGHT produce larger values than logits for all values higher than 1.55ng BGH cm^{-3}. This indicates that the curves are not parallel and that they intersect at about 1.56ng BGH cm^{-3}. In other cases LOGITS produced higher values than RIAPRT and WEIGHT at levels greater than the point of intersection. In brief, though the lines were not parallel, they were close together and near enough to parallel for practical purposes. Apparently both methods produced equally viable results and since the logits method involved less effort it was used to provide the data presented in Chapter 4.

3.8.4 Testing Parallelism in RIA's

The calculation of hormone concentrations in plasma samples was based upon the estimates of hormone concentration of the 2 levels of plasma (50 and 100µl dilutions) included in the assay. Logically the estimate obtained from the 100µl sample should be approximately twice the value obtained from the 50µl sample. This can be checked visually by plotting the levels of the unknown plasma sample adjacent to the standard curve so that they are the same distance apart as a doubling of hormone concentration on the X axis. An example of this can be seen in Figure 3.10. If the slope of the line joining the 50 and 100µl estimates is not parallel to the standard curve, then the 2 levels will provide differing estimates of hormone concentration. The importance of such a deviation from parallelism has been described by Midgley A.R., Niswender, G.D. and Rebar, R.W. (112) and by Bohn, G.M. (25). Such a deviation from parallelism can indicate:-

1) That the immunoreactivity of the standard hormone differs from that of the natural hormone in the plasma sample, or

2) that some factor in the assay is stimulating or depressing binding of antisera with the natural hormone compared to the standards (25).

Visual identification of some deviations from parallelism in the assays performed for this research made it necessary to check the significance of this result statistically. Twenty to thirty samples from each assay were chosen at random and the estimates of hormone concentration were converted to natural logarithms. The value of the 50µl level was effectively doubled by adding ln 2. An example of the values obtained can be found in Appendix 3.8.4, which shows the output of a microprocessor programme written by the author to test for parallelism errors. The logged estimates of hormone concentration at the 50 and 100µl levels should have been approximately the same but in some assays most of the values differed by about 0.5 i.e. the untransformed estimate of the hormone concentration in the 50µl sample was about half that of the 100µl sample e.g. analysis of variance, as shown in Table (3.9) demonstrated a significant ($P = 0.006$) difference in the mean estimates derived from the 50 and 100µl levels in the assay of GH in Experiment IX (using OGH standards).

TABLE 3.9: ANALYSIS OF VARIANCE OF ESTIMATES OF GH CONCENTRATION ($\log_{10}$ ng.cm^{-3}) DERIVED FROM 50 and 100µl LEVELS OF PLASMA IN EXPERIMENT IX USING OGH STANDARDS

Source	d.f.	Sum of Squares	Mean Square	P
Between plasma levels	1	5.938	5.938	P = 0.006
Between samples	29	219.862	7.581	P < 0.0001
Error	29	19.652	0.678	
TOTAL	59	245.452		

Such a deviation from parallelism would have resulted in errors in estimation of the absolute values of hormone concentrations in plasma samples. Although the source of error was discovered after all assays had been completed it was necessary to ascertain what effect it had on the final results and whether it affected the interpretation of the results.

In attempting to ascertain the effect of the deviations from parallelism it was necessary to consider the cause also. If the 50µl estimates were depressed by a variable amount relative to the 100µl estimates by some factor in the assay such as HP (which apparently had a larger effect at lower GH levels) then the absolute values of plasma hormone concentration might be distorted in relation to each other and comparisons might be invalid. If on the other hand the degree of depression was relatively constant (as they appeared to be) due to some factor such as differences in the immunoreactivity of the standard and natural hormones or a mathematical bias in the method of estimation, then it was possible that all samples were affected to a similar degree and that comparisons were therefore still valid.

Due to the low levels of HP used in the assays of experimental data it was initially assumed that any depressing effect of HP was minimal and as time was limited it was impossible to carry out further experimental work to investigate this possibility. Rather, since the data indicated a constant systematic bias it was decided that the error lay in the standards; either in their immunoreactivity or in the mathematical methods used to define the standard curves. It appeared

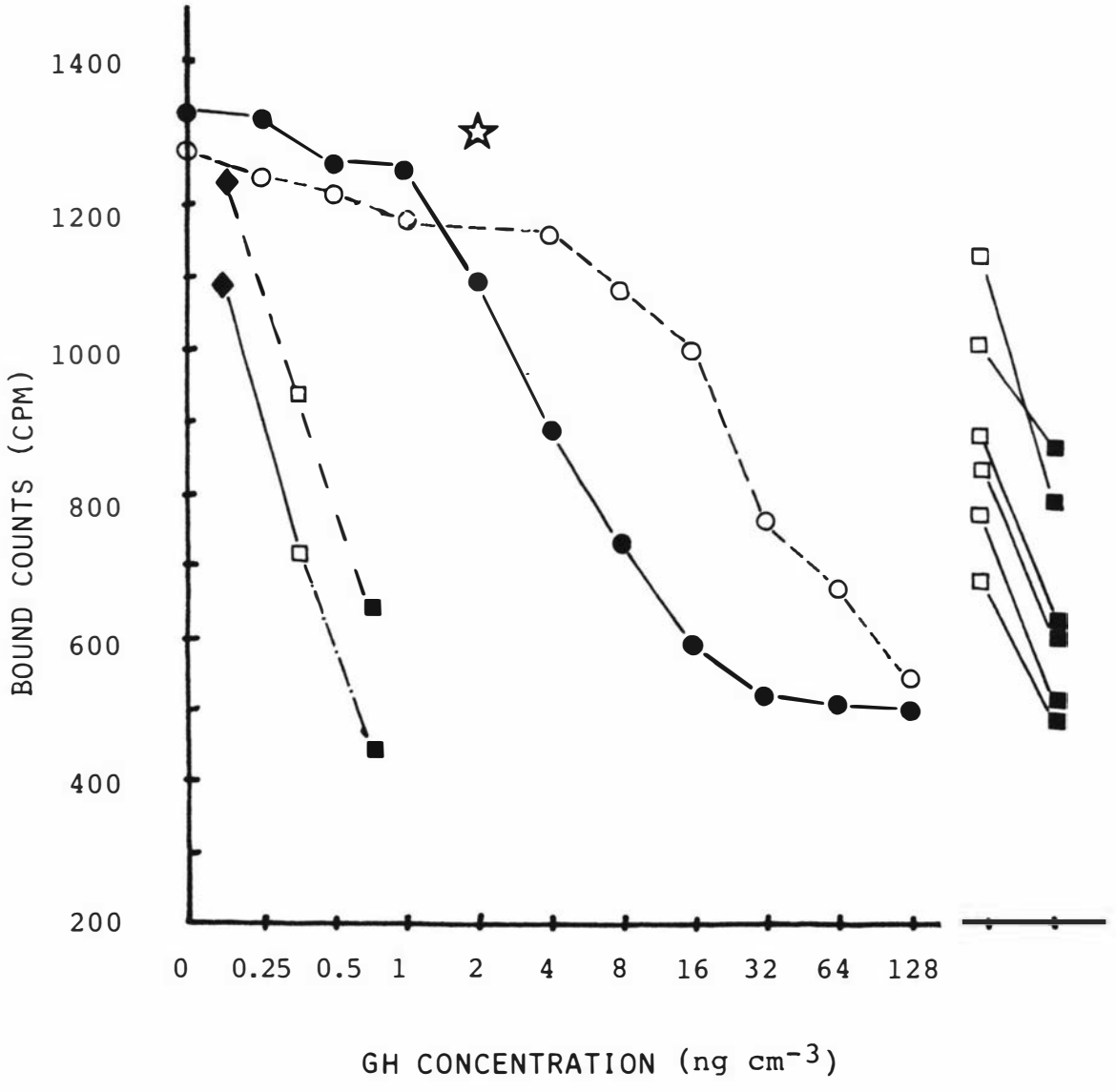

Figure 3.10 Parallelism investigation: slopes of lines joining bound counts of 2 or 3 levels of plasma samples compared to OGH & BGH standard curves from Experiment IX.

●——● BGH standard curve
○- - -○ OGH standard curve
- - - -■ Bulk ewe plasma standard BEPS
—·—·—■ Bulk lamb plasma standard BLPS
□——■ Ist 6 ewe plasma samples from Experiment IX
◆ 25ul plasma sample
□ 50ul plasma sample
■ 100ul plasma sample
☆ Rejected data (OGH standard)

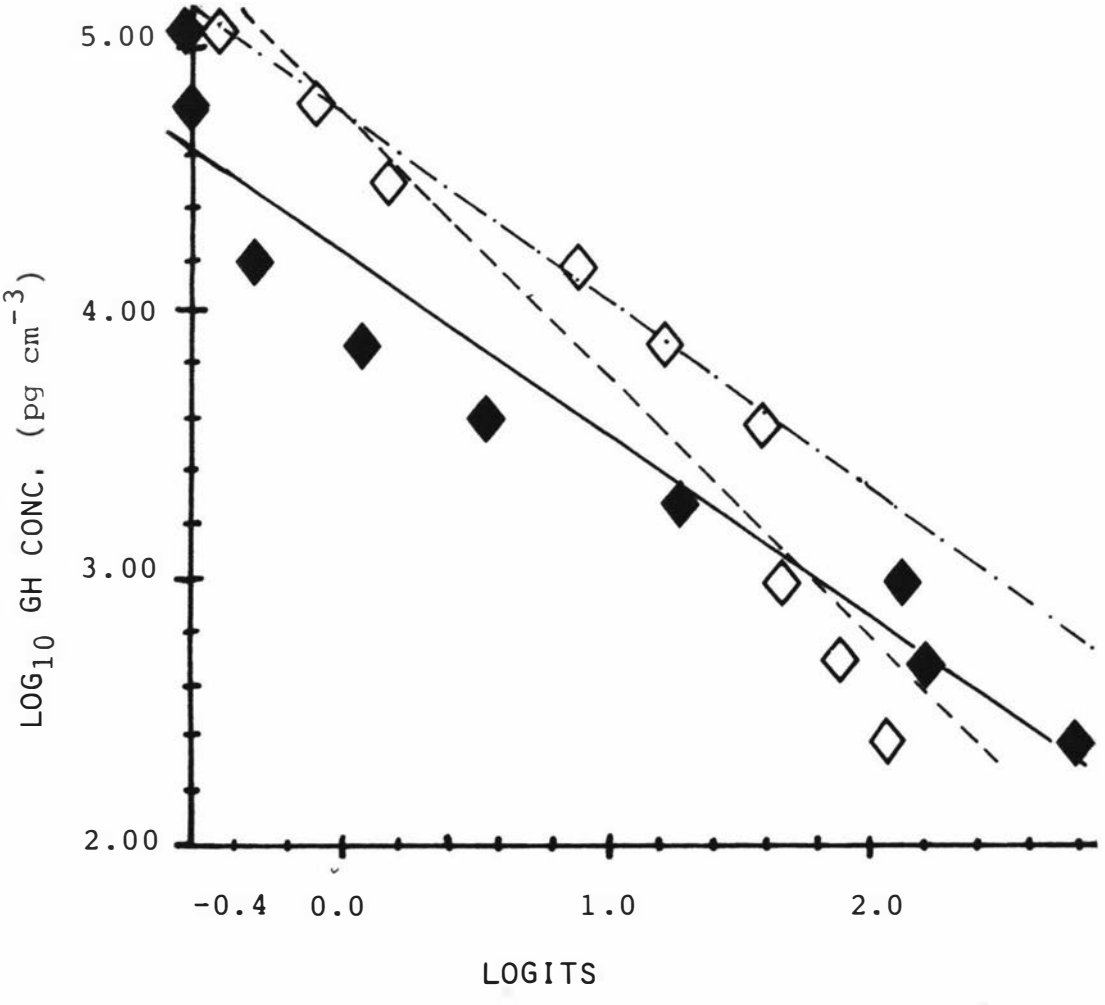

Figure 3.11 Comparison of OGH and BGH regressions in Experiment IX

Modified OGH regression Y=-0.693X + 4.740 r=0.99
OGH regression Y=-0.991X + 4.788 r=0.96
BGH regression Y=-0.674X + 4.226 r=0.96
OGH standards
BGH standards

in some assays (as in Figure 3.10) that the binding of BGH and OGH standards differed sufficiently to suggest that the problem may be due to differing immunoreactivity between the 3 sources of hormone in the assay (BGH and OGH standards and natural OGH). It was found that when the data for standards in Experiment IX were transformed and regressions fitted, that the regression lines of the OGH and BGH standards differed significantly in slope ($P = 0.02$) and had different means ($P = 0.08$). Because the slope of the BGH standard curve more closely paralleled the slope of the plasma samples it was this, rather than the OGH curve which was used to determine the final estimates of GH concentration. It was expected that the OGH standards would have been more suitable for estimation of ovine samples, but in fact deviations from parallelism were reduced when the BGH standard curve was used.

Nonetheless, the differing immunoreactivity of the standards is not thought to provide the whole reason for the deviations from parallelism. While testing the regressions it became abundantly clear that the slope of the regressions could be substantially modified by altering the value of T used in the calculation of logits (see formula in Section 3.8.1) or by rejecting data values at either end of the curve which did not contribute to the sensitivity or range of the assay. For example if the OGH standard values for 2ng cm^{-3} and those below (see Figure 3.10) were not included in the regression calculation then the modified regression obtained;

$$Y = -0.693\ X + 4.740 \quad \text{(see Figure 3.11)}$$

has a slope almost identical to the slope of the BGH regression

$$(Y = -0.674\ X + 4.226)$$

The low standard doses (<1ng GH cm^{-3}) were included to extend the sensitivity of the assay but the bound counts recorded were not much larger than those for 1ng cm^{-3} (i.e. the curve was relatively flat below 1ng cm^{-3} - see Figure 3.10). So while the low doses did not contribute much to the sensitivity of the assay their inclusion in the regressions of the standard curve had created a regression line with a slope differing significantly from the central, most important section of the standard curve (which part was parallel to the unknowns).

The effect of this was to decrease the estimates obtained from the 50µl samples and to increase the 100µl estimates as detected by the deviations from parallelism. Furthermore, due to the logarithmic nature of the scale (on the X axis) the overall result was an increase in the values of the estimates of the absolute hormone concentrations in the samples. More than 50% of the estimates were in excess of the highest standard dose (128ng cm^{-3}) and so could not readily be used.

So deviation from parallelism were a source of error in some assays and were affecting the hormone concentration results and probably also affecting the interpretation of those results. Because of this all RIA's were tested for such deviations, so that corrections might be made. The BGH standards were chosen to estimate the unknowns for reasons discussed earlier. Where parallelism problems occurred the BGH standard curves were modified by removal of data for some of the lowest and some of the highest doses, which had been causing the regression line to be somewhat less steep than it should have been. This is an acceptable procedure for 2 reasons. Firstly the assays are not capable of providing equal precision over the whole range of standards that were included; precision is much reduced at both ends of the range of hormone standards.

Secondly, the confidence bands (95% limits) for a population regression line are not straight lines but are in fact the 2 branches of a hyperbola (145) which curve away from the regression line in both directions at both ends. This recognizes the fact that population regression exhibit larger variance at each extreme. The result of this modification of the standard curves was to provide results free from significant deviations from parallelism in all assays except two. These were the GH and insulin assays of Experiment VII, which still had highly significant ($P < 0.001$) deviations from parallelism. They could not be satisfactorily improved by the removal of extreme values from the standard curve.

In order to remove the apparent error from these data it was necessary to hypothesise that some factor in the assay was suppressing bound counts, and in particular having a greater effect at lower standard

TABLE 3.10: SUMMARY OF TESTS FOR DEVIATIONS FROM PARALLELISM IN CORRECTED RIA DATA PRESENTED IN CHAPTER FOUR

RESULTS OF ANALYSIS OF VARIANCE OF RIA DATA OBTAINED FROM STANDARD CURVES SOME OF WHICH WERE MODIFIED TO COUNTERACT THE DEPRESSION OF HORMONE STANDARDS BY HP

Assay	Standard Hormone	Curve Modifications	P
Expt IX	BGH	not modified	0.77
Expt IX	Bovine insulin	24pg std rejected	0.29
Expt VII	BGH	expanded curve	0.10
Expt VII	Bovine insulin	expanded curve	0.85
Expt VI	BGH	not modified	0.08
Expt V	BGH	not modified	0.09

Expts I, II, III, IV not tested due to lack of usable results

Where P is the probability (calculated by ANOVA) that deviations from parallelism were chance effects. Values greater than 0.05 (5%) were considered satisfactory evidence that deviations from parallelism were not an important source of error in the assay.

hormone concentrations (thus making the standard curve more horizontal). Evidence to support this hypothesis was provided by Professor D.S. Flux (Animal Science Department, Massey University) (personal communication 1982) who found that 2% HP in the assay buffer depressed binding of insulin standards by about 20% compared to 2% ovine plasma (from a fasted sheep to ensure low insulin levels). This result was obtained in the same assay system used by the author (i.e. identical antisera and HP). Expanding the difference between bound counts of successive standards by a factor of 20% produced a new "expanded standard curve" which theoretically was directly comparable with the unknown plasma samples. When the expansion was applied to the two remaining assays in which parallelism errors persisted it effectively removed the errors. The expansion was applied to both insulin and BGH standards even though the original experiment upon which the correction factor was based only involved insulin. Evidence for the depressing effect of HP on BGH has been discussed earlier in this chapter and so it is essentially only the magnitude of the effect which is in doubt. It was not practical to carry out such a trial to determine the appropriate correction factor for BGH (due to time limitations). Accordingly the same 20% expansion factor was applied to the BGH standard curve (Expt VII) and as this produced a satisfactory curve (with respect to parallelism) it was accepted as a preferable alternative to the original standard curve.

So, while it was initially assumed that any depressing effect of HP (2% HP in assay buffer) was minimal, it became evident that this was probably not a fair assumption in at least 2 assays Corrections applied to counter this apparent effect provided results free from significant deviations from parallelism in all assays, as shown by the summary of analysis of variance tests shown in Table (3.10) which were carried out on data ultimately used to provide the experimental results presented in Chapter four.

3.9 EXPERIMENTAL DESIGN

The entire study involved 12 different studies which are outlined in Table 3.11. The design of each experiment is detailed along with the results of each experiment in Chapter 4.

TABLE 3.11: EXPERIMENTAL DESIGN: SUMMARY OF ALL EXPERIMENTAL TRIALS

Experiment Number	Ewes Involved (I.D. numbers)		Treatments Imposed	Factors Measured	Duration ($days.ewe^{-1}$)
Calorimetry 1	56 313	73 323	Fed normally (2)	O_2 consumption Back-fat depth Liveweight	3 or 4
I	56 313	73 323	Fasting & feeding	GH, FFA, glucose	3
II	313	323	Fasting & feeding	GH, FFA glucose	3
III	56	73			
Calorimetry 2	56 313	73 323	Fasting & feeding	O_2 consumption CO_2 production	4 or 5
IV	323		ACTH	GH, FFA, glucose	1
V (1)	57 142	116 186	ACTH	GH, FFA, glucose	5
VI	57 142	116 186	N.A.	GH, FFA, glucose	5
VII	57 142	116 186	Fasting & feeding	GH, insulin FFA, glucose	3
Calorimetry 3	57 142	116 186	ACTH & N.A.	O_2 consumption CO_2 production	3
VIII	142	186	BGH	GH, FFA, glucose	3
IX	142	86	BGH & N.A.	GH, insulin FFA, glucose	3

(1) The dividing line between Experiments IV & V marks the point where the initial group of ewes were replaced and consequent changes in objectives were necessary thereafter

(2) Backfat depth and liveweight were recorded for other trials but were only presented in Experiment I as no significant changes were detected during any experiments.

CHAPTER FOUR

EXPERIMENTAL

4.1 CALORIMETRY EXPERIMENT 1

4.1.1 Experimental Design

1) Objectives

One of the first tasks considered necessary was to determine if the differences in apparent fatness between sheep were due to differences in efficiency of utilization of dietary energy. Lean sheep might only have a higher metabolic rate and use energy for maintenance and/or anabolism less efficiently than fat sheep. To answer the simplest question first it was decided to;

> determine if the metabolic rates of fat and lean sheep differed under normal fed dietary conditions.

It was thought that perhaps the fat sheep had lower metabolic rates and managed to conserve more energy as adipose tissue than the lean sheep.

It was hoped that once this was known one way or the other, that the more specific questions regarding efficiency of energy utilization might be more effectively approached.

2) Method

The sheep used in this experiment were the four unmated ewes selected from the Massey - DSIR research flock and described in detail in Chapter 3 (Section 3.1). Assuming the previous measurements upon which selection was based were useful indicators of the phenotype of the animals and assuming that the phenotypic differences were not entirely due to environmental differences, it seemed that these sheep might represent 2 genotypes within each breed (see Table 4.1).

The aim of this experiment therefore was to determine firstly if these possible genotypes differed in their metabolic rate under the same environmental conditions.

The selected ewes were housed in metabolism crates at the animal physiology unit at the end of March 1977 and trained to eat hay and sheepnuts. McNatty and Thurley (110) found that adrenaline injected I.V. caused increased plasma cortisol levels proportional to the dose in housed and cannulated sheep.

The response of cortisol to adrenaline was larger when sheep were newly housed than when the sheep had been housed and sampled for 2 weeks. The response to ACTH also diminished over 2 weeks. Assuming that these findings were related to the stress caused by the different environment and handling by man it was decided to allow the sheep at least one month to settle down and to handle them frequently during that period so that they became accustomed to it.

To minimize experimental bias, attempts were made to ensure experimental conditions were as similar as possible for all sheep. To minimize carryover effects from previous unknown conditions all sheep were fed the same diet of good meadow hay and sheepnuts. It was decided that all sheep should be in "good" condition although it was not known how to define this. After an initial adjustment period of 3 weeks the sheep were weighed and their energy requirements for maintenance were calculated according to the method described by Coop (38).

For 3 weeks all were fed 1.5 x maintenance (M) in the form of 1 x M as sheepnuts and 0.5 x M as hay. Since refusals were variable and often high the ration was decreased to 1.25 x M with 1 x M as sheepnuts and 0.25 x M as hay. Requirements were not adjusted as bodyweights increased because it was not intended that the sheep should continue to put on condition. Rather it was intended that their weights would stabilize at a point were the constant intake provided only for maintenance at their new bodyweight.

Indirect calorimetry was used to estimate the metabolic rate of sheep. The calorimetry apparatus is described elsewhere by Holmes C.W. (83) and Holmes and McLean (84). Only 2 chambers were available so all 4 sheep could not be monitored simultaneously. The Romneys were tested together followed by the Southdowns. Results were recorded over 3 days for the Romneys and 4 days for the Southdowns. The latter group

had not been allowed as long to adjust and the data collected on the first day was suspect. Ambient temperatures were maintained at 15°C in each calorimeter and oxygen consumption was measured to estimate heat production which is directly proportional to metabolic rate.

The sheep were weighed and backfat measurements were taken both before and after calorimetry.

4.1.2 Results

The daily heat production of the 4 ewes is presented in Table 4.1.

TABLE 4.1: DAILY HEAT PRODUCTION OF FAT AND LEAN EWES (KJ $kg^{-0.75}$)

	Type Ewe	Lean R323	Fat R313	Lean SD73	Fat SD56
Day	1	312.70	280.39	230.21	267.26
	2	265.43	279.11	256.46	267.34
	3	294.76	255.63	258.66	255.01
	4			254.98	265.38
		290.96	271.71	250.08	263.75
	SD	±23.86	±13.01	±13.33	± 5.89

The data for each breed were analysed separately and a simple one way analysis of variance showed no significant differences in daily heat production between the sheep within the breeds.

Romneys	$P = 0.29$
Southdowns	$P = 0.11$

The complete analysis of variance is presented in appendix 4.1a.

Data obtained on each day was assumed to be an independent estimate of heat production i.e. time series effects were not allowed for in the analysis.

When data for the fat and lean genotypes were grouped their means were not significantly different (P = 0.97) (Table 4.2) (complete analysis is in appendix 4.1b.

TABLE 4.2: MEAN HEAT PRODUCTION OF FAT AND LEAN EWES (KJ $kg^{-0.75}.day^{-1}$)

	Fat	Lean
Mean	267.61	267.61
S.D.	±10.01	±27.52

Liveweight and back-fat thickness data are presented in Table 4.3.

TABLE 4.3: LIVEWEIGHT AND BACK-FAT THICKNESS OF EWES BEFORE AND AFTER CALORIMETRY

	Weight (kg)		Back Thickness (mm)*	
Ewe	Before	After	Before	After
R 323	67.5	64.3	12	11.5
R 313	52.2	50.2	12	12.5
SD 73	59.6	59.5	13	14.5
SD 56	54.0	53.2	12.5	12

* Data are the mean of 2 measurements recorded with an accuracy of ±0.5mm

Visual appraisal of the data indicates that changes in liveweight and back-fat thickness were small and no significant differences between individuals were evident.

4.1.3 Discussion

The southdowns were monitored an extra day mainly because the heat production result for SD 73 on day 1 was suspect. Although quite different from the other data recorded for Ewe 73, this result was

not rejected because it caused less variance than recorded in one of the Romneys (R 323).

The unequal numbers of records taken within the 2 breeds would have resulted in a complex analysis, and as there was no good reason to compare the breeds, the heat production data for each breed were analysed separately. In fact a cursory study of the data reveals that variance within the Romney data is greater than variance between the breeds so statistically significant differences between the breeds would not be found. No significant differences were found between fat and lean sheep within each breed, or over both breeds combined.

Thus it was assumed that there were no differences in the normal metabolic rate of the sheep sufficient to explain their apparent phenotypic differences. Each sheep used about the same amount of energy per kilogram of metabolic body weight so that if the fat sheep had an energy balance advantage it must be due to differences in efficiency of utilization of energy.

It was not possible to determine whether the fat sheep had such an energy balance advantage because the precision of measuring changes in weight and back-fat thickness was not sufficient to detect significant differences if they did in fact occur. Thus it was not possible to attribute an energy balance advantage to any of them and it was concluded that there were no significant differences in metabolic rate between the sheep and no detectable differences in the efficiency of utilization of dietary energy.

4.2 EXPERIMENT I

4.2.1 Experimental Design

1) Objectives

It has been suggested that there is considerable genetic variation in fat percentage in sheep (26) and it was thought that differences between fat and lean sheep might be due to relative differences in their ability to mobilise adipose tissue during periods of

restricted feed supply, rather than differences in lipogenic activity when fed.

While previous records have indicated that the ewes selected for this study (described in Calorimetry Experiment 1) were different in phenotype it was not known whether they were in fact genetically different with respect to adipocity. Having determined in the first experiment that the normal metabolic rates of the fat and lean sheep were not significantly different under normal (fed) conditions it was then proposed to find out if the two apparent phenotypes differed in their responses to submaintenance feeding and to determine what alterations in certain plasma metabolites and hormones accompanied such responses.

Specifically the objectives of this experiment were;

1. to determine the normal diurnal pattern of some plasma hormones and metabolites in the experimental sheep (both for the information per se and as a basis for comparisons with different experimental challenges).
2. to stimulate lipolysis by imposing fasting and ascertain the resultant changes in some plasma metabolites and hormones
3. to determine whether the fat and lean sheep of each breed differed in their response to this lipolytic stimulus.

2) Method

The same four ewes described in the first experiment, Calorimetry Experiment 1, were used in this study. Prior to obtaining the sheep they had all been grazed together under the same conditions, and it was thought that differences in feeding behaviour and aggressiveness might possibly have led to a nutritional advantage for the fatter sheep (79) so they were housed and penned individually in metabolism crates in the animal physiology unit (APU) to eliminate the possibility of such factors influencing the results of the present experiments. Over 10 weeks elapsed before the beginning of this experiment during which time they became accustomed to the routine, their diet and the general environment.

This experiment was the first half of a reversal trial (27) which was never completed. (Reasons discussed in experiments II and III). In this experiment (Exp. I), the lean member of each breed-pair was fasted while the fat ewes were fed normally. In Table 4.4 the whole of the planned "feeding and fasting" experimental programme is illustrated.

TABLE 4.4: PLANNED EXPERIMENTAL PROGRAMME OF FEEDING AND FASTING

	Experiment No.					
	I	II	III	X	Y	Z
SD56	FEED	REST	*FEED	FAST	REST	*FAST
Ewe SD73	FAST	REST	*FAST	FEED	REST	*FEED
No. R313	FEED	*FEED	REST	FAST	*FAST	REST
R323	FAST	*FAST	REST	FEED	*FEED	REST

SD denotes Southdown, R denotes Romney

* denotes concurrent calorimetry

Experiments I, II and III were attempted and are described in this thesis, while the experiments designated X, Y and Z were abandoned for reasons described later in experiments II, III and IV. The results of Experiment I, the design of which is shown in Column I of Table 4.4, are presented in this section.

The same steps taken in Calorimetry experiment I to minimize experimental bias were continued in this experiment (and in all experiments described hereinafter). The maintenance energy requirements for each ewe were calculated (as described in Calorimetry Experiment I) and the amounts fed are detailed in Table 4.5.

TABLE 4.5: WEIGHTS OF FEED GIVEN TO FED AND FASTED EWES (g)

Ewe No.	Phenotype	Treatment	Sheepnuts	Hay
SD 56	short/fat	feed	660	200
SD 73	long/lean	fast	180	55
R 313	short/fat	feed	660	200
R 323	long/lean	fast	190	60

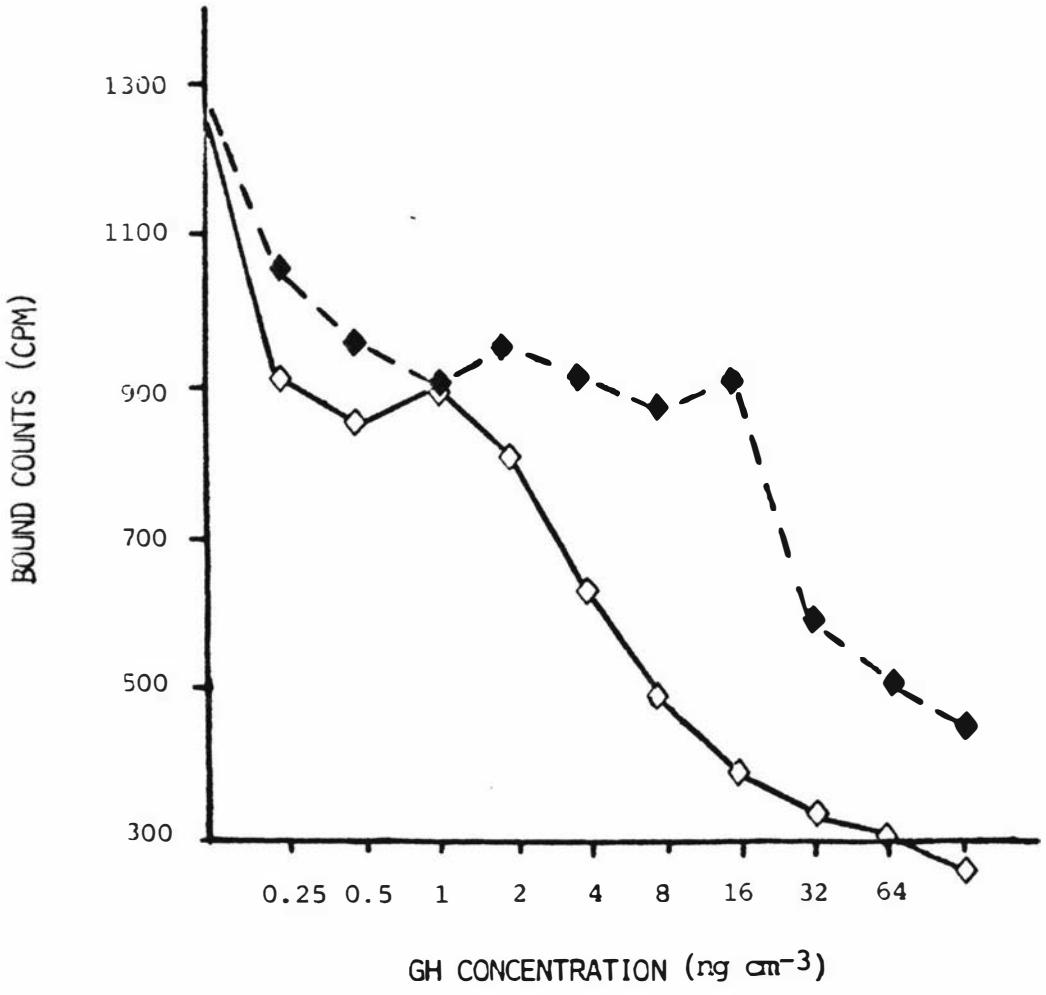

Figure 4.1 Standard curves produced in Growth Hormone assay of Experiment I

◇ BGH standards
◆ OGH standards

Fully fed sheep were fed 1.25 times their maintenance requirements (M) with 1 x M as sheepnuts and 0.25 x M as hay. Fasted sheep were not in fact starved but were fed a quarter of their normal 1.25 x M diet. Complete starvation is a relatively uncommon occurrence on commercial farms and results obtained from such treatment would be of limited practical value compared to results of submaintenance feed-in which simulates conditions more commonly encountered on farms. This is discussed further in Experiment VII.

The ewes were cannulated on the day prior to beginning the experiment. The cannulation method is described in Section 3.2. On this occasion local anaesthetic (xylocaine) only was used.

Blood sampling was carried out as described in Section 3.4, with 8 or 9 samples being taken on each day beginning about 8 a.m. and then at hourly intervals during the morning and 2 hour intervals in the afternoon.

Plasma samples were stored at about -4°C and later analysed for glucose, FFA and GH by the methods described in Chapter 3.

4.2.2 Results

Variation of Plasma Metabolites and Hormones

1. Growth Hormone

As can be seen in Table 4.6 most of the plasma GH determinations were indistinguishable from zero. Although about 25% of the results indicate GH concentrations considered to be within the operational range of the assay (0.5 - 64 ng cm^{-3}) no observable pattern could be detected. These results are unusually low compared to the values obtained in Experiments V, VI, VII, VIII and IX. The reason for this does not appear to be associated with the viability of the radioimmunoassay procedure: indeed all other factors indicate that the assay was functioning satisfactorily. Figure 4.1 shows the standard curves obtained in this assay, which, although far from perfect, are considered usable. The bulk ewe plasma standard (BEPS)

TABLE 4.6: PLASMA GROWTH HORMONE CONCENTRATIONS IN 2 FED AND 2 FASTED EWES (Experiment I)

Sample No	Time	Fed Ewe 56 ng cm^{-3}	Fasted Ewe 73 ng cm^{-3}	Fed Ewe 313 ng cm^{-3}	Fasted Ewe 328 ng cm^{-3}
1	8.15am	1.80	0	2.95	0
FED	8.30				
2	9.05	0	0	0	0
3	10.00	0	0	0	0
4	11.00	0	0	0	0
5	12.00	0	0	0.50	62.44
6	2.00	0	0	0	0
7	4.00	0	0	4.29	208.56
8	6.00pm	1.71	0	0.30	0.62
9	11.45pm	0	66.60	0	0.70
10	8.00am	13.21	0	1.822	1.161
FED	8.15				
11	9.00	0	0	0	0
12	10.00	1.73	1.90	0	0
13	11.00	0	0.46	0	22.95
14	12.00	3.24	0	0	0
15	2.00	0	0	18.72	31.11
16	4.00	0	2.03	4.10	0.60
17	6.00	0	2.07	2.63	0
18	11.25pm	1.45	0	0	0
19	8.00am	0	0.31	0.50	0
FED	8.30				
20	9.00	0	0	0	0
21	10.00	0	0	0	0
22	11.00	0	0	0	0
23	12.00	0	0	0	0
24	2.00	0	0	0	0.26
25	4.00	0	0	0.64	0
26	6.00	0	0	0	0.34
27	8.00	0	0	0	0

N.B. Estimates < 0.25ng cm^{-3} were considered indistinguishable from zero

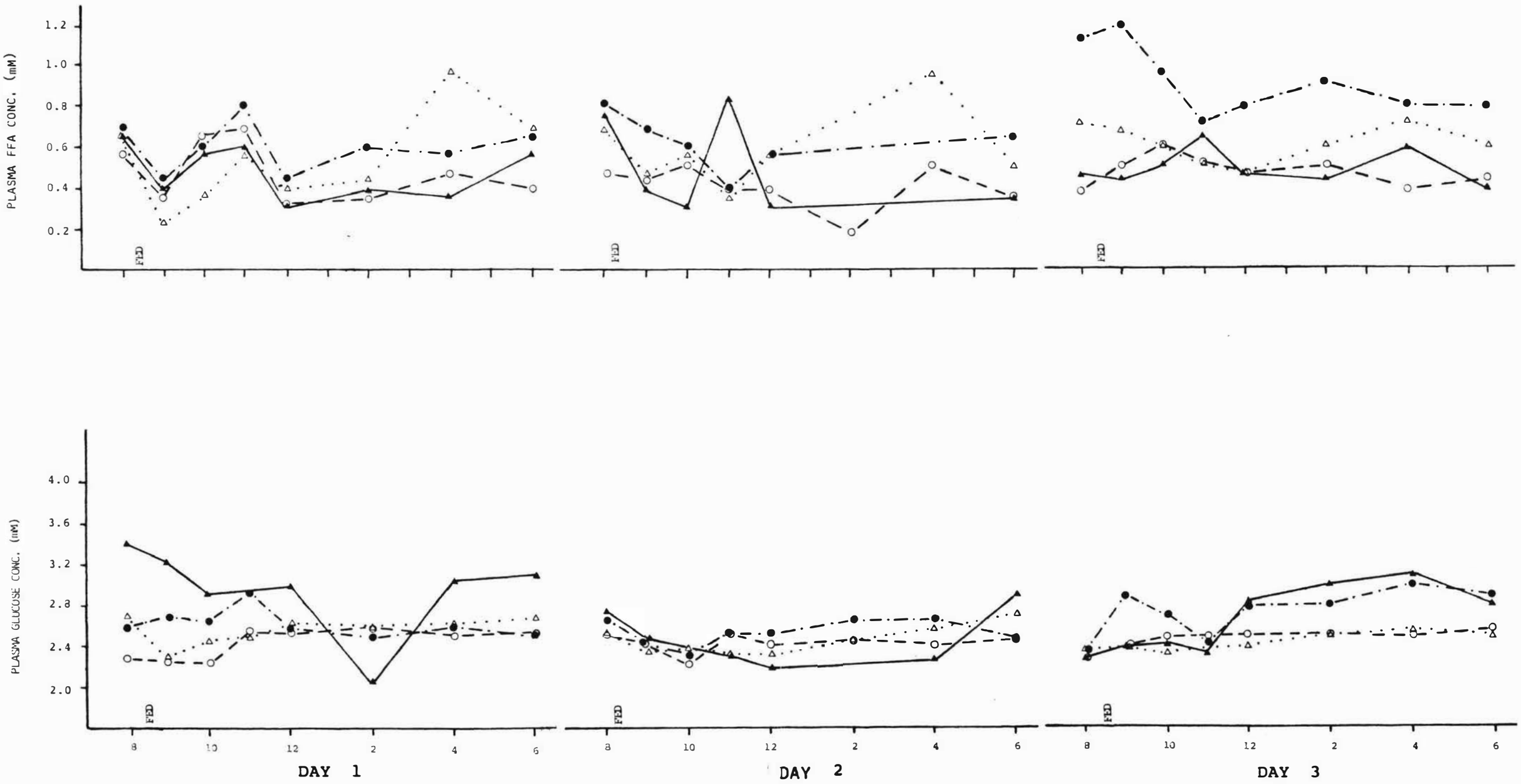

Figure 4.2 Plasma glucose and FFA concentrations in fed ewes of short/fat phenotype and fasted ewes of long/lean phenotype.

Short/fat ewes: ○—○ 56 ▲—▲ 313 Fed
Long/lean ewes: △·····△ 73 ●—●·—● 323 Fasted

used in the assay recorded a GH concentration of about 4.3 ng cm^{-3} and the bulk lamb plasma standard (BLPS) about 60 ng cm^{-3}, levels which are comparable with those obtained in other assays, and which indicate that the assay was indeed functional over much of its range. Nevertheless, the experimental values obtained are not considered reliable estimates of physiological GH levels, for reasons discussed later.

2. Glucose

Graphs of plasma glucose concentration versus time are presented in Figure 4.2. The results presented were obtained from a repeat glucose oxidase assay because about 20% of the samples in the first assay were affected by a technical hitch and were unusable. The repeat assay was done 2 months after the first and most of the values presented are about 10% lower than the original estimates (disregarding the unusable values in the first assay). However since the relative differences between samples within each assay are very similar it is thought that the results presented adequately show the size of differences between samples although the absolute values may not be accurate.

The glucose levels in this experiment show somewhat less variation than that exhibited by the fed and fasted sheep in experiment VII. In particular the Southdowns showed remarkably little variation with all samples falling in the range 2.25 - 2.70mM.

There were no apparent differences between the glucose levels of fed and fasted sheep or between lean and fat sheep, but there was a significant ($P < 0.005$) difference in glucose levels between the two breeds on the afternoon of day 3.

3. Free Fatty Acids

Graphs of FFA concentration versus time are presented in Figure 4.2. On day 1, before the fasting treatment was imposed on Ewes 73 and 323 prefeeding FFA concentrations were in the range 0.56 - 0.68mM for all four sheep, but by the beginning of the third day the range had widened to 0.39 - 1.13mM. Following

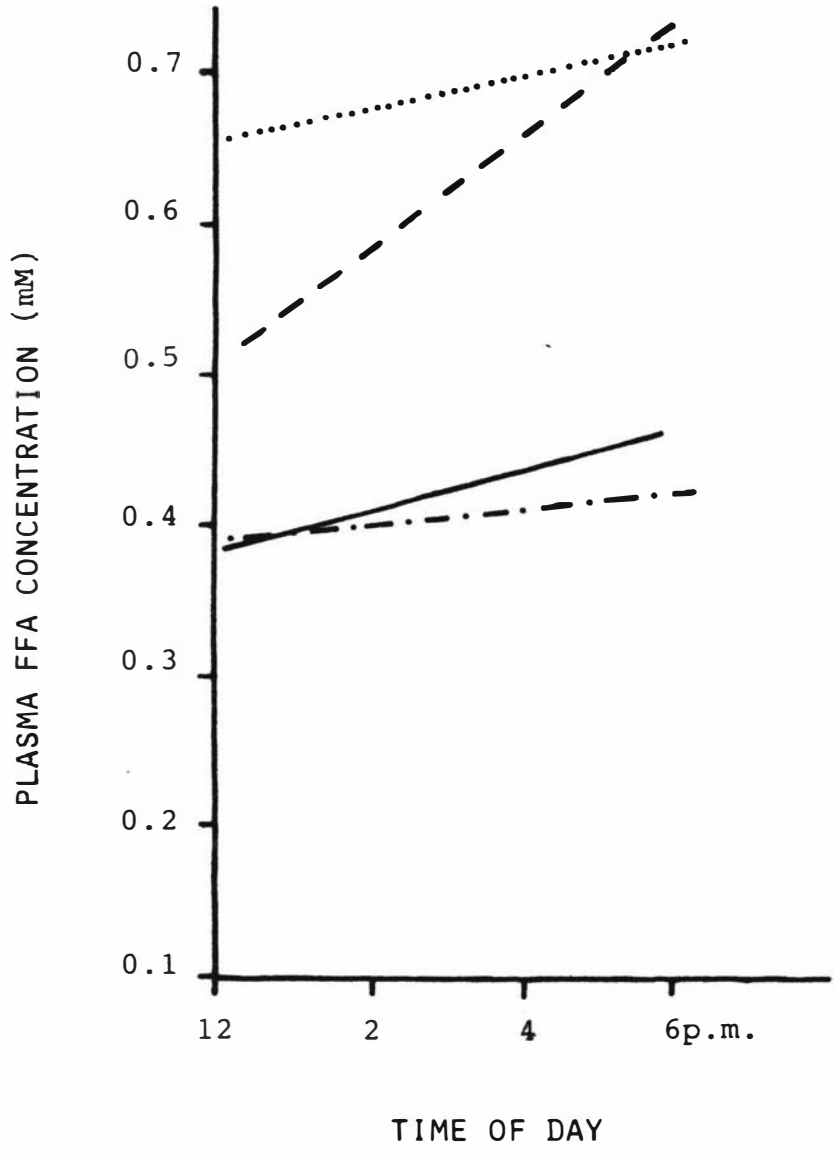

Figure 4.3 Slopes of average FFA levels in the afternoon for two fed and two fasted ewes.

—·— 57 fed
— — 73 fasted
—— 313 fed
···· 323 fasted

feeding plasma FFA levels decline in 10 out of the 12 cases. On day 1 the nadir in FFA levels occurred about 30 mins after feeding but on the following 2 days it occurred at quite different times for different sheep. In all sheep on day 1 FFA levels rose again to peak 2½ hours after feeding but on days 2 and 3 such a peak did not occur in the fasted sheep in 3 of the 4 cases and in the 4th case the size of the peak was small. Instead FFA levels in fasted sheep declined steadily from high prefeeding levels (on days 2 and 3) to nadirs about 3 hours after feeding. In fed sheep (on days 2 and 3) the peaks were recorded at the usual time, 2-3 hours post-feeding, after which they declined to levels about the same as those recorded in the post-feeding nadir.

In the afternoon FFA levels in all 4 sheep increased, but in fasted sheep they rose more rapidly to higher levels. Mean FFA levels of fasted sheep were significantly higher ($0.05 > P > 0.025$) than mean FFA levels of fed sheep in samples taken from noon till 6 p.m. (NB Possible time-series effects were not allowed for in this ANOVA). To minimize the effect of time series problems regressions were fitted to these data.

Analysis of variance of the regression coefficients showed no significant difference in the slopes of the lines between breeds ($P = 0.69$) or between treatments ($P = 0.55$). However the elevation of the regression lines were found to be different ($P = 0.065$) between treatments though not between breeds ($P = 0.32$) (see Figure 4.3).

4.2.3 Discussion

As described in Experiment II, difficulties were encountered in developing the GH radioimmunoassay. It was initially thought that the assay was not sensitive enough at low hormone levels (<1ng cm^{-3}) to accurately estimate levels in the plasma samples, but it was eventually concluded that the problem lay in the samples rather than the assay.

The same assay procedure successfully detected a wide range of GH levels in samples taken during Experiments V, VI, VII, VIII and IX and in fact the Experiment I samples were assayed at the same time as Experiment VIII samples using the same reagents. Hence it was concluded that the GH estimates obtained in the assay of Experiment I samples were good estimates of the actual levels in those samples. However it is thought that the samples themselves did not accurately reflect in vivo plasma GH levels as the recorded levels were much lower than those obtained in Experiments V, VI, VII, VIII and IX. Rather it is believed that the samples may have deteriorated in storage. Samples from Experiment I were 42 months old before analysis while those from Experiment VIII were a relatively young 30 months of age. Furthermore, it is felt that the speed and efficiency of the sampling procedure improved with practice and time and it is likely that the earlier samples were exposed to more contamination and were not frozen as quickly as later samples. The failure of freezers on at least one occasion resulted in partial thawing of samples which may have accelerated activity in the vials. Long term storage in common freezers at about -4°C (with unknown fluctuations) was probably a mistake. In future experiments it is thought that increased precautions taken to chill the samples during their preparation might be beneficial and that storage at -20°C should be considered if they are to be kept for an extended period.

Despite these suspicions regarding sample deterioration it is interesting to note that samples from Ewes 57, 116, 142, 86 and 186 taken in Experiments V, VI, VII, VIII and IX yielded GH estimates that are considered good while those taken from Ewes 56, 73, 313 and 323 in Experiments I, II, III and IV yielded estimated values considered poor. It is indeed possible that all the GH estimations were accurate and that real differences existed in the GH levels in the 2 groups of ewes. However, no possible reason for such a difference can be envisaged by the author.

As mentioned in Section 4.2 glucose levels in this experiment showed less variation than those in Experiment VII (in which ewes were also fed and fasted) but the present results show similar diurnal fluctuations. Variation in plasma glucose levels cannot be attributed directly

to any one other factor, and as discussed in Experiment VII, such variation is probably due to a combination of other effects (11, 64, 157). It was therefore not unexpected that no treatment differences were detected. However the apparent difference between breeds noted on day 3 was unexpected and no explanation for this result can be profered.

The difference in glucose concentrations between the two assays may have been due to metabolic activity or deterioration in the samples during storge or during thawing and refreezing in which case this might lend weight to the considerations discussed regarding the many very low GH estimates. The observation that glucose estimates in this experiment are about 1mM lower on average than those in Experiments V-IX might also be due to such deterioration. Alternatively the differences may have been due to differences in reagents and/or standards used in the various assays. Bulk ewe plasma standards were run in each glucose assay in an attempt to estimate such variation between assays but deterioration of this bulk standard invalidated any such comparison. The bulk plasma sample should have been stored in small aliquots as was done later in the RIA's. A third alternative is, again, that there were real differences between the 2 groups of sheep in the different experiments.

Variation in FFA levels in this experiment were similar to those obtained in Experiment VII, where the implications of such changes are discussed. It is considered preferable that such changes be discussed in relation to levels of the other plasma metabolites and hormones, and more and better data is available on these in Experiment VII.

The FFA response to fasting observed in this experiment has a higher degree of statistical significance ($0.05 > P > 0.025$) than the response found in Experiment VII ($P = 0.074$). It would appear that the fasted ewes in this experiment are quite capable of mobilizing body lipids and it should be noted that in doing so they appeared to suffer no adverse effects.

In light of the apparent lipolytic response it is unfortunate that more information could not be gleaned from the GH estimates. Insulin was not assayed because of the reservations held regarding the condition of the samples and this too may have contributed some insight into the mechanisms of the lipolytic response. Nevertheless it would seem that it has been shown that the lean phenotypes are indeed capable of mobilizing fat during undernutrition and it is most unfortunate that the reverse experiment could not be carried out to determine whether the fat phenotypes were equally capable of mobilizing fat reserves.

The failure to complete the reversal was due to, amongst other problems, the inability to maintain patent jugular cannulae in the sheep (as discussed later) and because of the death of Ewe 56 (Fat Freddy R.I.P.)

4.3 EXPERIMENTS II AND III

4.3.1 Experimental Design

1) Objectives

Experiments II and III were part of the design illustrated in Table 4.4. The objectives of these experiments were the same as those of Experiment I with the additional aim of obtaining calorimetry measurements. The objectives of the calorimetry were to determine whether the fat and lean sheep differed in BMR or responses to fasting. This aspect of the work is detailed in calorimetry Experiment 2 described later in this chapter (Section 4.4).

It would have been preferable to obtain calorimetry data during Experiment I concurrently with the blood samples, thus eliminating the need for Experiments II and III. However due to practical problems associated with maintaining cannulas and sampling sheep while they were within the calorimetry chambers it was decided to initially carry out the fasting and feeding treatments without attempting calorimetry so that blood samples could be collected with a minimum of difficulty. Once this was achieved it was intended to repeat the treatments, this time with the sheep in the calorimeters, at the same time attempting to collect blood samples in order to check that the same plasma metabolite and hormone responses occurred on both occasions. Thus Experiments II and III were basically repeats of Experiment I with the additional aim of obtaining calorimetry measurements, but because there were only 2 calorimetry chambers available the repeat had to be carried out in 2 parts designated Experiments II and III (see Table 4.4).

In Experiments II and III the prime objective was to obtain the calorimetry data while at the same time obtaining plasma to confirm the results of Experiment I and to allow correlation of the plasma metabolite and calorimetric results. When blood samples proved difficult or impossible to collect this was not allowed to compromise the calorimetry. In this way it

was hoped to obtain complete usable sets of data on both calorimetry and blood parameters.

Following Experiment III it was planned to reverse the treatments applied to the sheep in each breed pair and repeat the procedures as Experiments X, Y and Z (see Table 4.4). However, blood sampling was abandoned following Experiment III due to the failure of cannulae and only calorimetry aspects of Experiments Y and Z were completed. Because the number of blood samples collected during Experiments were insufficient to provide meaningful profiles of blood parameters the calorimetry results have been considered separately. They are designated Calorimetry Experiment 2 and are described in full later in this chapter (Section 4.4).

2) Method

The sheep used in these experiments are the same ones used previously and described in Experiment I and Calorimetry Experiments 1 and 2. The 2 sheep of each breed were placed in 2 open-circuit respiration calorimeters as described by Holmes C.W. (83) and Holmes and McLean (84). The Romneys were studied during the first period and the Southdowns during the second, designated Experiments II and III respectively.

All ewes were cannulated (as described in Section 3.2) 2 or 3 days before the experiment started. Due to the thickness of subcutaneous fat and the presence of haematomas on the necks of the sheep extreme difficulty was encountered in cannulating them. The sheep were anaesthetized with about 1.5cm^3 of Rompun and when necessary surgical incisions were made in order to find the jugular vein. In some cases the jugular vein could not be found; it appeared that the jugular had collapsed and had been superceded by anastamoses of smaller veins. In such cases one such vein was cannulated rather than risking further damage by seeking an intact portion of the jugular.

Each pair of ewes was placed in the calorimeter chambers on the day prior to beginning the experiment in order to become accustomed to the apparatus. They had previously been trained to accept collars which were used to tether them by a chain within the chambers. This, combined with barricades consisting of various items of suitable size placed beside and behind them, was found necessary to prevent the animals from excessive movement which resulted in damage to the cannulae. Some slight modifications were made to the feed boxes to allow the sheep to reach all their food and water.

Extensions of small diameter plastic tubing 3-4 metres in length were attached to the cannula and led to the top of the sheeps neck then tied under the wool along their back to the centre of their backs. From here the tubing extended vertically to the roof of the chamber and thence to the outside. About 1 metre of slack was incorporated in the tubing to allow for movement of the sheep. This was secured to a length of elastic which ensured that the tubing could not dangle down where it could be damaged, but which would stretch during feeding or lying.

The sheep were fed and watered each morning about 8.30 a.m. The ration given is detailed in Experiment I Table 4.5. The feed was put into the chambers as quickly as possible through a small hatch above the feed bins in order to minimize disturbance of the gaseous equilibrium within the chambers. Fresh water was added through a special pipe directly into the bottom of the water trough.

Blood samples were collected from outside the chambers through the extensions on most occasions, but on some occasions when the cannulae or extensions were damaged or failed to operate, entry was made to the chambers briefly to obtain the sample and repair the cannulae. It was intended to collect the samples at times and intervals corresponding to those in Experiment I but this was not alwasy possible and many samples were missed altogether.

The treatment period lasted 3 days in each experiment.

4.3.2 Results

Variation of Plasma Metabolites and Hormones

1) Growth Hormone

As was the case in Experiment I most of the plasma GH determinations were indistinguishable from zero. The RIA determinations of the unknown sheep plasma samples produced bound counts about the same as (and often in excess of the zero standard (0ng GH cm^{-3}). Possible reasons for this, and attempts to correct the problem are described in Chapter 3. Suffice to say here that no usable estimates of plasma GH concentrations were ever obtained for these samples.

2) Glucose

Plasma glucose samples were assayed on 2 occasions and levels were found to be in the same range as those reported in Experiment I. As was found in Experiment I the levels recorded during the second assay were depressed. However due to the small number of samples obtained (A total of 40 in Exp II and 8 in Expt III) it is felt that meaningful profiles of glucose levels cannot be obtained from these data and they will not be presented in this thesis.

3) Free Fatty Acids

FFA determinations showed a similar range of levels as reported in Experiment I but for the reason described above it is felt they have nothing to contribute and are not included herein.

4.3.3. Discussion

The number of blood samples collected in these trials was insufficient to provide meaningful data. All that could be gained from them was that the range of concentrations of the plasma parameters approximated the results obtained in Experiment I.

The failure to obtain adequate blood samples was primarily due to the frequent blocking of cannulae, the additional problems associated with the long extensions, and to the resourcefulness of the sheep in performing gymnastics to destroy the cannula or to see the back of the chamber. This latter problem was thought to have been due to boredom and irritability on the part of the sheep. Although the chambers contained glass windows in front of the sheep, the view was of a blank wall and provided no sensory stimulus to the sheep. During the course of experimental work for this thesis, the sheep were found to have different "personal" characters and all were inquisitive, alert and prone to boredom. While in metabolism crates they often turned around, stamped their feet, scratched the floor, spilt their water, banged their feed bins (with their heads), threw food on the floor (much like children) and when no one was looking chewed up or otherwise destroyed their cannulae or that of any other sheep they could reach. In Calorimetry Experiment 3 they were better behaved and this was partly attributed to the clear and relatively interesting view afforded by the window in the hood.

An additional factor that contributed to the failure of cannulae was the quality of the cannulae themselves. In these early experiments inexpensive plastic tubing was used, mainly Silastik, which often blocked and also tended to collapse when a vacuum was applied due to the soft flexible nature of the walls. In later experiments it was found that the more expensive cannulas provided markedly better service.

Another possibility that was considered likely was that some blood characteristics of these very fat sheep led to rapid and effective blocking of the internal end of the cannula. The inability to withdraw samples while still able to easily inject saline suggested that some form of "flap" had formed over the end of the cannula forming an effective one-way valve, although no evidence for this was ever found when blocked cannulae were removed.

4.4 CALORIMETRY EXPERIMENT 2

4.4.1 Experimental Design

1) Objectives

Having established in Calorimetry Experiment I that the metabolic rates of the sheep were not significantly different under normal fed conditions it was then proposed to determine whether their;

1. basal metabolic rates (BMR)
2. response to fasting

differed between the fat and lean sheep.

The data from calorimetry experiment I showed that the metabolic rates of the sheep did not differ on a metabolic body weight basis, so that if there were real differences in their ability to produce adipose tissue (which was thus far not determined) it had to be due to differences in the efficiency of utilization of energy for maintenance and/or anabolism and perhaps involved differences in the partitioning of energy between maintenance and growth. It was thought that perhaps the BMR was lower for the fat animals leaving more energy available for growth; or perhaps the lean sheep responded to periods of feed deficit by mobilizing fat more quickly or in larger amounts than did the fat sheep.

2) Method

The experiment was a reversal or switchback trial (27) in which the same sheep as were used in Calorimetry Experiment I were alternately fasted and fed while in the calorimeters. Only 2 sheep could be monitored at once so one sheep of each breed was fasted while the other was fed, then after a restabilization period of at least 10 days the treatments were reversed.

Period	Chamber 1	Chamber 2
1	323 FASTED	313 FED
2	73 FASTED	56 FED
3	73 FED	56 FASTED
4	313 FASTED	323 FED

The fed sheep were fed 1.25 x maintenance (M) requirements, with 1 x M as nuts and 0.25 x M as hay. The fasted sheep were not in fact starved but fed 0.25 x M i.e. one fifth of their normal ration. The rationale behind this is discussed in Experiment VII (Section 4.8).

Each treatment period lasted 4 or 5 days as dictated by availability of calorimeters, experimental problems, convenience and the authors health.

The indirect calorimetry equipment was used to measure O_2 consumption and CO_2 production by the sheep, and the ratio of CO_2 production : O_2 consumption (RQ) was calculated. Since ruminants utilize acetate and glucose as their major substrates for energy storage and oxidation in the fed state (11) the respiratory quotient (RQ) characteristic of the fed state should be about 1.0 since the RQ associated with the complete oxidation of either glucose or acetate is 1.0 (see Table 4.7). The oxidation of long chain fatty acids is associated with an RQ of about 0.7, the RQ decreasing as the chain length of the fatty acid increases (see Table 4.7).

Assuming that lipolysis results in the mobilization and oxidation of long chain fatty acids, the RQ of a fasted animal should move towards 0.7. With this in mind the RQ was calculated to express the fasting response.

TABLE 4.7: THEORETICAL RESPIRATORY QUOTENTS FOR THE OXIDATION OF GLUCOSE AND FATTY ACIDS

			$\frac{CO_2}{O_2}$	RQ
OXIDATION OF GLUCOSE				
		$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$	$\frac{6}{6}$	1
OXIDATION OF FATTY ACIDS WITH n CARBONS				
acid	n			
acetic	2	$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O$	$\frac{2}{2}$	1
propionic	3	$CH_3CH_2COOH + 7O_2 = 6CO_2 + 6H_2O$	$\frac{6}{7}$	0.86
butyric	4	$CH_3(CH_2)_2COOH + 5O_2 = 4CO_2 + 4H_2O$	$\frac{4}{5}$	0.80
valeric	5	$CH_3(CH_2)_3COOH + 13O_2 = 10CO_2 + 10H_2O$	$\frac{10}{13}$	0.77
caproic	6	$CH_3(CH_2)_4COOH + 8O_2 = 6CO_2 + 6H_2O$	$\frac{6}{8}$	0.75
	⋮			
palmitic	16	$CH_3(CH_2)_{14}COOH + 23O_2 = 16CO_2 + 16H_2O$	$\frac{16}{23}$	0.696
stearic	18	$CH_3(CH_2)_{16}COOH + 26O_2 = 18CO_2 + 18H_2O$	$\frac{18}{26}$	0.692

$$RQ = \frac{n}{1.5n - 1}$$

Regression and covariance analysis according to the method of Snedecor and Cochrane (145) were used to evaluate the data.

4.4.2 Results

As can be seen in Table 4.8 the RQ's of the fed sheep remained about 1 with a mean of 1.066 ± 0.032. The fasted sheep had a lower mean RQ value of 0.950 ± 0.074. The difference between the means was statistically significant ($0.025 < P < P0.01$).

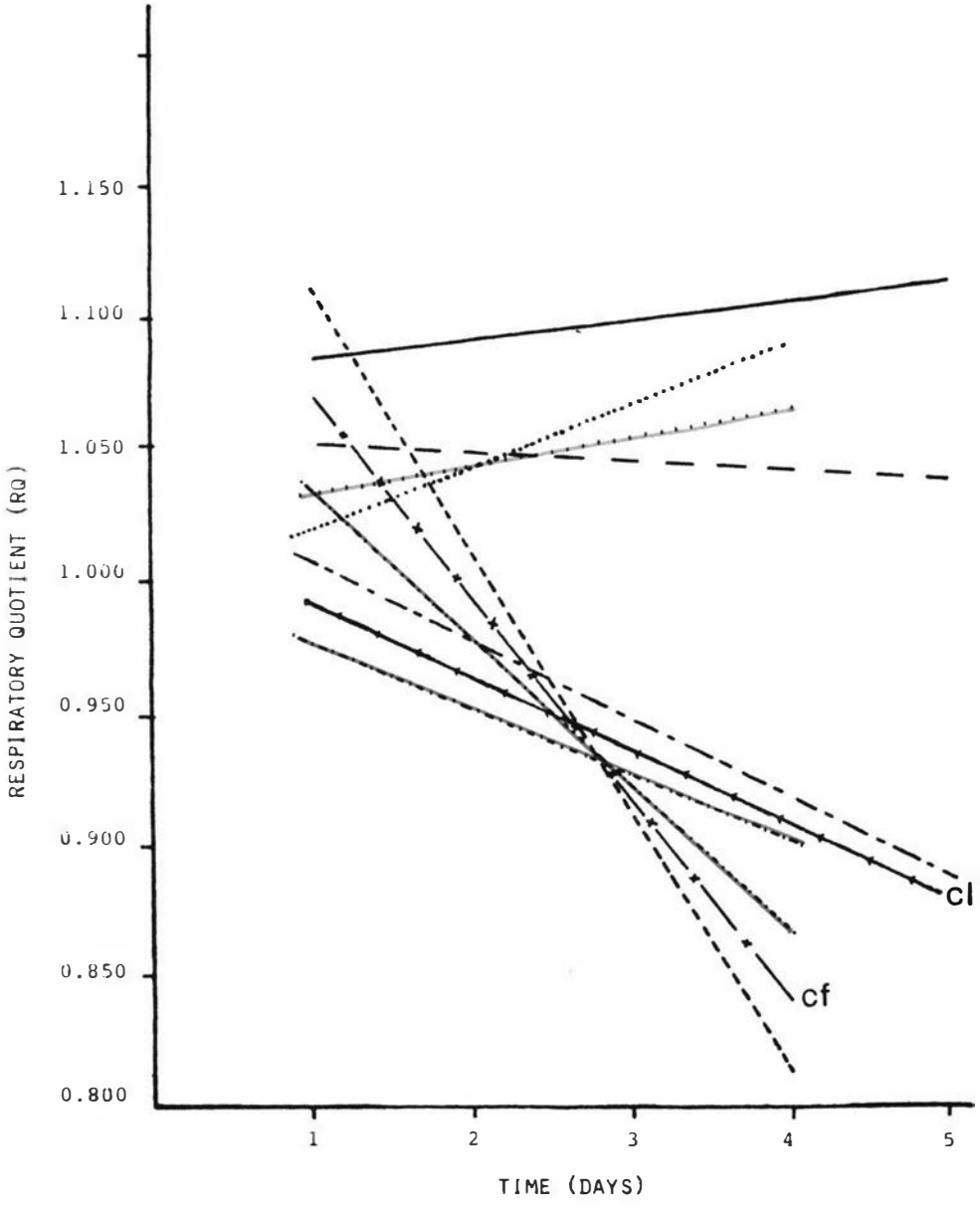

Figure 4.4 Slopes of regressions of RQ on time for four sheep when fasted or fed.

Ewe 56 Fed ——— Fasted - - - - -
Ewe 73 Fed – – – – Fasted — – —
Ewe 313 Fed ..... Fasted
Ewe 323 Fed Fasted

Common regression of fat ewes when fasted —x— cf

Common regression of lean ewes when fasted cl

TABLE 4.8: DAILY RESPIRATORY QUOTIENTS OF 4 EWES WHEN FED OR FASTED FOR 4 OR 5 DAYS

Ewe	Day	Fasted	Fed
313	1	1.0331	1.0314
	2	0.9785	1.0366
	3	*	1.0582
	4	0.8662	1.1045
323	1	0.9753	1.0331
	2	0.9588	1.0412
	3	0.9217	1.0623
	4	0.9044	1.0621
73	1	1.0363	1.0333
	2	0.9341	1.0801
	3	0.9391	1.0415
	4	0.9181	1.0353
	5	0.8868	*
56	1	1.1238	1.0878
	2	1.0002	1.0755
	3	0.8946	1.1019
	4	0.8298	1.1410
	5	*	1.0925

* denotes missing data

It will also be noticed in Table 4.8 and in the graphs (Figure 4.4 that the RQ's of the fasted sheep declined with time, while those of the fed sheep did not. When regressions were fitted the slopes of individual regression lines of fed sheep did not differ significantly ($P > 0.25$) from the slope of a common regression line for the fed sheep. The slopes of individual regression lines of fasted sheep did differ significantly ($P < 0.005$) but nevertheless a common regression line for fasted sheep was derived and found to have a significantly different slope ($P < 0.005$) from that of the fed sheep.

The RQ response to fasting was a straight line relationship with recorded correlation coefficients of -1.000, -0.987, -0.888 and -0.991. Correlations in the fed sheep were not as good, with values of 0.934, 0.941, -0.191 and 0.475.

The fat group, comprised of Ewes 313 and 56 exhibited significantly ($P = 0.002$) steeper regression slopes when fasted, than the lean group. Within the fat group Ewe 56 had a significantly steeper regression slope $P = 0.03$. (Complete regression analysis and analysis of covariance can be examined in appendix 4.4).

4.4.3 Discussion

As was expected, the RQ's of fed sheep remained about 1, while the values for fasted sheep declined in a straight line with time. Since the RQ's of fasted sheep did not stabilize at a lower level, but were still falling linearly, it was assumed that a steady state of fasting metabolism was not reached and so the BMR's could not be calculated. Extrapolation of the regressions indicated that 3 or more extra days of fasting may have been needed to reach a steady state of fasting metabolism in the lean sheep, assuming the responses remained linear and straight and that an RQ of about 0.7 is characteristic of fasting metabolism.

Modyanov (113) starved sheep for 144 hours and found that heat production decreased until the end of the third day, then became somewhat stable and remained at the same level subsequently. The non-protein RQ after 72 hours of starvation was 0.72 which Modyanov said was typical of fat combustion. The fact that the sheep in the present experiment did not decline below 0.8 after 4 or 5 days can most likely be attributed to their intake of a quarter of their maintenance requirements daily compared to Modyanov's sheep which were starved. Nevertheless it indicates that the sheep in the present study did not reach a basal metabolic state.

Annison et al (2) reported RQ's for fed sheep of 1.03 and for 24 hour fasted sheep of 0.94, but pointed out that these values were more than usually difficult to interpret in ruminants where a significant

proportion of the total CO_2 output arises by anaerobic fermentation in the rumen.

The 2 groups of ewes differed in their response to fasting as measured by RQ. The fat pair had steeper regression slopes which possibly indicated a greater rate of mobilisation of fat; i.e. a more accelerated lipolysis than the lean sheep. Note that this does not indicate an earlier response.

Sidhu et al (141) found that basal lipolysis increased with fatness in homogenates of lamb adipose tissue and postulated that factors affecting deposition of fat must compensate for the increased lipolysis (see Section 2.4.5).

Few conclusions can be made since BMR's were not determined. Nevertheless it is evident that the response to fasting was an extremely straight linear decrease in RQ with respect to time, which was apparently steeper in the fat ewes especially, in the Southdown (Ewe 56).

In relation to the theoretical fasting RQ of about 0.7 it is noted that Annison et al (2) measured RQ's no lower than 0.85 during infusion of ^{14}C-labelled fatty acids into 24 hour fasted sheep fitted with tracheal cannulas. They reported RQ's for stearate of 0.87, 0.89 and 0.90 (3 individual observations from 2 ewes). The physiological significance of this result is difficult to understand in relation to "normal" fasted sheep. Nevertheless the value of 0.72 achieved by Modyanov (113) is some indication that the method used in the present study was suitable.

4.5 EXPERIMENT IV

4.5.1 Experimental Design

Objectives

The objectives of this experiment were;

1. to determine whether or not ACTH is lipolytic in sheep
2. to determine the effects of exogenous ACTH on plasma metabolites and hormones in fed sheep
3. to determine whether the "fat and lean" sheep differed in their response to ACTH.

The rationale behind the study of ACTH is outlined in Experiment V later in this chapter (Section 4.6).

4.5.2 Discussion

The sheep to be studied in this experiment were the "fat and lean" ewes used in the previous experiments. However only Ewe 323 was successfully cannulated, and so the experiment was commenced with only Ewe 323. This situation was taken as an opportunity to test the safety and effectiveness of the proposed dose and preparation. 30iu of ACTH (ACTHAR) in physiological saline were injected 10 mins after feeding but 1 hour later no blood samples could be obtained. The cannula was not blocked and withdrawal only produced air. It was concluded that the jugular was empty and that the blood was taking an alternative route. The experiment was abandoned at this point and 4 new ewes were obtained (as described in Experiment V). It was intended to attempt Experiment IV again at a later date after the initial 4 ewes had rested and recuperated.

Unfortunately 3 weeks later Ewe 56 (Fat Freddy) became ill and the following day she collapsed and died. Post mortem examination indicated that she had died of copper poisoning. This was attributed to the high proportion of sheep nuts in the diet. The sheep nuts were known to contain lucerne although the suppliers (Farm Products, Palmerston North) would not reveal the proportions of various components of the

ration or the chemical analysis. Lucerne is known to commonly contain high levels of copper and low levels of molybdenum . Imbalance of the 2 metal ions over an extended period results in copper being sequestered in the liver until critical levels are reached and acute copper poisoning results in a rapid death (A.W.F. Davey: Personal Communication 1977). The remaining 3 of the original 4 sheep were assumed to be suffering from chronic copper poisoning and so were not fed any more sheep nuts. The amount of nuts fed to the new Romneys was decreased to half their maintenance energy requirements and their hay ration was doubled to maintenance levels (a total of 1.5 x M).

The loss of Fat Freddy, the supposed chronic copper poisoning of the 3 remaining original ewes along with the damage to their jugulars led to the complete abandonment of studies using these sheep. All subsequent work used cull ewes obtained from Massey flocks and as a result the initial objective of studying lipolysis in fat and lean genotypes had to be abandoned.

The 17 plasma samples obtained from Ewe 323 in Experiment IV were a useful source of ovine plasma for use in the development of assays. They were insufficient in number to be of any value with respect to the initial objectives of the experiment, especially since the collection of blood samples failed only an hour after ACTH injection. It did appear however that the ACTH injection had no adverse effects on the ewe and it was assumed that the preparation and dose were safe to use in a later trial.

4.6 EXPERIMENT V

4.6.1 Experimental Design

1) Objectives

1. To determine whether ACTH is lipolytic in sheep
2. To determine the effects of exogenous ACTH on plasma metabolites and hormones on fed sheep.

ACTH showed lipolytic activity in adipose tissue from rabbit, guinea-pig, hamster and rat, but not in pig and dog tissue, in a study by Rudman (1965) cited by Luthman and Jonson (102).

In their review, Lebovitz and Engel (96) described the in vivo adipokinetic actions of corticotropin as:

1) increased release of FFA from adipose tissue
2) increase in plasma FFA
3) increase in liver fat
4) ketosis

However they made the point that at that time a physiological role for ACTH in lipid mobilization had not been demonstrated. Published in vivo effects had only been elicited by pharmacological doses while lipolytic effects of endogenous ACTH had not been observed (96).

Radloff and Schultz (130) found that ACTH caused a significant increase in plasma FFA in female goats, but had no effect on castrate males. The highest FFA levels occurred after 8 hours and Luthman and Johnson (102) questioned whether the increase could be attributed to a direct effect of ACTH on adipose tissue. They (Luthman and Jonson) found no FFA response to ACTH in sheep within 6 hours of administration (102). However it should be noted that the two groups of experiments utilized different methods of hormone administration and different doses. Radloff and Schultz used intramuscular injections of 80 units of ACTH in goats of unstated weight (130) while Luthman and Jonson infused intravenously $0.5u\ Kg^{-1}$ into sheep weighing from 38 to 54kg (102). It is unlikely that the goats used by the former workers differed greatly in weight from the sheep used by the latter (102).

With evidence of lipolytic responses to ACTH in several species and conflicting results amongst the meagre ruminant studies, it therefore seemed appropriate to test the lipolytic activity of ACTH in sheep.

2) Method

Four adult Romney ewes about 5 years of age were obtained from a small group of non-pregnant ewes culled from Massey sheep farms on 4th August 1977. The selection of these sheep is discussed more fully in Experiment VII. The sheep were housed indoors in metabolism crates at the animal physiology unit. They were fed and watered and had their crates cleaned daily and in addition they were handled and visited frequently to ensure that they became accustomed to their new environment as rapidly as possible.

The sheep were weighed and their maintenance requirements calculated by the method of Coop (38). They were fed their respective maintenance allowances in the form of sheep nuts (Farm Products, Palmerston North) and an additional 0.5 x M in the form of good quality meadow hay. They were given the whole amount each morning between 8.30 and 9.30 a.m. depending upon difficulties encountered with blood sampling and ate the nuts and much of the hay within the first hour.

All ewes were cannulated (jugular vein) using only local anaesthetic on the day prior to beginning the experiment.

The basic experimental design was a switchback or reversal trial (27).

TABLE 4.9: DESIGN OF EXPERIMENT V

Day	1	2	3	4	5
Treatment	none	ACTH	none	ACTH	none
Ewes treated	-	142 + 116	-	57 + 186	-

On day 1 no treatments were administered. Blood samples were collected to determine the normal diurnal plasma profiles of these sheep.

On day 2 (9.30 a.m.), 30 i.u. ACTH (ACTHAR) was injected subcutaneously into Ewes 142 and 116. On day 3 no treatments were administered, though blood samples were collected to determine whether plasma profiles were normal i.e. a day was allowed to ensure that any treatment effects did not carry over into the next treatment period.

Following the experimental period on day 3 the first 2 samples taken from all sheep on day 1 and the last 2 taken from each on day 3 were analysed for plasma FFA levels. This confirmed that plasma FFA levels had either returned to normal or remained unchanged and indicated that the second treatment period (day 4) could be commenced with minimal fear of carry-over effects.

On day 4 (9.30a.m.), 30 i.u. ACTH was injected subcutaneously into the other 2 sheep, Ewes 57 and 186. On day 5 no treatments were administered but blood sampling was continued to detect any prolonged or delayed treatment effects.

A control injection to untreated sheep was inadvertently omitted from the experimental design.

Blood sampling was carried out as described in Section 3.4 beginning with a prefeeding sample between 8.30 and 9.00 a.m. each morning. Six to eight samples were taken on each of the 5 days at various times up until 7.00p.m. Feeding, treatment and sampling times could not be standardised due to extreme difficulties encountered with the cannulae, which often blocked. At least 16 samples were taken by venepuncture, most from Ewe 116, when attempts to withdraw from the cannulae failed.

Sheep weights recorded prior to the experiment were in the range 40-50kg.

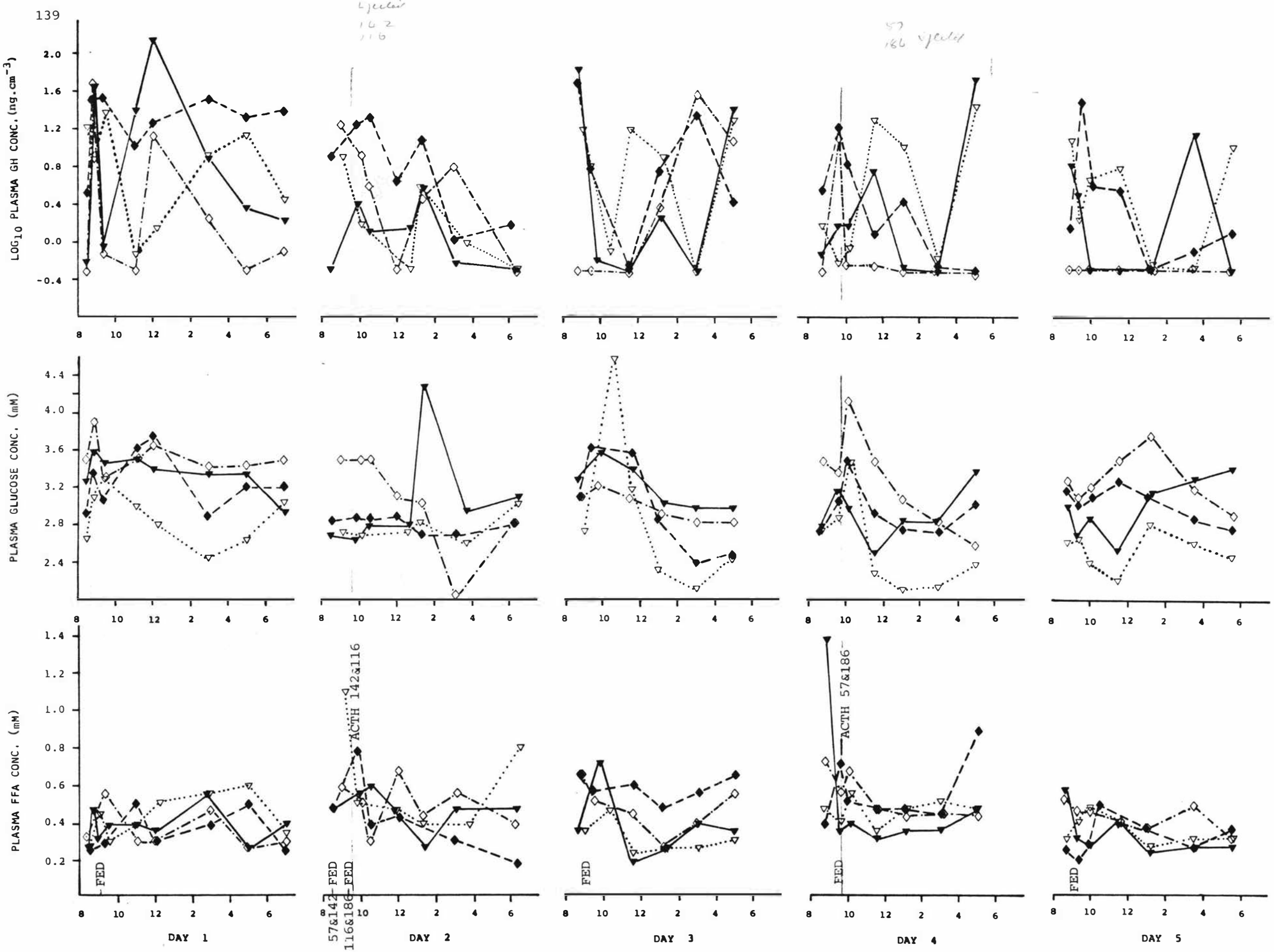

Figure 4.5 The effect of ACTH on plasma GH , insulin , glucose and FFA concentrations in four Romney ewes.

Ewe 57 ♦--♦ Ewe 116 ▼····▽ Ewe 142 ▼—▼ Ewe 186 ◇-·-◇

4.6.2 Results

Variation of Plasma Metabolites and Hormones

1) Growth Hormone

There was much variation in prefeeding plasma GH levels as can be seen in the graphs in Figure 4.5. Concentrations were in the range between undetectable levels (< 0.5ng cm^{-3}) and 71ng cm^{-3}. In 6 out of sixteen cases untreated sheep exhibited rapid peaks within about an hour after feeding which is unusual when compared to the results in Experiments VI, VII and IX in which only 4 out of 38 cases showed deviation from the more common finding of a sharp fall from prefeeding levels. Nevertheless all sheep in this experiment (V) recorded a depression in plasma GH levels at some stage in the 3 hour period after feeding and approximated the diurnal variation described in Experiment VII (Section 4.8).

Immediately following ACTH injection 3 out of the 4 ewes recorded peaks in plasma GH, but these are indistinguishable from the peaks in other cases following feeding described above.

All 4 sheep had undetectable (< 0.5ng cm^{-3}) GH levels at the last sample taken on the treatment days, while this occurred in only 3 out of 16 cases in untreated sheep.

Following ACTH injection GH levels in Ewe 186 fell to almost undetectable levels (0.58ng cm^{-3}) within 30 mins and from 1 a.m. until the end of the experiment on day 5 (over 28 hours later) GH levels remained undetectable. No carry-over effects were evident in other sheep.

2) Free Fatty Acids

Prefeeding FFA concentrations were in the range of 0.23 - 1.38mM but only 2 records were in excess of 0.72mM (see Figure 4.5). Nine out of 16 untreated ewes showed FFA peaks in the

90 mins following feeding. A further 3 untreated ewes showed later peaks (up to 3 hours post feeding) and one (Ewe 142) had an extremely high FFA titre, just prior to feeding, leaving only 3 that did not show an apparent FFA peak in response to feeding. 17 of the 20 cases exhibited troughs in FFA levels between 11 a.m. and 1.30 p.m. in the range 0.27 - 0.47mM of which 14 values were the minimum recorded for each sheep on that day. Following this 19 of the 20 either showed little change or gradually increasing FFA levels throughout the remainder of the afternoon.

On days in which they received ACTH Ewes 116 and 57 exhibited unusually high FFA levels ($\geq$ 0.8mM) at the last sample of the day (corresponding to uncharacteristically undetectable ($<$ 0.5ng cm^{-3}) GH levels). This response was not detected in the other 2 ewes.

3) Glucose

Prefeeding plasma glucose concentrations were in the range 2.60 - 3.50mM (see graphs in Figure 4.5). There was greater variation in glucose levels between days than between sheep on each day, i.e. glucose changes showed a similar pattern for all sheep on each day but this pattern was less similar between days. Untreated ewes showed marked increases in glucose levels in the 90 mins following feeding on days 1, 3 and 4 but none did so on days 2 and 5. Plasma glucose changes in the afternoon were quite variable between sheep and between days and no definable pattern could be detected.

No definite glucose responses to ACTH were recorded, but 1 or 2 unusual values might have been attributable to ACTH treatment. Four hours after ACTH injection Ewe 142 exhibited a single result in excess of the highest standard (4.00mM) used in the assay with an estimated value of 4.3mM. On day 3, 25 hours after ACTH injection Ewe 116 had an extremely high glucose concentration in one sample. The concentration was estimated by extrapolation of the standard curve to be about 6mM.

On day 4, 30 mins after ACTH, Ewe 186 exhibited a high glucose concentration with an estimated value of 4.15mM, but this is less remarkable in light of the 3.90mM result recorded for that sheep at a comparable time on day one.

4.6.3 Discussion

Many problems were encountered in obtaining blood samples in this experiment. In particular, cannulae often would not allow withdrawal of blood and cannulae were damaged or removed by the sheep so that many samples were obtained late and some missed altogether and in general much more fuss occurred around the sheep than was normal or desirable. Ewes 116 and 57 had to be recannulated during the course of the experiment and the aberrant glucose result obtained on day 3 at 10.30 a.m. can most likely be attributed to the cannulation of Ewe 116 just prior to that time. Ewe 116 subsequently removed her cannula and was sampled by venepuncture for the last 2 days of the experiment. What effect this had on her plasma hormones and metabolites is unknown, but she was minimally disturbed by the venepuncture and did not even stand up during sampling. She apparently preferred the rapid and apparently painless venepuncture to the continual annoyance of the cannula and tedious attempts at sampling from it.

Additional problems were encountered with coagulation and/or haemolysis of samples. On day 2 the incidence of coagulation in samples from both the ACTH treated sheep led to the suspicion that the ACTH had altered or increased the clotting mechanism in some way so that the concentration of EDTA used as anticoagulant was inadequate. No evidence for this was gained however. Hameoglobinaemia was evident in some samples despite stringent precautions taken against entry of hypotonic solutions to the samples or to the circulation of the animals. This led to the suspicion that some factor in vivo was causing haemolysis. No particular significance could be attributed to the observations of coagulation and haemolysis with respect to the results of the experiment.

McNatty and Thurley (110) reported on the adaptation of sheep to a new environment, and reported a "tendency for mean cortisol levels to fall with longer housing". The response of plasma cortisol to adrenaline and ACTH was diminished over 2 weeks. On the basis of their results it was decided that 2 weeks was a minimal but acceptable time to allow the sheep to adjust to their new environment. However in light of the extraordinary commotion caused by the above-mentioned problems it is probable that stress could have influenced the experimental results to a degree.

The dose of ACTH used in this experiment (30iu) was intermediate between the doses used by Radloff and Schultz (130) and those used by Luthman and Jonson (102). The dose used by the former authors was criticised by the latter, and it is expected that the dose used in the present trial was an effective compromise.

The effect of the ommission of a control injection in untreated sheep is not known, but in view of the fact that no distinct rapid responses to the ACTH injection were observed it is thought that the results were not significantly affected.

Administration of ACTH had no clearly defined effect on the 3 parameters measured. The elevated FFA levels exhibited by Ewes 116 and 57, eight and nine hours respectively after ACTH injection may indeed have been a lipolytic response like that recorded by Radloff and Shultz (130) in female goats, but the other 2 ewes showed no such response. Since only 2 samples are involved in the possible response no attempt was made to establish statistical significance to them. Nevertheless, since they occurred at the precise time one might expect from the results of Radloff and Schultz it would be improper to ignore them outright. Further circumstantial evidence for the reality of those results is afforded by their concurrence with markedly depressed GH levels, but such conjecture is weakened by the observation that the 2 other sheep recorded the same low ($< 0.5 ng\ cm^{-3}$) GH levels at the same time (following ACTH treatment) and they did not exhibit lipolysis. In retrospect it is apparent that more extensive blood sampling would have been expedient around the time in question. If

the response was indeed a real lipolytic response, and GH levels were depressed as a result of ACTH, it could be postulated that ACTH might surplant the lipolytic role of GH in times of stress or other ACTH stimulation.

Something, presumably the ACTH injection, completely depressed the GH levels in Ewe 186 to undetectable levels apparently over the whole of the last 25 hours of the experiment. This is perhaps the strongest evidence of some role for ACTH in lipid metabolism in sheep.

Extrapolating this conjecture further, it would seem by the time scale of the action that it is unlikely to be a direct effect of ACTH on adipose tissue (the same comment made by Luthman and Jonson (102) about the results of Radloff and Schultz (130)) but is more likely to be a tropic effect of ACTH involving protein synthesis.

However all this conjecture is based on but a few samples and unless the sheep used in this study were of 2 distinct strains (one like the female goats of Radloff and Schultz and one like the sheep of Luthman and Jonson) it is more probable that ACTH did not produce a lipolytic response in the sheep. This is supported by Vernon (156) who in his extensive review cited only 4 cases in which the effect of ACTH on FFA levels in sheep was studied (with one case, Manns and Boda (105), apparently in error) but in each case no lipolytic effect was recorded.

4.7 EXPERIMENT VI

4.7.1 Experimental Design

1) OBJECTIVES

1. To stimulate lipolysis in fed sheep using a catecholamine
2. To determine the effects of exogenous catecholamine on plasma metabolites and hormone levels in fed sheep.

In his extensive review of the sympathetic regulation of metabolism Himms-Hagen (80) stated "the sympathetic nervous innervation of adipose tissue and the metabolic effects of stimulation of these nerves indicate a major role of the sympathetic nervous system in the control of lipid metabolism. Both brown and white adipose tissue contain noradrenaline (N.A.) in nerve endings around arteries and arterioles and lose this N.A. after denervation". He also pointed out that the injection of adrenaline or N.A. into intact animals resulted in accelerated lipolysis in all species tested (prior to 1967) except the chicken. Two studies involving sheep were cited in this context.

He further stated that the lipolytic effect of N.A. an adipose tissue is equal to or greater than that of adrenaline. Bassett (6) however, in a later paper (1970), found it difficult to speculate on the sensitivity of the various metabolic processes to the effects of noradrenaline relative to adrenaline due to the absence of information on blood levels of catecholamines, and for other reasons discussed by himself (6) and Himms-Hagen (80).

Thus there was no basis on which to select adrenaline or noradrenaline as the appropriate lipolytic stimulus for the present study and so a readily available preparation was used (see Method below).

2) METHOD

Four Romney ewes were obtained from a small group of non-pregnant ewes culled from Massey sheep farms on the 4th August 1977. The selection of the ewes is discussed in more detail in Expt. VII.

The ewes were housed in metabolism crates at the A.P.U.

Two months elapsed before the start of this experiment during which time the ewes became accustomed to their environment and were used in Experiment V.

The experimental design was a "reversal trial" (27).

TABLE 4.10: DESIGN OF EXPERIMENT VI

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
Control	All sheep	Ewes 116, 142	All sheep	Ewes 57, 186	All sheep
N.A.	-	Ewes 57, 186		Ewes 116, 142	

All four ewes were cannulated two days prior to the start of this experiment - Day 1 involved no experimental treatment but was devoted to blood sampling to obtain estimates of the normal diurnal metabolic profiles of the sheep.

- Day 2 involved N.A. injections into Ewes 57 and 186 with control injection into Ewes 116 and 142.

- Day 3 was a control day similar to day 1. No treatments were imposed and all sheep were sampled to ensure blood metabolite levels had returned to normal levels following the treatment on day 2.

- Day 4 involved N.A. injections into Ewes 116 and 142 and control injections into Ewes 57 and 186.

- Day 5 was a control day like days 1 and 3 to monitor the return of any treatment effects to normal levels.

The preparation of noradrenaline used was "Bronkephrine" a brand of ethylnoradrenaline hydrochloride produced by Winthrop Laboratories (Sydney) and chemically known as racemic 1-(3,4-dihydroxyphenyl)-2-amino-1-butanol hydrochloride. Each cm^3 of Bronkephrine solution contained 2mg ethylnoradrenaline hydrochloride (0.2% W/V) in a

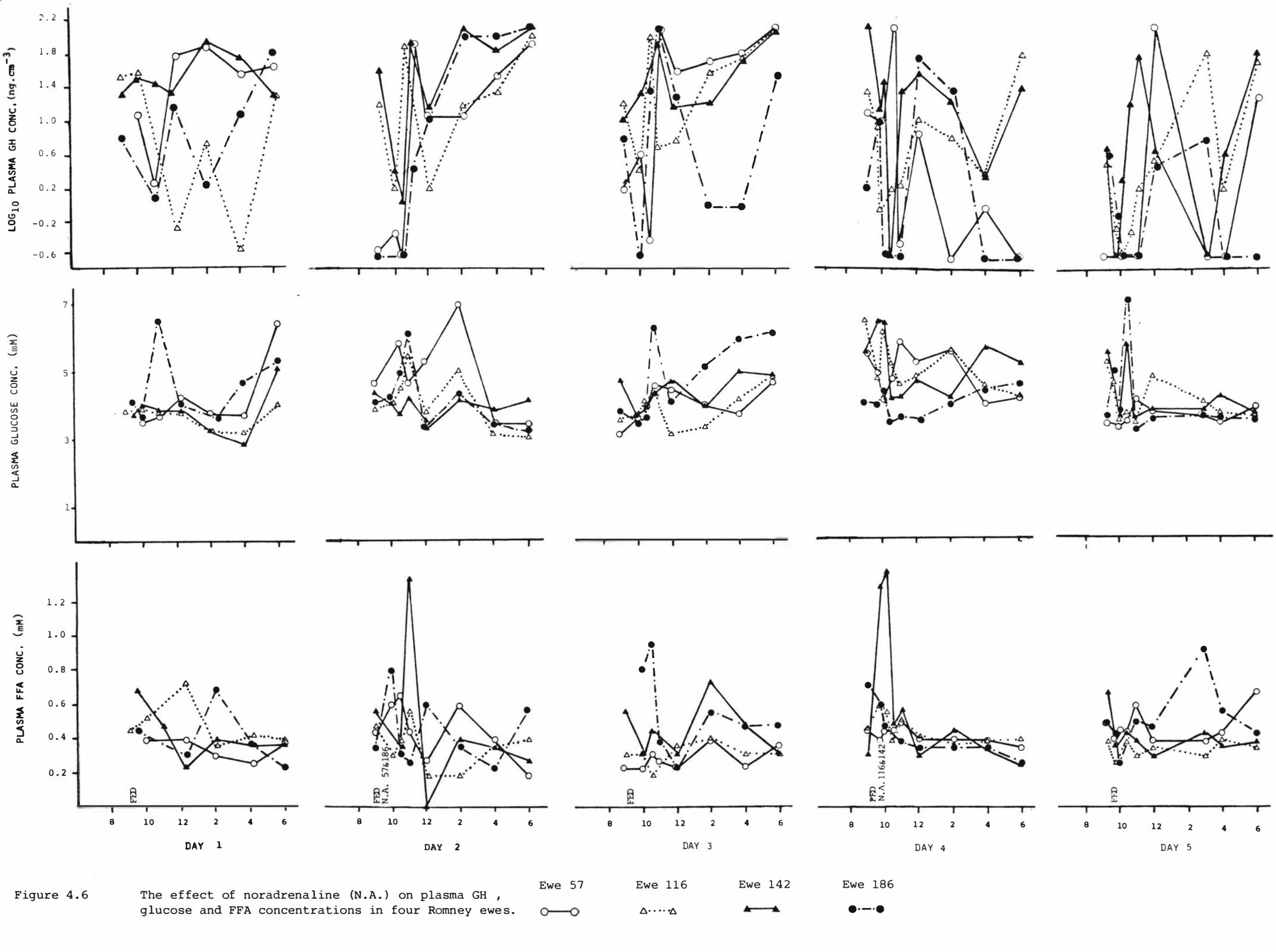

Figure 4.6 The effect of noradrenaline (N.A.) on plasma GH , glucose and FFA concentrations in four Romney ewes.

sterile isotonic solution with sodium acetone bisulphite 0.2% W/V and chlorbutol 0.25% W/V as preservative. Administration by subcutaneous or intramuscular injection was indicated (Makers information sheet).

The dose of N.A. was 1cm³ injected subcutaneously in the neck. On treatment days a 1cm³ control injection of chlorbutol 0.25% W/V in sterile isotonic saline was administered to control sheep immediately after N.A. was administered to the treatment sheep.

Sheep were fed 1.5 x M consisting of 1 x M as hay and 0.5 x M as sheep nuts. Feeding was done each morning between 9.15 and 9.45. On days 2 and 4, the treatment days, feeding took place at 9.15 a.m., followed by the N.A. and control injections at 9.30 a.m.

Blood samples were collected from 9 a.m. - 6 p.m. daily with the most frequent sampling in the morning.

4.7.2 Results

Variation of Plasma Metabolites and Hormones

1) Growth Hormone

As can be seen in the graphs in Figure 4.6 N.A. injections had no detectable effect on plasma GH levels, which followed the same patterns as is described in Expt VII and so these results will not be dwelt upon. In passing it is worth noting several unusual inexplicable results. On day 2 Ewes 57 and 186 recorded very low GH levels ($< 0.5ng\ cm^{-3}$) prior to and immediately following feeding and N.A. injection. Since no anticipation of N.A. injection can be envisaged this result must be considered to be a chance occurrence. On day 4 the same pair (Ewes 57 and 186) recorded unusually low ($< 1ng\ cm^{-3}$) GH levels in the late afternoon. On days 5 Ewe 57 had undetectable GH levels prior to 12 noon and at 3 and 4 p.m. samples. Ewe 186 had undetectable levels also in the late afternoon.

2) Insulin

Plasma insulin levels were not assayed due to apparent lack of effects in the other results.

3) Glucose

Prefeeding plasma glucose concentrations were in the range 3-5.5mM. Graphs of plasma glucose versus time are presented in Figure 4.6. At the next sampling time after feeding 50% of samples showed a rise in plasma glucose and 50% fell, although 4 of the 20 records would have been affected by N.A. injections within the period between feeding and the next sample. Sixteen of the twenty records showed an increase in glucose levels which peaked 1-2 hours after feeding but again 4 of these cases were subject to influence from N.A. injections. The magnitude of these increases varied between sheep and between days as did the time of the peaks. No attempt was made to establish whether these rises were significant due to time series problems and because of large variation between sheep, but visual inspection leads to the belief that they are real effects e.g. Ewe 186 showed increases in glucose levels of 50-100% on 4 out of 5 days.

Any responses to N.A. injection would have been confused with the apparent response to feeding. It should be noted however that N.A. treatment ewes peaked at higher glucose concentrations and at an earlier time than control treated ewes. No attempt was made to establish whether this was a significant result because of large variation in glucose levels around that time.

After these major peaks, plasma glucose levels declined to approximately prefeeding levels by 2-3 hours after feeding. Following this glucose levels in all sheep showed a distinctly similar pattern of changes on 4 out of the 5 days but variation between days was such that no definable pattern could be discerned.

4) Free Fatty Acids

FFA levels are shown in graphs in Figure 4.6. No discernable pattern of plasma FFA levels could be found due to large variation within and between days. With respect to N.A. treatment all four sheep showed peak levels of FFA's following injection at times when their control counterparts exhibited low or declining FFA levels.

However 2 apparently spontaneous peaks were recorded in each of Ewes 142 and 186 at times supposedly unassociated with experimental treatment. Thus any attempts at establishing the significance of responses to N.A. were considered futile.

4.7.3 Discussion

This experiment has not shown any significant effects of Bronkephrine on the three parameters measured. However it would be improper to assume from this that N.A. does not affect fat metabolism in sheep. Rather, all that can be deduced is that Bronkephrine had little or no effect under these experimental conditions. Any one of several factors in the experimental design, or some combination of them, could have been responsible for the apparent lack of response.

Firstly, it is possible that the brand of N.A. used (Bronkephrine) had little or no physiological effect on the metabolic parameters measured in the sheep. Shortly after the present experiment was completed Young and Landsberg (163) published a review in which they pointed out that catecholamine-stimulated lipolysis in adipose tissue is mediated by the beta-receptor while the alpha receptor is antilipolytic. Mayes (109) indicated that the lipolytic actions of adrenaline and noradrenaline are blocked by beta-adrenergic blockers. Fain (54) stated that the initial step in catecholamine action (in lipolysis) is binding with the beta-1 receptor. The manufacturers of Bronkephrine describe it as a synthetic sympathomimetic amine, largely beta-active in effect, but lacking glycogenolytic activity. (Source; manufacturers information sheet). The glycogenolytic actions of adrenaline and

noradrenaline are mediated by the beta-receptors (163). Since Bronkephrine is not glycogenolytic (beta-mediated) this casts some doubts upon its lipolytic activity (also beta-mediated). Nevertheless there is no evidence to show that it is not lipolytic. The lack of a hyperglycaemic response in the present experiment is not surprising in light of the lack of glycogenolytic acitivity of bronkephrine. Also noradrenaline is not glycogenolytic in muscle anyway (while adrenaline is) and generally has a lesser hyperglycaemic effect than adrenaline (80).

Secondly, the timing of N.A. injections in relation to feeding may have altered or diminished the effect of the N.A. The effects of feeding on plasma hormones and metabolites can be seen, in this experiment and in Experiment IX, to continue for a number of hours after feeding. The nutritional state of the animal may alter not only regional blood flow to adipose tissue, but also the pattern of response observed to the catecholamine stimulus (163). In addition there is a feeding-related response, as distinct from a food-related response (99) which must be taken into account. In sheep fed once daily it is clear that major changes occur as a result of feeding and food, and that these changes may have obscured any treatment effect. Lindsay (99) warned against blood sampling within a short period before or after feeding. In retrospect it seems clear that the N.A. treatment should have been imposed at sometime well removed from feeding, but the selection of such a time must take into account other diurnal changes in plasma parameters and might involve trials with N.A. treatments being imposed at various times after feeding. Alternatively the sheep could be provided with a constant supply of food, but diurnal variation would still need to be considered.

The method and route of administration of N.A. is another factor which may have altered the response. Bassett (6) reported that intravenous infusion of 1.5mg of adrenaline over 30 min into adult Merino wethers, increased glucose, lactate, and FFA concentrations in plasma much more than did a single rapid intravenous injection of the same amount. He subsequently employed slow intravenous infusions lasting 60 min for all catecholamines used. Nevertheless the rapid intravenous injection did result in marked increases in glucose, lactate and FFA. The

rapid subcutaneous injection used in the present experiment (Expt. VI) was a cautious approach designed to allow a slower entry rate than i.v. injection and avoid rapid changes in the circulatory system. Thus one might assume that the subcutaneous injection approximated a slow infusion. Since the makers indicated subcutaneous or intramuscular injection as the method of administration and warned against intravenous administration of Bronkephrine, then it was assumed that the subcutaneous route employed was satisfactory and indeed preferable.

The concentrations of catecholamines required to stimulate lipolysis in vitro are in general higher than levels of circulating catecholamines in vivo (163) and this may indicate a role for locally released N.A. If this is so, then the dose of N.A. administered may have been too low to obtain a lipolytic response. Bassett (6) obtained a rise in plasma FFA following 1.5mg of adrenaline (i.v.) so one might expect that the 2mg dose of Bronkephrine used in the present experiment was sufficient if the subcutaneous route did not alter the effective dose. McNatty and Thurley (110) administered 1 and 3mg adrenaline (i.v.) and believed that at least the 1mg dose was within physiological limits.

It appears then that the failure of Bronkephrine to stimulate a lipolytic response cannot be proven to be attributable to any aspect of the experimental design. Had a positive lipolytic response been achieved it could have been justified physiologically and perhaps statistically. However a negative result is much harder to interpret, and the reason for this result can not be determined without further trials.

Methods for the determination of catecholamines are not commonly available so that the usual method of studying the metabolic effects of these hormones is by administration of exogenous catecholamines. Since there is some doubt about the relative importance of circulating levels of catecholamines and locally released N.A. it may be that attempts to alter the circulating levels of catecholamines by administration of exogenous sources is a futile method of studying their normal metabolic effects in adipose tissue in vivo. Put another way, administration of catecholamines is carried out to alter plasma catecholamine levels that are difficult to measure and which have uncertain

effects on adipose tissue metabolism relative to locally released N.A. It may be that physiological circulating catecholamine levels are involved in the "fight and flight" emergency response and in tissues involved in short term energy metabolism, while locally released N.A. plays a part in adipose tissue control. Young and Landsberg (163) supported the hypothesis that sympathetic innervation and locally released N.A. are important in controlling lipolysis while in muscle for example they cited a paucity of information supporting sympathetic innervation and suggested that circulating adrenaline may play an important role in that tissue. They sum up the state of knowledge (as at 1977) in the following statement. "Although substantial evidence indicates that the sympathetic nerves can stimulate lipolysis, the relative importance of locally released noradrenaline and circulating adrenaline in various physiological and pathophysiological states is not known with certainty". Fain and Shepherd (55) are a little more confident in stating that "lipolysis is directly activated by catecholamines and the sensitivity to catecholamines released at nerve endings is moderated by thyroid hormones, glucocorticoids, growth hormone, and insulin".

4.8 EXPERIMENT VII

4.8.1 Experimental Design

1) Objectives

1. To determine the "normal" diurnal pattern of some plasma metabolites and hormones in the experimental sheep (both for the information per se and as a basis for comparisons with different experimental challenges).

2. To stimulate lipolysis by imposing fasting and ascertain the resultant changes in some plasma metabolites and hormones.

3. To determine whether individual sheep differed in their responses to this lipolytic stimulus.

2) Method

Four Romney ewes were obtained from a small group of non-pregnant ewes culled from Massey sheep farms on the 4th August 1977.

The basic design was a switch back or reversal trial (27). The 4 sheep were divided into 2 groups (see Table 4.11).

TABLE 4.11: DEISGN OF EXPERIMENT VII

	Ewe No.	Period 1 Wed Thur	Period 2 Fri Sat	Period 3 Sun Mon
Group A	(57 (186	Feed	Fast	Feed
Group B	(142 (186	Fast	Feed	Fast

Sheep in Group A were fed normally for the first period of 2 days while those in Group B were fasted during this period.

Blood samples were taken on the second day only in each case. During the second period of two days Group A was fasted while Group B was fed. Again blood samples were taken only on the second day of the period i.e. day 4 of the experiment.

During the third period (days 5 & 6) Group A was fed while Group B was fasted and blood samples were taken only on day 6.

Thus period 3 was a repeat of period 1. Sheep were allocated to treatment groups at random.

The sheep were housed in metabolism crates indoors in the animal physiology unit. The experiment began in December so the sheep were well trained and acclimatised to their environment.

DEFINITION OF FASTING

The fasted sheep were not completely starved. They were provided with a small amount of hay and sheep nuts (see Table 4.12) at the same time as the control animals were fed. This was for two reasons

i) to avoid possible psychological stress of "missing out" on food while others are fed.

ii) to avoid gut stasis, ketosis and other problems associated with complete fasting.

The small amount of feed provided was not expected to provide sufficient nutrition to prevent the mobilization of adipose tissue reserves but it was hoped that this did ensure that the animals were more nearly normal than completely starved experimental creatures.

Fully fed sheep were fed 1.5 x maintenance while fasted sheep were fed only 0.25 x maintenance requirements i.e. fasted sheep were fed one sixth of their normal ration.

TABLE 4.12: WEIGHT OF FEED GIVEN TO SHEEP ($g.d.^{-1}$)

Treatment		Feed		Fast	
	Feed Type	Hay	Nuts	Hay	Nuts
	57	750	310	150*	77
Ewe	186	700	280	175	70
Nos	142	660	270	165	70
	116	780	320	192	80

* Error in calculation detected later. Amount should have been 188g.

Sheep were fed the whole amount of hay and sheepnuts at 1.30 a.m. each morning. Refusals were very small and not recorded.

Blood sampling began at 10.00 a.m. and was carried out at intervals increasing from 15 mins to 120 mins until 6.30 p.m. (or 8.10 p.m. with a 240min interval on the last day). Blood sampling was carried out as described in Section 3.4.

4.8.2 Results

Variation of plasma metabolites and hormones

1. Growth Hormone

As shown in Figure 4.7 the plasma GH levels fell sharply on 10 occasions out of 12 from a mean prefeeding value of 10.5 ± 5.3ng cm^{-3} to levels of 3.7 ± 1.4ng cm^{-3} after feeding in both fed and fasted sheep. The only exceptions were Ewe 142 which peaked about 30 mins

FED 57 & 186
FASTED 116 & 142

FED 116 & 142
FASTED 57 & 186

FED 57 & 186
FASTED 116 & 142

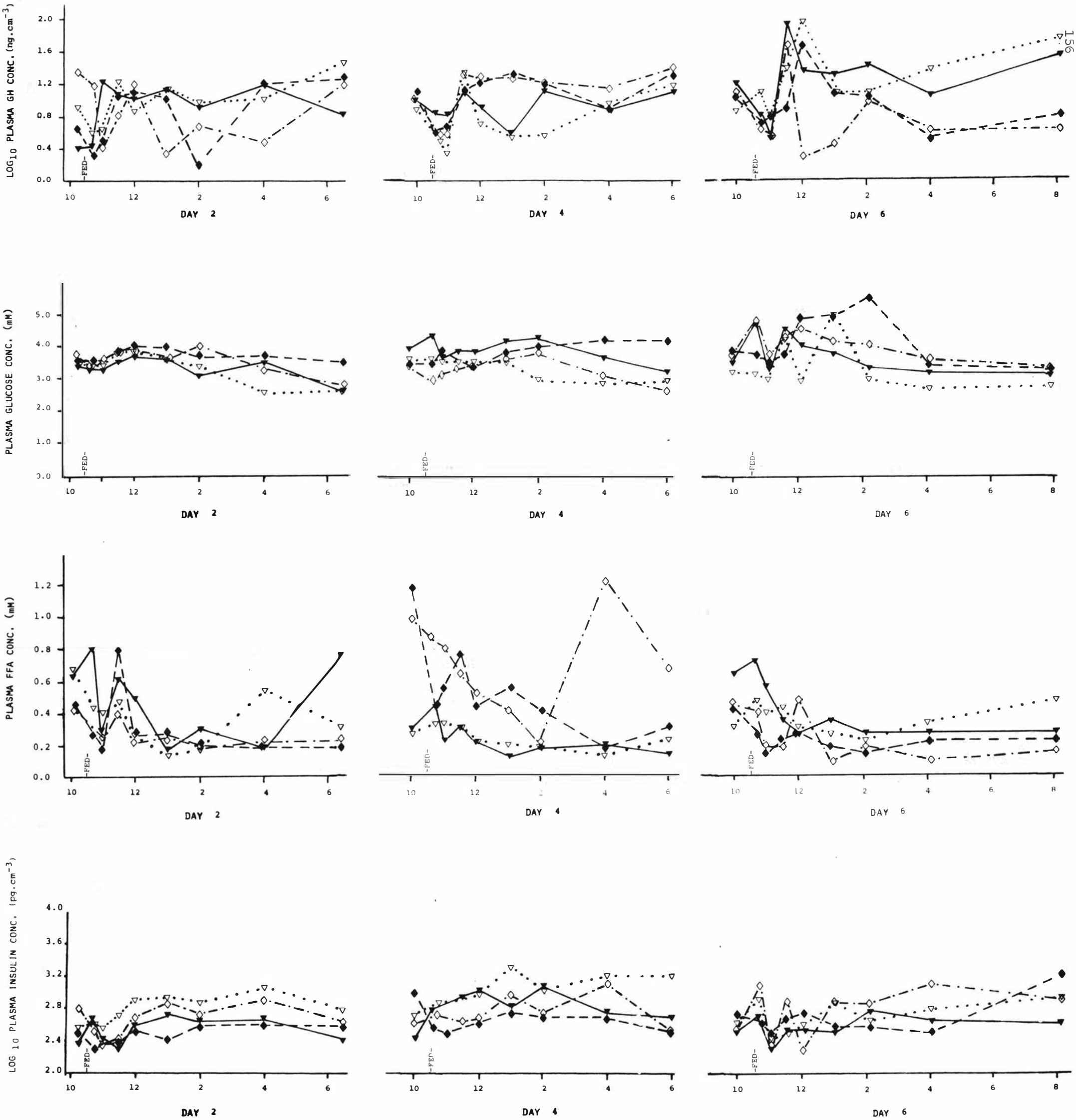

Figure 4.7 The effect of fasting on plasma GH, insulin glucose and FFA concentrations in 4 Romney ewes

Ewe 57 Ewe 116 Ewe 142 Ewe 186

after feeding on day 2 and Ewe 116 which peaked about 15 mins after feeding on day 3. In the other cases GH stayed depressed for up to 30 mins then rose rapidly to peak 1-2 hours after feeding. The mean concentration of the peaks was 33.7 ± 28.1ng cm^{-3} and the range was 12.3 - 93.4ng cm^{-3}. In period 3, in which were recorded the highest levels in all sheep, all peak samples were more than twice the size of such peaks recorded on previous days.

In fed sheep the plasma GH levels fell quickly to about prefeeding values in the next 1-2 hours. The mean of 1 p.m. samples in fed sheep was 5.9 ± 4.4ng cm^{-3}. In fasted sheep GH levels remained high (the mean value at 1 p.m. was 16.9 ± 3.9ng cm^{-3}) although they also decreased slightly in the early afternoon they then increased fairly rapidly throughout the remainder of the afternoon until the last evening sample which had a mean value of 29.7 ± 16.5ng cm^{-3}, compared to the mean in fed sheep of 12.7 ± 6.1ng cm^{-3}. The difference in mean afternoon samples between the fed and fasted sheep was tested (see appendix 4.8.2) and GH levels in the fasted ewes were found to be significantly greater at the 3% level than the GH levels in the fed sheep.

2. Insulin

Plasma insulin levels were less consistent between periods than GH levels (see Figure 4.7). Prefeeding insulin concentrations varied from 233 - 957pg cm^{-3} with a mean value of 443 ± 189pg cm^{-3}. On 8 ocassions out of 12, levels were slightly higher at the next sampling time, 15 mins after feeding. After this peak insulin decreased to approximately prefeeding levels 30 mins after feeding. During the second experimental period several values obtained 30-60 min after feeding were unusually large (in excess of the highest insulin standard of 6400pg cm^{-3}) and so were rejected.

The nadir which occurred 30-60 minutes post-feeding was followed by a general rise in insulin which often continued to increase gradually throughout the afternoon to reach highest levels as late as 4 p.m. Levels were lower at the last sample of the day, comapred to the 4 p.m. sample, in 10 of the 12 cases.

Within each period the plasma insulin levels of both treatment groups, on average, followed a remarkably similar pattern. No significant differences between treatments were detected, but during the last 2 periods the fasted group recorded lower average insulin levels at all sampling times after feeding.

3. Glucose

Prefeeding plasma glucose levels were in the range 3.40mM to 4.00mM (see Figure 4.7). In some cases higher glucose levels (4.40 - 4.80mM) were recorded at the first sampling time after feeding, but most showed a gradual decline to levels in the range of 2.98 to 3.70mM, 15-30 mins after feeding. This was generally followed by a definite gradual rise to levels of about 4mM 1.5 - 3.5 hours after feeding. In period 3 however this general rise was confused by marked fluctuations in plasma glucose levels. Throughout the rest of the sampling period plasma glucose levels declined to levels in most cases slightly below prefeeding levels.

No significant differences were detected between treatment groups (see Appendix 4.8.2). This was also the case in Experiment I.

4. Free Fatty Acids

Prefeeding plasma FFA levels were in the range of 0.27 - 1.17mM (see graphs in Figure 4.7). After feeding FFA levels were usually about half their prefeeding level 30 mins after feeding denoting a steep decline, but some sheep (especially ewe 142) exhibited an increase of 0.1 - 0.2mM preceeding the fall. Very similar observations were made in Experiment I (see Section 4.6.2). Following this nine out of the twelve cases exhibited a peak 60-90 mins after feeding which was extremely variable in size. After the peak FFA levels usually fell sharply to pre-peak levels in 30 mins after which they declined gradually over about 2 hours to about 0.2 ± 0.1mM.

Fed sheep maintained a fairly constant plasma FFA level for the remainder of each sampling period but fasted sheep were much more variable and rose to higher levels later in the day. The difference was significant at the 7% level (see Appendix 4.8.2).

4.8.3 Discussion

Although some of the data appears conclusive to the eye, the low numbers of animals used and consequent low degrees of freedom prevent greater confidence than indicated in the statistical analysis. Since this was designed as a pilot study small numbers of animals were used so that they could be studied intensively, although it was recognised that the results would need cautious interpretation.

Variation in responses by individual sheep were apparent, especially Ewe 142 which often did not follow the changes exhibited by the other ewes. The method of selection of the 4 sheep from the 6 culled ewes might be criticised. This was by necessity not choice and for this reason, although the selection of the ewes from within the cull ewe class was random, because they were cull 5 year ewes they can hardly be accurately classed or representative of normal or average ewes. Nevertheless the choice of any 4 ewes could result in bias (due to such small numbers). So the problem was recognised and results are discussed in relation to these animals with minimal extrapolation to other sheep. Since one of the objectives was to determine if sheep differed in their response to lipolytic challenges it is fortunate that Ewe 142 appears to differ, but it was not possible to determine the reasons for the difference.

The error in the amount of feed given to Ewe 57 when fasted was small (see Table 4.12) and should not have had an important influence on the results, but it may have contributed to variation between the sheep in group A during period 2.

In retrospect, the 2 day period of fasting imposed on the ewes was possibly insufficient to obtain a marked lipolytic response. A 3 day period is now considered to be the minimal time before nutrient uptake from the reticulo-rumen reaches fasting levels. Even in sheep fed once daily the flow of digesta through the tract distal to the rumen is continuous (11). However, since full fasting or starvation were not intended in the present experiment, this oversight may have been valuable in ensuring that the treatments were not too abnormal.

Feeding had a dramatic effect on plasma GH levels which fell very sharply and remained depressed for about 30 mins. This result was also observed by Bassett (9) in 1974 but in his review presented the same year (11) he cites depressions in plasma GH lasting 2-4 hours. The fall in plasma GH levels follows feeding very closely and may be associated with a very rapid large peak in plasma insulin levels (lasting only a few minutes) which was observed by Bassett (9) and which can be clearly seen in the result of Experiment IX (see Figure 4.11). Such short term changes could not be detected in Experiment VII because of the 15 minute interval between feeding and the next sample. This experiment showed an apparent rise in plasma insulin levels 15 mins after feeding, which occurred at the same time as plasma GH levels were depressed. These results are in some agreement with another report by Bassett (10) who recorded that eating resulted in a rapid increase in plasma insulin and a rapid decrease in plasma GH. In that trial Bassett sampled at 45 minute intervals and so those results cannot help explain the large short term changes seen in the present experiment. The large sustained insulin peak seen in period 2 is in line with Bassetts findings (10) and many others reviewed by Bassett (11) but in period 1 and 3 the present results show only a relatively small insulin peak followed by depressed plasma insulin levels for about an hour.

This depression in insulin may have been related to or caused by the large and consistent GH peaks which span the same period. In Experiment IX GH infusions were associated with temporarily depressed insulin levels. However the literature contains no evidence that GH depresses insulin. In fact Wallace and Bassett (157) and Davis et al (43) reported that administration of OGH increased plasma insulin levels. Thus it is likely that the GH and insulin levels were controlled by another factor(s), probably the autonomic nervous system.

The decline in plasma FFA's associated with feeding is not unexpected, especially in fed animals. With the usual resultant increase in plasma metabolites following normal feeding one might assume that body reserves need no longer be mobilized. In this case the early decrease in plasma GH is not surprising since it is generally accepted to be lipolytic in the sheep (13, 42, 43, 93, 102, 105) and cow (75, 92, 118, 119).

Similarly the rise in insulin is not unexpected (especially the very early rapid peak described in Experiment IX) since insulin is reported to be antilipolytic (3, 17, 42, 88, 102). Vernon (156) attributed the FFA decline to the rapid rise in insulin on feeding. The stimulus leading to the early insulin peak is discussed in Experiment IX, but the stimulus to the rises in insulin recorded in this experiment may be attributed to VFA production in the digestive tract. Horino et al (86) found that insulin secretion was stimulated by propionate, butyrate and valerate while glucose was much less effective. Manns and Boda (106) stimulated insulin release using butyrate but found glucose, propionate and acetate much less effective. On the other hand Bassett, Weston and Hogan (15) found that plasma insulin was positively correlated ($r = 0.74$) with the DOM and amount of crude protein digested ($r = 0.74$) but was less clearly related to VFA.

The simultaneous occurrence of large peaks in both GH and insulin 60 minutes after feeding is much harder to explain if we expect their actions to be opposed. Bassett and Wallace (13) recorded an early insulin-like antilipolytic phase of GH when injected into sheep, as did Goodman in rat adipose tissue (64, 65). This may mean that the function of GH as a lipolytic agent can be changed by some factor near feeding to take an antilipolytic role more consistent with the anabolic metabolic environment expected in a recently fed animal. The anabolic or protein sparing role of GH should be remembered in this context (43, 105) (see Section 2.4.3.6).

While most of the changes recorded in the fed sheep can be justified it is difficult to understand why fasted sheep should follow the same initial response to feeding. It may be that the responses to feeding are inherent and not aquired. That is to say that the animal can make no short term adjustment to compensate for being grossly underfed. Such compensation only became evident an hour or more after feeding when the fasted sheep appear to have maintained a higher average plasma GH concentration than the fed sheep. In his review Bassett (11) stated that fasting does not cause any consistent increase in plasma GH levels. However in this experiment the difference between GH levels in fed and fasted sheep became progressively larger throughout the later part of the sampling period. It can be argued that this is in agreement

with some of Bassetts own results (10, 11) in which sheep on higher levels of feeding recorded lower GH concentrations especially later in the day, while the completely fasted sheep maintained high average values, although with marked fluctuations. This, in conjunction with the FFA results of Experiment VII, supports the concept of a lipolytic role for GH (at this later time) since, although plasma FFA levels showed an overall decline after feeding in both fed and fasted sheep, they remained, on average, higher in the fasted sheep and as could be expected they increased significantly more quickly to higher levels later in the experimental periods. In this later period one might expect insulin levels to be lower and these data indicate they may be, but the difference was not significant. Bassett (10) recorded significantly lower levels of insulin in fasted sheep, than in fed sheep.

Variation in plasma glucose cannot be attributed directly to any one other factor. It was probably due to a combination of other effects (11, 64, 157). In his review, Bassett (11) indicated that changes in glucose play little part in determining the initial increase in insulin levels after feeding, although the eventual increase in glucose levels may play some part in the maintenance of high insulin levels. In this experiment glucose levels followed insulin more closely than they did any other recorded factor, but the largest variation occurred when GH was most variable (Period 3 see Figure 4.7).

Wallace and Bassett (157) postulated that GH may inhibit the effect of insulin on glucose uptake, but not its repressive effect on FFA release. Thus after feeding the concurrent peaks of insulin and GH would ensure repression of lipolysis as well as defence of blood glucose and amino acid levels, leaving dietary VFA's as the preferred energy and precursor source for anabolism. The later fall in insulin in fasted sheep would remove suppression of lipolysis (still stimulated by high GH levels) and further protect blood glucose and amino acid levels, leaving FFA's as the preferred energy and precursor source.

It appears then that neither FFA nor glucose are controlled directly by either GH or insulin alone. Peel, et al (119) postulated that an increased ratio of GH to insulin may stimulate lipolysis in lactating

dairy cows. Bassett et al (15) found that plasma GH was negatively correlated with plasma insulin (r = -0.71) in adult sheep on a range of diets fed about ad libitum over 3 weeks, and Bassett (10) recorded negative correlations between insulin and GH in sheep fed at different levels. The relationship between $\log_{10}$ GH and $\log_{10}$ insulin in Experiment VII was examined by regression analysis and analysis of covariance, but no significant relationship was found (see Appendix 4.8.3). Examination of the ratio of $\log_{10}$ GH to $\log_{10}$ insulin for late afternoon samples revealed a consistent marked increase in the ratio for both fed and fasted animals. However it was felt that such a finding was not consistent with the absolute hormone levels in the fed sheep, and that the ratios calculated had little or no biological significance. Due to the inherent problems of using and interpreting ratios this line of inquiry was discontinued. Examples of the ratios calculated can be seen in Appendix 4.8.3.

4.9 CALORIMETRY 3

4.9.1 Experimental Design

1) Objectives

In Experiments V and VI the effects of ACTH and N.A. on blood parameters were investigated. It was hoped to obtain information on the effects of these hormones on the metabolic rate and RQ of the sheep, but it was found to be impractical to obtain blood samples from the sheep while they were in the calorimetry apparatus. Also the calorimeters were required for other experiments at the same time as Experiments V and VI, so it was decided to carry out the calorimetry studies at a later time independent of blood sampling.

It was expected that the metabolic rates of the sheep would be altered by the administration of the hormones. It was hoped to determine the magnitude of any such changes in metabolic rate and to determine whether the sheep responded differently to either hormone. In addition it was hoped that any lipolytic responses would be reflected in a decrease in RQ.

Figure 4.8 A ewe in the ventilated hood of the open-circuit calorimetry system used in Calorimetry Experiment 3.

Note that the lid has been removed from the chamber to allow better lighting for the photograph. Normally the only entry for air was under the leather flap at bottom front then up through the floor under the feed. Air from the hood left via the green hose on the left and travelled to the gas meters and analysers.

The objectives of this experiment then were to investigate the effects of 1) ACTH

2) N.A.

on both a) heat production (metabolic rate)

and b) RQ

2) Method

The same four Romney ewes used in Experiments V and VI were used in this experiment. They were allowed a month to recover from the previous experiments during which time all were fed their normal ration of 1 x M (maintenance requirement) as hay plus 0.5 x M as sheep nuts.

Due to the unavailability of the large calorimetry chambers used in the previous calorimetry trials, and for convenience of use, oxygen consumption and carbon dioxide production were measured in a ventilated hood in an open-circuit system. This can be seen in the photograph of Figure 4.8 and has been described elsewhere by Holmes (82). The chamber had advantages in that only a relatively short time was required for re-equilibration of the air inside the chamber after opening for feeding and in addition the hormones could be injected into part of the sheep outside the chamber thereby avoiding opening the chamber and disturbing the air composition. Thus it was thought that any short term responses to the hormones could be detected.

Air was drawn through the hood at a controlled rate, between 46 and 52 litre min^{-1} which ensured that the exhaust air contained O_2 and CO_2 concentrations within the operating range of the gas analysers. The flow rate of exhaust air was measured by dry gas meters and gas volumes were converted to STP dry. Small samples could be taken from fresh air in the room and from exhaust gases leaving the hood; these samples were drawn through silica gel and through an automatic CO_2 analyser and an automatic oxygen analyser in series. The output of each analyser was connected to one channel of a two channel recorder. Both gas analysers and the recorder were calibrated daily by pumping through them two compressed gas mixtures of known composition.

The recorded traces were used to calculate the difference in O_2 and CO_2 content between the ingoing and outgoing gases over 5 min intervals. These values were multiplied by the corrected ventilation rate to obtain the O_2 consumption and CO_2 production for each of the 5 min intervals. More detailed information on the gas analysis equipment and method has been published elsewhere by Holmes (83) and Holmes and McLean (84).

Heat production was calculated using the formula:

$$HP = \frac{O_2 \times 4.90 \times 60}{BW}$$

Where HP = heat production (K cal kg^{-1} hr^{-1})
O_2 = O_2 consumed (l min^{-1})
BW = bodyweight (kg)

RQ was calculated from the formula:

$$RQ = \frac{CO_2}{O_2}$$

where RQ = respiratory quotient
CO_2 = CO_2 produced (l min^{-1})
O_2 = O_2 consumed (l min^{-1})

Both formulae supplied by C.W. Holmes (pers. comm. 1977).

Prior to starting the experiment all four ewes were placed in the hood (at different times) for 2-3 days to accustom them to the equipment (The chamber was not sealed and no measurements were taken during this training).

The experiments were carried out in November-December 1977 and the average daily ambient temperature (indoors) varied between 18.5-22.5°C. Each sheep was placed in the hood for at least a day before commencing measurements to allow the ewe to become reaccustomed to it. Each sheep remained in the hood for at least four days.

TABLE 4.13: DESIGN OF CALORIMETRY EXPERIMENT 3

	Day 0	Day 1	Day 2	Day 3
TREATMENT FOR EACH EWE	Put in hood to settle down	Control	N.A. Injection	ACTH Injection

On the next day recordings were taken to determine the normal changes in heat production and RQ and to observe any effects of control injections. Days 2 and 3 were devoted to studying the effects of N.A. and ACTH injections respectively (see Table 4.13).

Recording generally began between 9 and 10 a.m. following the calibration of equipment. The recording traces were observed until a stable output was achieved. A minimum of 15 mins of stable output was recorded before opening the chamber and giving each ewe her normal rations. 15 min later the appropriate injections were administered subcutaneously in the neck or shoulder region. Recordings were continued for about 90 mins after the injections. Observation of the response to injection sometimes led to a change in dose and/or additional injections later in the day. A full summary of the injections given can be seen in Table 4.14.

TABLE 4.14: TREATMENTS AND DOSES APPLIED TO EACH EWE IN CALORIMETRY EXPERIMENT 3

Ewe No	Day 1	Day 2	Day 3
186		N.A. 2mg N.A. 2mg	30iu ACTH
142		N.A. 2mg N.A. 2mg Chlorbutol 1cm^3 N.A. 4mg	40iu ACTH
116	Chlorbutol	N.A. 2mg N.A. 2mg	40iu ACTH
57	Chlorbutol	N.A. 2mg	40iu ACTH

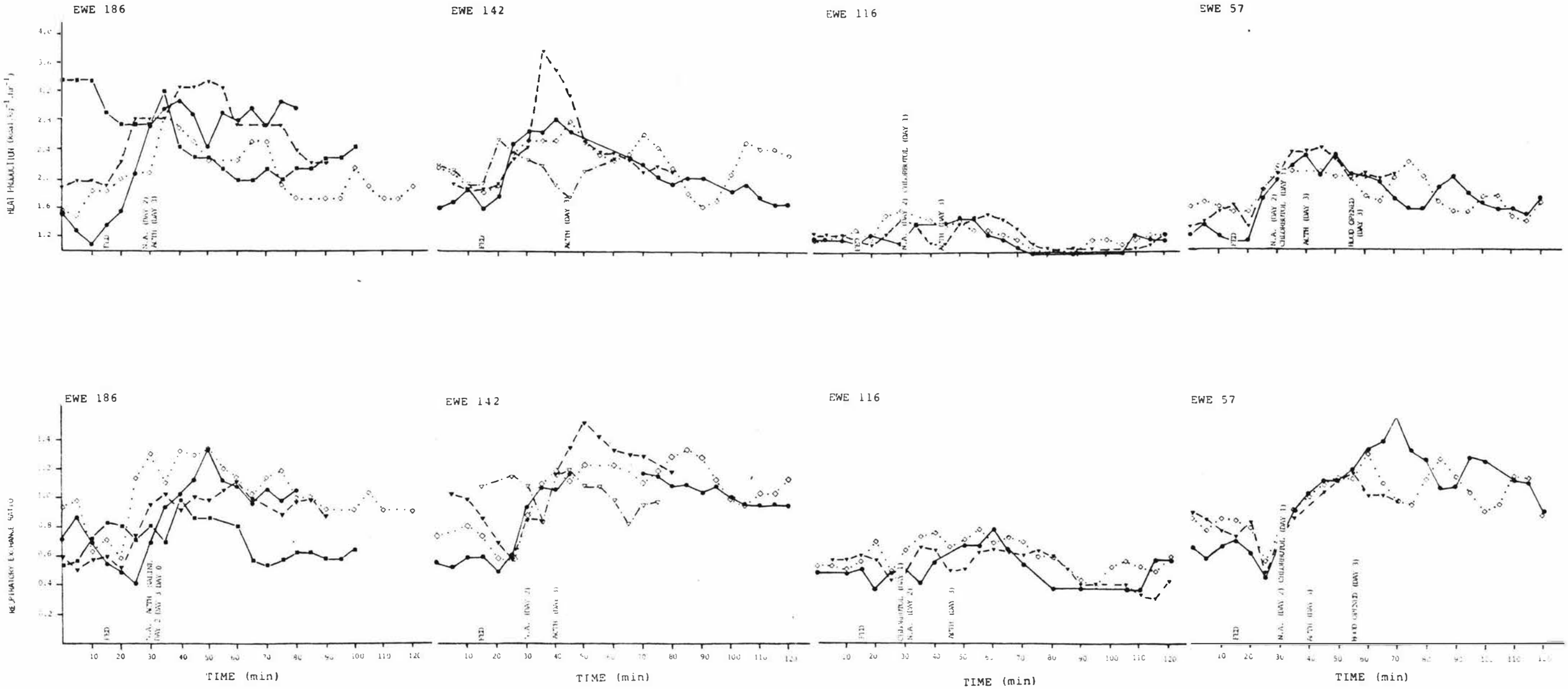

Figure 4.9 Heat production and repiratory exchange ratio (R) in four Romney ewes showing responses to injections of noradrenaline , ACTH and control solutions.

Note the changes associated with feeding.

Day 0 Day 1 Day 2 Day 2 (p.m.) Day 3

The preparation of N.A. used was "Bronkephrine" which is described fully in Experiment VI (Section 4.7). As in Experiment IV chlorbutol 0.25% was used as a control to the N.A. The preparation of ACTH was the same as that used in Experiment V (Section 4.6). Since the ACTH was dissolved in isotonic saline solution it would have been appropriate to use saline injections as a control to the ACTH injections. However to avoid having two control injections the chlorbutol injection on Day 1 was considered to be the control for both N.A. and ACTH. The chlorbutol solution used was 0.25% w/v in isotonic saline. Ewe 186 in fact received isotonic saline as a control injection.

4.9.2 Results

Heat production and RQ versus time for each sheep are graphed in Figure 4.9. Heat production was in the range 1.0-2.2 Kcal/kg/hr prior to feeding. Following feeding the heat production increased in all sheep to peak about 30 mins post feeding. The size of this response differed between sheep, but was similar for the same sheep on different days. This is best seen in the graphs of Ewe 186 and 116 which show responses quite different in size. Ewe 116 exhibited a highest peak of 1.6 Kcal/kg/hr while Ewe 186 exceeded 2.8 Kcal/kg/hr on all days tested. Heat production returned to prefeeding levels 60-90 mins after feeding in all cases in which recordings were continued long enough to observe this.

Prefeeding RQ's were in the range 0.52-1.02. Following feeding in all cases RQ decreased to a nadir within 5-10 mins. In all sheep except Ewe 116 this was followed by a rapid rise to peak values in the range 1-1.6, 15-60 minutes after feeding. RQ values then declined, but were still about 1.0 ± 0.2 at the end of the recorded period. Ewe 116 showed the same pattern of changes but varied within a much smaller range of values (0.3-0.8).

No changes in either heat production or RQ could be attributed to the actions of the hormones. Ewes 57, 142 and 186 recorded their highest heat productions following N.A. injection, but showed such large variation within each day that differences between days would not be significant. Ewe 142 showed a very large RQ peak in excess of 1.5 following

N.A. injection but Ewe 57 produced a similar value following the control chlorbutol injection. It should be noted that 2 days of control data are presented for Ewe 186, i.e. day 0 and day 1. On day 0 a saline control injection was administered 15 minutes after feeding and an immediate increase in oxygen consumption was observed. This can be seen in the graph of heat production. However the ventilation rate was considered too high (881 min^{-1}) on that day resulting in very small percentage changes in CO_2 levels which were too different from the standard gases used and had to be estimated by extrapolation of the standard curve. So it was intended to discard the data for that day (day 0) and repeat the measurements with a ventilation rate of about 501 min^{-1} on the next day (day 1). Unfortunately the control injection was omitted on day 1, so day 0 data is presented to show the extent of the effect of the saline injection on heat production.

It is evident from a cursory examination of the graphs that any responses to hormone injections would have been confused with the large changes following feeding. In addition, the unexpected range of RQ values (0.37-1.58) raised questions about the validity of the experimental technique (discussed later), so no attempts were made to analyse the RQ data statistically. Nevertheless, the heat production data was considered usable and polynomials were fitted to the heat production curves of each ewe on each day (using Genstat on the Massey Prime computer).

The polynomial equation was of the form

$$Y = t_1x + t_2x^2 + t_3x^3 + t_4x^4 + C$$

The regression coefficients and constants derived for each curve are listed in Table 4.15. In 7 of the 11 cases the fitted curves accounted for more than 70% of the variance e.g. the polynomial fitted to the data for Ewe 57 day 1 accounted for 94% of the variance (see Table 4.16).

TABLE 4.15: REGRESSION COEFFICIENTS OF POLYNOMIAL CURVES OF THE FORM $Y = tx + t_2x^2 + t_3x^3 + t_4x^4 + C$ FITTED TO HEAT PRODUCTION DATA OF 4 EWES

	Day 1	Day 2	Day 3
	Ewe 57		
Constant	0.9808	2.0676	1.3652
T	-0.0540	-0.8687	0.0529
T_2	0.1429	0.3833	0.0614
T_3	-0.0215	-0.0507	-0.0110
T_4	0.0009	0.0021	0.0005
	Ewe 116		
Constant	1.1888	0.7076	1.1133
T	-0.0651	0.5231	0.1204
T_2	0.0249	-0.1577	0.0040
T_3	-0.0013	0.0185	-0.0035
T_4	-0.0001	-0.0007	0.0002
	Ewe 142		
Constant		2.6579	1.7045
T	Incomplete	-1.4593	-0.0273
T_2	Data	0.7032	0.1211
T_3		-0.0987	-0.0207
T_4		0.0042	0.0009
	Ewe 186		
Constant	1.1367	1.3721	2.2900
T	-0.0741	0.5661	-0.7541
T_2	0.2459	-0.0654	0.3626
T_3	-0.0425	0.0049	-0.0516
T_4	0.0020	-0.0002	0.0023

TABLE 4.16: COMPARISON OF HEAT PRODUCTION VALUES OBSERVED IN EWE 57 (DAY 1) WITH THOSE PREDICTED BY THE FITTED POLYNOMIAL

Time after feeding (min)	Time Interval	Observed	Fitted	Residual
0	1	1.11	1.049	0.061
1	2	1.11	1.286	-0.176
10	3	1.71	1.594	0.116
15	4	1.93	1.898	0.032
20	5	2.15	2.143	0.007
25	6	2.30	2.293	0.007
30	7	2.23	2.337	-0.107
35	8	2.30	2.283	0.017
40	9	2.23	2.161	0.069
45	10	2.00	2.021	-0.021
50	11	1.93	1.936	-0.006

In order to determine whether the heat production curves differed between treatments or between sheep the regression coefficients (and constants) were analysed in a two-way analysis of variance. The analysis of the regression coefficient t_1 is presented in the Appendix 4.9.2 as an example. No significant difference was found between any of the coefficients and so it is assumed that there were no significant differences in heat production between sheep or between treatments.

4.9.3 Discussion

Three points of major interest can be noted in the results:

a. the apparent lack of treatment effects
b. the trough in RQ levels following feeding
c. the unexpected range of RQ values obtained.

These shall be discussed in turn.

There are 2 possible reasons for the apparent lack of treatment effects; either none occurred or they were not detected by the techniques used.

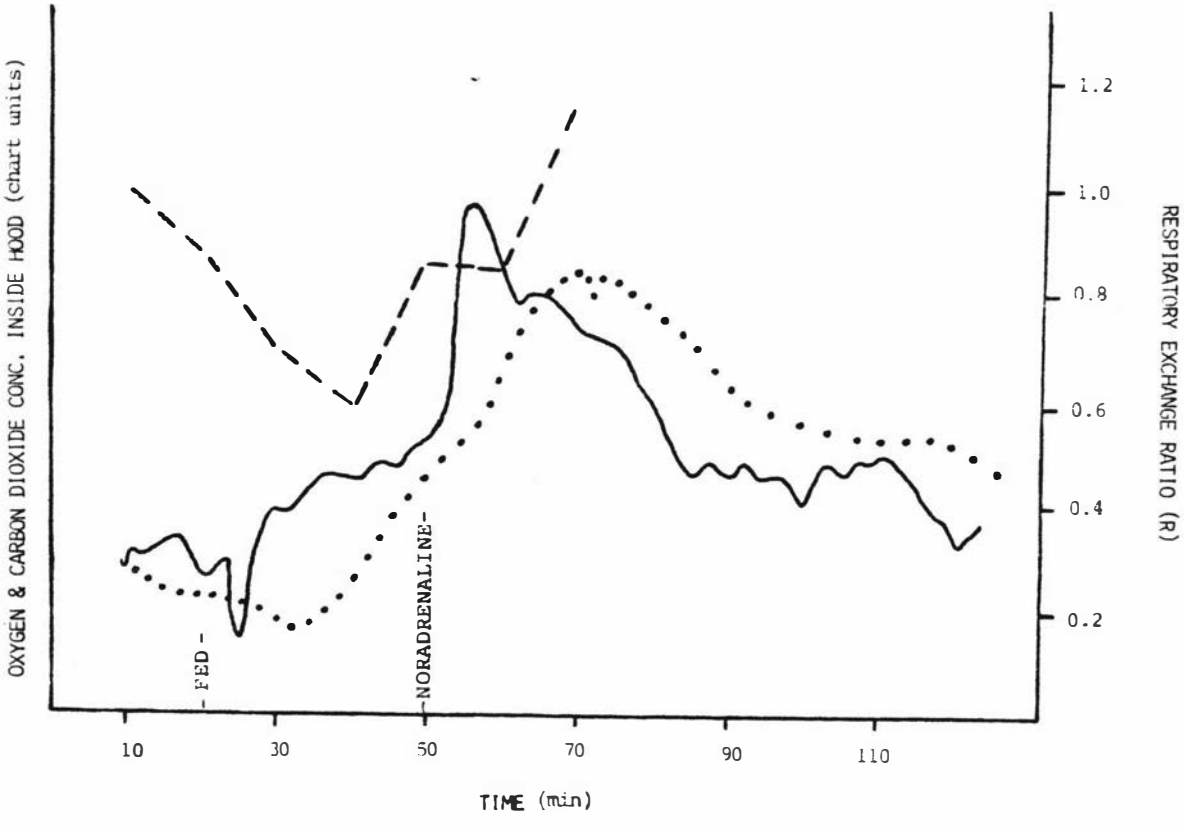

Figure 4.10 The interrelationship between between O_2 production , CO_2 production and respiratory exchange ratio (R) in a Romney ewe , showing a typical response to feeding and a response to **N.A.** injection.

------- Respiratory exchange ratio (R)
..... Carbon dioxide levels
——— Oxygen levels.

The former reason, the absence of a treatment response, can only lead to speculation with no conclusion, however it is possible to make some conclusions regarding the validity of the techniques used. In retrospect it is evident that the time of injection, in the immediate post-feeding period, was poorly chosen. The reasons for this are discussed more fully in Experiment VI. The length of time for which recordings were taken might be considered to be too short. While one might expect effects of N.A. to be rapid in onset, the response to ACTH appeared to be considerably delayed (in light of the conclusion in Experiment V) and would not have been recorded in this experiment. Nevertheless it is unlikely that such considerations are of much importance because it is now considered that the technique of indirect calorimetry itself was not a suitable method with which to study the short term effects of the hormones on lipolysis. The reaons for this will become clear later in this discussion.

In the absence of apparent responses to the hormones, the response to feeding became a point of major interest, especially the decline in RQ following the beginning of feeding. Initially it was thought that this was entirely due to the disturbances of gas equilibrium in the chamber when the lid was opened to put in the feed, however it was noted that the timescale of this effect did not coincide with the time apparently taken for re-equilibration of the Chamber. The trough in RQ lasted a timespan of about 20 mins while it can be seen in Figure 4.10 that re-equilibration of oxygen levels in the chamber took only slightly longer than 2 mins, and CO_2 levels were not noticeably altered. A further possibility arose from the suggestion that the CO_2 gas was delayed in the silica gel during drying and that it was subsequently analysed and recorded about 3 minutes after the corresponding oxygen sample (C.W. Holmes pers. comm. 1978). If this were so the RQ ratio could have been altered for a longer period that that apparently required for oxygen re-equilibration. In such a case the error should be removed by calculating the ratio using CO_2 levels recorded about 3 minutes after any given oxygen level. To check this, RQ's were calculated using the average oxygen concentration for each five minute period and the average CO_2 concentration for the following 5 minute period: the effect still remained, although the individual RQ values were slightly altered. It appeared then, to be a real effect and the decline

in RQ was attributed to a shift from carbohydrate and VFA metabolism to the oxidation of long chain fatty acids. The evident reason for such a response to feeding was thought to be the sudden increased requirement for energy for digestive work in the alimentary canal. It was postulated that the initial intake of feed would require energy for digestion in excess of that being produced by absorption and that this was provided by oxidation of long chain FFA reflected in the decreasing RQ. Once digestion provided sufficient energy for its own needs then lipolysis was no longer necessary and indeed when digestion produced energy precursors in excess of the requirements of digestive work then lipogenesis began. These changes from lipolysis to lipogenesis would explain the recorded increase in RQ to values up to 1.

It was not possible however, to explain the values of recorded RQ which were below 0.7 or in excess of 1.0 in terms of carbohydrate or lipid metabolism. These levels would seem to be physiologically impossible results for the oxidation of lipid or carbohydrates or proteins (24). The accuracy and precision of the calorimetry equipment was not in doubt and it was concluded that the results obtained were real physiological changes, but which did not reflect changes in the balance of carbohydrate, lipid and protein (alone).

It was thus necessary to invoke other physiological mechanisms to explain these data.

In order to produce an RQ > 1 the sheep must have been releasing CO_2 at a faster rate than they were consuming oxygen. This is known to occur during respiratory compensation to metabolic acidosis and during respiratory alkalosis (41). Egan (49) warned that CO_2 washed out of the body CO_2 pool may constitute a bias in short term measurements. However it would seem that it is unlikely that sufficient CO_2 would be ventilated from the ECF to produce the results observed (see Appendix 4.9.3) under normal conditions. The source of most of the extra CO_2 is undoubtedly from rumen fermentation (37, 49). CO_2 forms about 60% of the gas present in the rumen but may vary from 20-65% in cattle fed once daily (120). The production of 2l of CO_2 in the hour after feeding is quite possible and indeed likely. This provides an explan-

ation of the high RQ values.

In order to produce an RQ < 0.7 the sheep must have been releasing CO_2 at a slower rate than they were consuming oxygen. To do this they must have been sequestering CO_2 within their bodies; most likely in the form of bicarbonate. This is known to occur during respiratory acidosis and metabolic alkalosis (41).

Large amounts of VFA, mainly acetic acid, are produced in the rumen during normal microbial fermentation (120, 153) and the rumen fluid is well buffered against acid within the usual pH range of the rumen, by HCO_3^- and phosphate (153). The absorption of these acids into the circulation is buffered by the normal blood buffering systems of which the carbonic acid-bicarbonate system is an important component. Thus, a source of HCO_3^- is required, and in sheep fed once-daily, as in the present experiment, a large amount will be required at the time of feeding compared to grazing or continuously fed sheep. In the fasting rumen, the concentration of HCO_3^- is high, tending towards the levels in saliva (153). Turner and Hodgetts (153) recorded rumen fluid HCO_3^- concentrations of $50mEql^{-1}$ and $32mEql^{-1}$ after 16 and 18 hours fasting respectively. (The sheep had previously been fed differing diets). In fed sheep they recorded $4mEql^{-1}$ of rumen fluid. Before feeding the urine of sheep was markedly alkaline (106, 138, 142) and HCO_3^- urinary excretion rates were recorded by Simpson (142) in the range of 4-5 mEq per 30 min prior to feeding in sheep fed once daily. The onset of feeding results in a decrease in rumen pH (37, 121, 153) rumen [HCO_3^-] (153), urine pH and HCO_3^- excretion rate (106, 138, 142) total blood CO_2 content (142) and in blood pH (37, 103, 138, 142). All these changes can be attributed to the acidification of the rumen fluid and the consequent need for HCO_3^- to buffer changes in rumen pH. The buffering action involves the neutralization of fatty acids by the formation of CO_2 and H_2O, from HCO_3^- and a proton (120)

$$HCO_3^- + H^+ \rightleftharpoons CO_2 + H_2O$$

HCO_3^- is contributed to the rumen by a high rate of salivary flow during feeding (106, 138, 142), through diffusion across the rumen wall from the plasma (142), and by the synthesis of HCO_3^- during the transfer

of acetic acid across the rumen epithelium into the plasma (120, 121). The high salivary flow during feeding by lowering plasma HCO_3^- concentration initiates a post-prandial acidaemia (106, 138, 142) to which the kidneys respond by retaining HCO_3^- and lowering urine pH (138). (Thus ruminants tend to produce acid urine following feeding unlike "the alkaline-tide" seen in most non-suminants (106, 138)).

The upshot of all this is that sheep appear to sequester CO_2 as bicarbonate prior to feeding to cope with the impending acidaemia. The uncompensated alkalaemia prior to feeding could explain how low RQ values (< 0.7) as CO_2 is retained for HCO_3^- production. In fact CO_2 production in the present experiment continued to decline for about 5 minutes after feeding had begun (see Figure 4.10) even though O_2 consumption increased immediately. This probably occurred because CO_2 continued to be sequestered until organic acid production began in the rumen. This provides an explanation for the decline in RQ following feeding. Presumably rumen CO_2 production and respiratory compensatory CO_2 losses then increased resulting in the rapid rise in CO_2 production seen in Figure 4.10. Ten minutes after feeding, CO_2 production was increasing faster than O_2 consumption, resulting in the upward change in RQ.

It would seem then that all the changes in RQ observed can be explained in terms of the mechanisms of acid-base homeostasis. It is abundantly clear that RQ values obtained using short term measures of total CO_2 production are meaningless. In fact it is a misnomer to call these values respiratory quotients (RQ). In fact they should be termed respiratory exchange ratios R. Only under steady state conditions does R = RQ (149). It is unfortunte that the large influence of microbially produced CO_2 was not forseen as it is possible to correct the RQ by partitioning expired CO_2 into fermentation and metabolic components. This partition has been based upon the ratio of CO_2 to methane produced by in vitro fermentation, and on the ratio of these gases found by analysis of rumen gas (41). Thus measurement of methane would have enabled correction of the RQ values. Nevertheless, the time around feeding is not a steady state period and the metabolic homeostatic mechanisms already described would surely invalidate interpretation of RQ values in terms of cellular metabolism.

While fermentation in the rumen does produce CO_2, it apparently does not use oxygen (41). It would therefore appear that the values of heat production in the present experiment, derived from oxygen consumption are a valid indicator of metabolic rate.

The data shown in graphs of heat production versus time in Figure 4.9 predominantly show the response to feeding. The increased heat production following feeding is a reflection of the specific dynamic effect (SDE) (24) of the food, otherwise called the specific dynamic action (SDA), thermogenic effect, calorigenic effect or heat increment (41, 49). This has been adequately described by others (24, 41, 49) and will not be discussed further.

The hormones administered showed no apparent effect on heat production. Although the injection of N.A. to Ewe 142 (see Figures 4.9 and 4.10, day 2 (a.m.)) was followed by a rapid increase in heat production this cannot be directly attributed to the action of the hormones alone as a control chlorbutol injection (on the same day) produced an increase in heat production also. Furthermore an injection of twice the dose of N.A. (4mg) later in the day produced an effect no larger than the control injection. In addition a similar response was recorded to a saline injection in Ewe 186 on day 0.

In conclusion it is evident that the experimental design was not suited to all the objectives of this experiment. In particular the RQ measurements cannot be used in the way intended, i.e. to show changes in lipid metabolism. Nevertheless, since the heat production estimates were apparently a viable measure of metabolic rate, it would seem fair to say that N.A. and ACTH do not cause short term (< 100 minute) changes in metabolic rate which are different to those resulting from control injections.

4.10 EXPERIMENT VIII

4.10.1 Experimental Design

OBJECTIVES

1. To stimulate lipolysis in fed sheep using BGH
2. To determine the effcts of BGH on plasma metabolite and hormone

levels in fed sheep

3. To identify short term hormone and plasma metabolite changes in response to feeding
4. To determine the half life of GH in fed sheep

METHOD

The objective of this experiment and the experimental design were essentially the same as those of Experiment IX (described later in this chapter) with the exception that injections of N.A. were added to the design of Experiment IX. Ewes 142 and 186 were used in this experiment (VIII) which went without a hitch. Unfortunately it was later discovered that Ewe 186 was pregnant at the time of the experiment.

4.10.2 Discussion

Since one ewe (Ewe 186) was pregnent during the study and the other (Ewe 142) was not it was deemed to be an unsatisfactory experiment due to the additional variables which may have been present due to hormones associated with pregnancy. Hence the results of Experiment VIII are not included in this thesis. The basic procedure was repeated using a replacement ewe (Ewe 86) and was designated Experiment IX described below.

4.11 EXPERIMENT IX

4.11.1 Experimental Design

1) Objectives

1. To stimulate lipolysis in fed sheep using BGH and noradrenaline (NA)
2. To determine the effects of exogenous GH and NA on plasma metabolites and hormone levels in fed sheep
3. To identify short term hormone and plasma metabolite changes

in response to feeding

4. To determine the half life of GH in fed sheep.

The attempts to stimulate lipolysis in this study had thus far been unsuccessful. GH has often been reported to be lipolytic in the sheep (13, 42, 43, 102, 105) and it was felt that infusions of GH offered a good chance of stimulating lipolysis in the sheep. When calculating the size of GH dose to be infused a record of the half-life of GH in sheep could not be found. It was discovered after the completion of the experiment that Wallace and Bassett (158) had reported that the half-life of GH in adult sheep was 7-8 minutes. Hence an additional objective of this trial was to determine the half life of infused GH in sheep. Success in stimulating lipolysis might lead to differing responses to GH in different sheep and this in turn might have enabled study of the original objective regarding the metabolic differences between fat and lean sheep.

The calorimetry data from calorimetry Experiment 3 indicated the need for more detailed information regarding the events immediately following feeding and it was decided to sample more intensively at this time.

2) Method

Two non-pregnant Romney ewes were obtained from Massey farms. One was obtained on 4th August 1977 (Ewe 142) and was used in Experiments V, VI, VII and VIII described elsewhere. The other, Ewe 86 was obtained on 7th August, 1978.

The sheep were housed in metabolism crates in the animal physiology unit. The new ewe settled in very quickly and both were healthy and friendly when the experiment began on August 21st 1978.

For 2 weeks prior to, and throughout the experimental period, both sheep were fed a ration of hay and sheep nuts equivalent to twice their maintenance requirements. About half the ration was eaten in the first hour and the remainder throughout the day

and night. They were fed the whole amount at 9.00 a.m. each morning. Refusals were small and were not recorded.

The basic design was a switchback or reversal trial (27).

TABLE 4.17: DESIGN OF EXPERIMENT IX

Ewe No	Day 1	Day 2	Day 3	
142	GH	*PBS Control	9.30	NA
			10.30	Chlorbutol control
86	PBS Control	GH	9.30	Chlorbutol
			10.30	Control

* PBS Phosphate buffered saline

The experiment was conducted in two parts: Days 1 and 2 were devoted to the GH infusions and Day 3 to NA. The infusions were not begun until 2½ hours after feeding so as not to compromise the study of the metabolic responses to feeding.

On day 1 ewe 142 received GH infusion while 86 received a control infusion of PBS. On day 2 the treatments were reversed. On day 3 at 9.30 a.m. 2cm³ NA (for specifications see Experiment VI) was injected subcutaneously into Ewe 142 and the control solution (see also Experiment VI) into Ewe 86. At 10.30 a.m. the treatments were administered in reverse. In this experiment twice the amount of both solutions was administered compared to experiment VI as the earlier 1cm³ dose was apparently safe and the objective of this experiment was to achieve a larger and clearer response than that previously obtained.

Both sheep were cannulated the day prior to the beginning of the experiment. They were anaesthetized with a short acting general anaesthetic (Nembutal) and cannulae inserted in both jugular veins of both sheep.

Both sheep recovered about 30 minutes after commencement of the operation.

Blood sampling began at 8.55 a.m. on day one and continued around the clock at varying intervals, with 24 samples being collected per sheep on each of the first two days. To investigate rapid changes in response to feeding blood samples were taken 5 minutes prior to feeding and at 5 minute intervals after feeding for 15 minutes. The frequency of sampling was then gradually decreased to 2 hour intervals. To investigate the half life of GH blood samples were collected at 20 minute intervals for 2 hours after the cessation of infusions . Blood samples were collected as described in Section 3.4.

Infusions began at 11.30 a.m. on days 1 and 2. The aim in the GH infusion was to maintain the plasma GH concentration at about 60ng cm^{-3}. Assuming the half life of GH in ewes to be about 20 minutes based upon work with goats (D.S. Flux, personal communication 1977) (to be confirmed in this experiment) it was calculated that this would require an infusion rate of 180ng GH cm^{-3} plasma hr^{-1}. Assuming a blood volume of about 2.8l the required infusion rate was 4.8×10^{5} ng hr^{-1}. The GH solution was 5×10^{6}ng $100cm^{-3}$ in phosphate buffered saline (PBS), so the required infusion rate was $9.6cm^{3}$ hr^{-1}.

It was decided to attempt to infuse $10cm^{3}$ hr^{-1}. Infusions were made using LKB peristaltic pumps. It was found that these were impossible to calibrate accurately if the tubing was removed from and replaced in the pumps at any stage. Since the pumps were used elsewhere just prior to this experiment it was impossible to calibrate them in the time available and so the actual infusion rate achieved was $14.2cm^{3}/hr$.

4.11.2 Results

Variation of Plasma Metabolites and Hormones

1) Growth Hormone

As shown in graphs in Figure 4.11 plasma GH levels in Ewe 86 responded to feeding in the same manner as most ewes in experiment

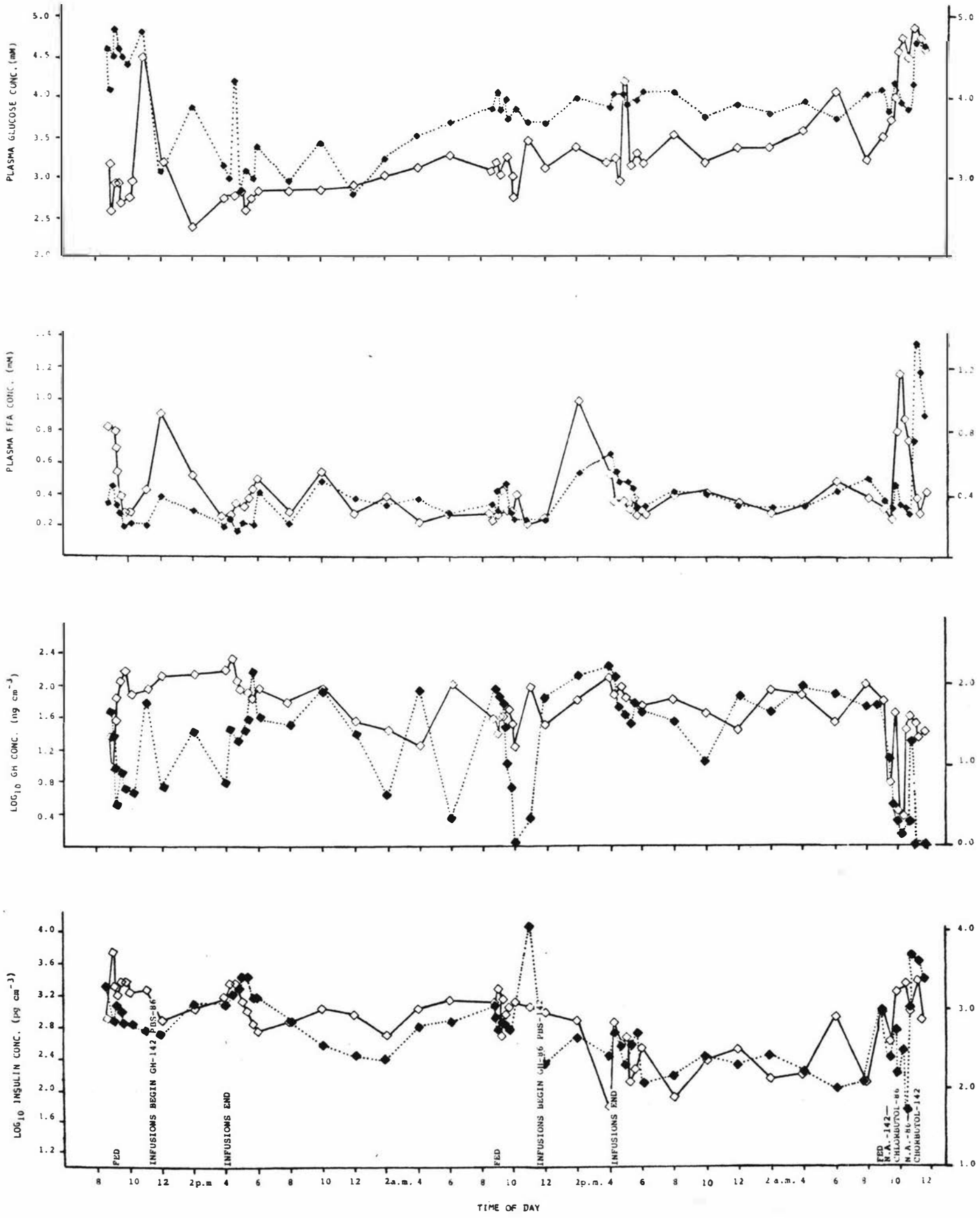

Figure 4.11 The effect of BGH and N.A. on plasma GH , insulin , FFA and glucose in 2 Romney ewes.

BGH or PBS were infused on the first 2 days for 4.5 hrs. beginning at 11.30 a.m. N.A. or chlorbutol (control) were injected after feeding on the third day.

◆···◆ Ewe 86

◇——◇ Ewe 142

VII (see Section 4.8). GH levels fell from prefeeding values of 42, 82 and 59ng cm^{-3} on days 1, 2 and 3 respectively to less than 3ng cm^{-3} and remained depressed for about an hour. Ewe 142 however exhibited a differing response as she did in experiment VII. Her GH levels fell only slightly 5 mins after feeding then rose to peak 30-45 mins after feeding. Thus GH levels in 142 were already high (about 86ng cm^{-3}) before the GH infusion began and so plasma GH levels were 128ng cm^{-3} half an hour after infusing began. They stayed above 128ng cm^{-3} until the infusion was terminated when they declined rapidly over a period of 80 mins (see Figure 4.11). Because the first GH concentration determined 20 min after the end of the infusion was still considerably higher than the highest standard dose of 128ng cm^{-3}, that value could not be used in calculating the half life of GH in this sheep. The first value that could be used was 105ng cm^{-3} obtained from the sample taken 40 mins after the infusion terminated. GH levels had not fallen to half that level an hour later when they increased once more. From this it can be estimated that the half life of GH in Ewe 142 is more than one hour and probably about 70 mins, assuming that no endogenous GH was secreted during that period.

GH levels in Ewe 86 during the control PBS infusion were considerably lower and were recorded at levels of 5, 26 and 6ng cm^{-3}. After the cessation of the PBS infusion GH levels climbed steadily to peak 100 mins later at a very high level in excess of 128ng cm^{-3}. This was concomitant with the rise in insulin concentrations. GH levels returned rapidly to about 30ng cm^{-3} but varied between 2ng cm^{-3} and 88ng cm^{-3} throughout the night.

On the second day Ewe 86 was the subject of GH infusion and her response was much the same as that of Ewe 142, except that following feeding her GH levels were very low (about 2ng cm^{-3}) and so increased rapidly over the first 30 mins of infusion to 71ng cm^{-3}. After 2½ hours plasma GH levels were greater than 128ng cm^{-3} and remained so until some time after infusion ceased. Twenty minutes after the end of the infusion the GH concentration was only slightly above the highest standard, and so the estimated concentration of 135ng cm^{-3} was used in determining the half life of GH in Ewe

86. As can be seen in figure 4.11 GH levels decreased rapidly and the half life of GH in Ewe 86 was estimated at between 17 and 45 minutes.

The PBS infusion in Ewe 142 also resulted in a marked increase in GH; but this occurred more slowly and was of smaller magnitude. After 2.5 hours of PBS infusing the plasma GH concentration had reached 69ng cm^{-3} and by the end of the infusion period it was about 133ng cm^{-3}. When the infusion was stopped GH levels declined rapidly over a period of 2 hours as was the case after GH infusions.

Injections of NA were closely followed in both ewes by small, transitory peaks in GH after which GH fell to very low levels. The chlorbutol control injection had the same effect in Ewe 142 but caused no apparent response in Ewe 86.

2) Insulin

Insulin levels rose rapidly to peak levels (shown in Table 4.18) at or just before feeding in Ewe 86 and in the case of Ewe 142, 5 mins after feeding, then fell in both cases to levels less than half of the peak values within 15 mins.

TABLE 4.18: EARLY INSULIN PEAK LEVELS ASSOCIATED WITH FEEDING (pg cm^{-3})

	Ewe 142	Ewe 86
Day 1	5412	1942
Day 2	1901	1279

This was followed closely by another rise to peak values of about 1200pg cm^{-3} in 3 cases and 2140pg cm^{-3} in the fourth case, with maxima occurring 15-20 mins after feeding.

Following the onset of both GH and PBS infusions, insulin levels decreased markedly to concentrations less than those recorded by each sheep at any stage previously. These nadirs are recorded in Table 4.19.

TABLE 4.19: LOWEST INSULIN LEVELS DURING BGH INFUSION PERIOD ($pg\ cm^{-3}$)

	Ewe 142	Ewe 86
Day 1	754	481
Day 2	57	210

However, insulin levels did not remain depressed throughout the infusion periods. In 3 of the 4 cases insulin concentrations increased, to values about twice as high as the respective minimum values, before the infusions were terminated. In the fourth case (Ewe 142 with PBS) insulin levels were slow to fall and did not reach their lowest point until the end of the infusion period.

In all cases the termination of infusions resulted in further rapid rises in insulin levels. As can be seen in the graphs in Figure 4.11, insulin concentrations in both sheep peaked at high levels on day 1 before falling quite rapidly to the lowest levels of the day. They remained low through most of the night, then increased gradually from 2 a.m. as feeding time approached. On day 2 when the infusions finished insulin levels rose to small peaks twice in 2 hours before settling at relatively stable low levels around 200pg cm^{-3} for the next 12 hours, with little sign of activity even as feeding time approached.

Injections of both N.A. and the control chlorbutol ellicited large brief peaks in insulin reaching about 2200pg cm^{-3} in Ewe 142. In Ewe 86 however the control injection had very little effect while N.A. injection brought about a massive peak in insulin concentration reaching 4600pg cm^{-3}.

3) Glucose

Prefeeding plasma glucose levels were quite different in the two ewes. Ewe 142 recorded about 3mM each morning prior to feeding while Ewe 86 recorded about 4mM. The responses to feeding were somewhat larger on day one than day two but nonetheless there were apparent similarities. In 3 cases out of 4 feeding was followed by a brief rise in glucose levels followed by an equally sharp fall. A second small peak occurred 15-30 mins after feeding. Following this all 4 records show a decline in glucose levels then a gradual rise and then in 3 out of 4 cases a sharp rise to a peak 2 hours after feeding (see Figure 4.11).

The start of infusions masked any further normal diurnal changes and no clearly discernable pattern of glucose levels was evident during the infusion period. The termination of PBS infusions was followed in both cases by a very large increase in glucose concentration from 3.0mM to peak at 4.2mM 40-60 minutes later. Within 20 mins levels returned to about 3mM in both sheep.

Following the GH infusion in Ewe 142 two-hourly samples throughout the night showed remarkably little variation in plasma glucose levels apart from a gradual rise over the period from 6pm to 6am. Ewe 86 however exhibited comparatively large changes in glucose concentration until midnight after which levels increased steadily until feeding time.

On day 2 the pattern was not as clear although following PBS infusion Ewe 142 exhibited much more variation in glucose levels than she did after GH infusion on day 1. On the other hand the magnitude of variation in Ewe 86 was only half as large on day 2 following GH infusion as it was on day 1 following PBS infusion.

Following N.A. and chlorbutol injections glucose levels increased in both ewes. N.A. appears to cause a response about three times larger than that following chlorbutol, however these changes cannot adequately be distinguished from the response to feeding.

4) Free Fatty Acids

Plasma FFA levels are graphed in Figure 4.11.
Prefeeding plasma FFA levels were within the range 0.29mM - 3.6mM on 5 out of 6 occasions but Ewe 142 recorded 0.8mM on day 1. The response to feeding was very similar between sheep on the same day but was quite different on each of the three days. On day one FFA levels declined over an hour and had just begun to increase in Ewe 142 after 2 hours. On day 2 levels marginally declined for 5 mins then both ewes produced small peaks after 15-30 mins and Ewe 142 peaked again at 60 mins. Levels in both sheep were just below 0.25mM 2 hours after feeding. On day 3 FFA levels had declined slightly 30 mins after feeding but the injections altered subsequent diurnal changes.

Infusions of GH and PBS in Ewe 142 were both followed by large peaks in FFA levels of 0.90mM and 0.98mM respectively but concentrations were back to normal levels before infusions ended. PBS infusion in Ewe 86 was followed by a small peak after 90 mins but GH infusion was associated with FFA levels which increased from 12 noon to peak at the end of infusion at 4 p.m.

Following the end of infusions and throughout the night until feeding the next day FFA levels in the two sheep followed a remarkably similar pattern which was somewhat different on each day; i.e. variation between sheep was small when compared to the differences between days.

N.A. injections evoked large peaks in FFA levels reaching 1.16mM in Ewe 142 and 1.34mM in Ewe 86. Chlorbutol control injections had little or no effect.

4.11.3 Discussion

As was previously observed in Experiment VII feeding caused rapid changes in GH levels. Ewe 86 followed the pattern exhibited by 3 out of 4 ewes in experiment VII and which was recorded by Bassett (19), but Ewe 142 again proved to be different. There was no apparent reason or explanation for the difference.

The more frequent sampling in this trial enabled detection of the short-lived but large early peak in insulin which was also observed by Bassett (9). In Ewe 142 this peak occurred 5 mins or sooner after feed was presented to the sheep, and in Ewe 86 it occurred before feeding, suggesting an anticipatory response. Further studies involving frequent rapid sampling in the 30 mins prior to feeding are needed to confirm this hypothesis. Bassett (9) found no such insulin peak in control sheep which were not fed but he recorded the insulin peak during active sham-feeding in a sheep fitted with an oesophageal fistula. The speed of the insulin response to feeding and indeed to possible anticipation of feeding suggests a neurally mediated response.

Bassett (11) reviewed evidence for such a reflex pathway and concluded that "there is considerable evidence for parasympathetic innervation of both α and β-cells in the pancreatic islets" and that "direct reflex stimulation of insulin and glucagon release by eating is therefore possible and may be important in initiating the hormonal responses to food in ruminant". However he did not preclude an indirect stimulation of their release by reflex release of gastrointestinal hormones.

Lindsay (99) discussed the possibility that higher centres in the CNS could be involved in the responses to feeding and cited evidence of the control of pancreatic secretion by the CNS, and also of a gluco-receptor in the CNS which can inhibit glucose output and facilitate glucose uptake by the liver.

Martin et al (108) reported that the mouse and rat ventro-lateral hypothalamus released a humoral factor which stimulated insulin secretion by isolated islets. The finding that the region of the hypothalamus in which the feeding area is located (148) also released into the circulation an islet-stimulating factor suggests a close relationship between feeding behaviour and insulin secretion (108). Such a mechanism if found in sheep could be responsible for producing the very early insulin peaks. Bassett (12) recently reviewed evidences for the GEP system (see Section 2.4.3.4) which encompasses both neural and endocrine mechanisms.

Whatever the mechanism is that is responsible for the early insulin release it almost certainly has a neural component. Since the early insulin peak is the first recorded response to feeding in plasma hormones or metabolites it may be tentatively assumed that it is the first widespread metabolic signal which evokes the metabolic responses necessary for the efficient utilization of incoming food. The subsequent early decrease in GH and FFA must be considered to occur in response to a rapid early signal such as the insulin peak and not to changes in circulating plasma metabolites or other factors depending upon digestive processes. Only once the intake of food ellicits changes within the digestive tract can such mechanisms be invoked as the mediators of metabolic responses to feeding.

The earliest changes in plasma glucose and FFA levels were probably also associated closely with the early insulin "signal". The brief early increases in glucose were paradoxical because insulin inhibits gluconeogenesis and increases peripheral uptake of glucose (11) so that a decline in glucose was to be expected at that time. Nevertheless this does not take into account the possible release of glucagon at the same time (11). No data are available on early changes in plasma levels of glucagon for reasons described by Bassett in his review (11).

The rapid early decline in FFA's is in accordance with the antilipolic role of insulin (3, 16, 17, 33, 88, 102, 155). Declining FFA levels indicate utilization exceeds lipolysis and the changes in RQ described in Calorimetry Experiment 3 may have indicated the utilization of plasma lipids for energy during the initial period of digestive work.

The initiation of eating in animals fed infrequently marks a major requirement for metabolic change. The sheep in this case must prepare to utilize the incoming food, and as the process of eating, digestion and absorption is assumed to have a high initial energy cost before it provides an energetic return, the sheep must meet this cost from plasma metabolites and body stores. Ruminants depend almost totally on gluconeogenesis for provision of glucose (11) and the gluconeogenic substrates (propionate and amino acids) are not available from the digestive tract in large amounts at this early stage and so FFA's rather

than glucose can be expected to provide the energy for the work of digestion. This hypothesis is supported by the decline in RQ at feeding described in Calorimetry Experiment 3. Consequently plasma FFA levels decline rapidly as FFA's are utilized for energy and the rate of lipolysis decreases following the antilipolytic insulin "signal" and decreasing levels of the lipolytic GH.

Once the products of digestion begin to increase, plasma VFA concentrations increase to reach maximum values 2-4hr after feeding (10) and the rate of gluconeogenesis is highest when the availability of precursors is highest (11). Bassett (11) stated that the "hormonal responses to nutrient intake in ruminants should therefore favour acetate oxidation or incorporation into fat and the maintenance of high rates of glucose synthesis to preserve metabolic homeostasis". He further stated that "one function of the endocrine alterations in fed ruminants might be to ensure maximum rates of gluconeogenesis from propionate while at the same time conserving absorbed amino acids for synthetic purposes".

The hormonal environment that achieves these anabolic goals appears to be the concurrent high levels of GH and insulin which are effective 30-60 mins after feeding, as discussed in Experiment VII. Between feeding and the initiation of the anabolic environment then, is the period of change, probably initiated by an early neurally mediated transient insulin peak and characterised by falling or depressed levels of insulin, GH and FFA's concurrent with the trough in RQ during which time the animal is utilizing endogenous metabolites to meet the energy costs of digestion and absorption, and changing the hormonal environment in readiness for the anabolic utilization of the products of digestion.

In Experiment IX this anabolic environment was in full effect when the GH and PBS infusions began. As GH was expected to be in its insulin-like anabolic phase at this time it was thought that the exogenous GH should have acted in the same role, but the imposition of unnaturally high GH levels at that time appeared to alter the normal diurnal pattern.

The fact that both GH and PBS infusions were associated with insulin and FFA responses of similar magnitude means that little significance can be placed upon any cause and effect relationships which might be considered. The possibility that the PBS infusions themselves caused physiological responses must be taken into account. Wallace and Bassett (158) found that injection of saline alone caused a brief rise in plasma GH in lambs and in some ewes. Changes in plasma volume, osmolarity and the effects of stress may have played a part in producing the results recorded. Nevertheless it must be remembered that during the absorptive period under consideration one would expect high levels of GH and insulin and the imposition of higher GH levels may not have greatly modified the diurnal variation in these sheep. Perhaps GH receptors in adipose tissue were saturated or less responsive.

The excessive GH may have been responsible for the initial decline in plasma insulin and the resultant hormone imbalance could have brought about the high FFA levels. But insulin did not remain suppressed and increased to high levels while the infusions continued. It has been shown (144) that GH can act as a trophic hormone and cause insulin release in dogs but it is thought that in this case the insulin rise is in response to endocrine or neural signals from the GEP system.

The stimulus to this later release of insulin is not necessarily the same as the stimulus to the early insulin peak. Many workers have studied the later release of insulin as part of the normal diurnal pattern and have variously implicated VFA's, dietary protein, gastrointestinal hormones and vagal reflexes. Bassett (11) reviewed this area of research and while he did seem to champion the role of vagal reflex neural loops and gastrointestinal hormones he did not reject any of these alternatives. Given that any or all of these mechanisms would be stimulating or potentiating the release of insulin in the experimental case in hand it is not surprising that insulin levels increased, even during the GH infusion.

The responses to N.A. injections were similar to those previously obtained in Experiment VI. Proximity of feeding had again obscured the responses. However the FFA responses were clearer on this occasion.

The large FFA peaks following N.A. injections did not occur after control injection.

This result supports a lipolytic role for N.A. GH probably was not involved in the lipolytic response since GH declined to low levels while FFA increased. This may have been a feedback action of FFA on GH release or a direct inhibition of GH release by N.A.

CHAPTER FIVE

SUMMARY, DISCUSSION AND CONCLUSIONS

5.1 INTRODUCTION

Very few of the differences in this study were significant i.e. responses apparently did not occur in the parameters measured, or differences were not statistically significant. However in a pilot study of this nature, with small numbers of animals this was not unexpected. While positive results are usually explained relatively easily in terms of the treatment, a lack of detectable effects may or may not indicate the effect of an experimental treatment. It may indicate that the experimental design or method were not effective in detecting changes or it may indeed reflect the ineffectiveness of the treatment on the factors measured.

5.2 SUMMARY AND DISCUSSION OF EXPERIMENTAL RESULTS

This section summarises and discusses the effects of treatments imposed in the various experiments and the responses to feeding observed in those studies.

1. Calorimetry showed that there were no differences in the normal fed metabolic rate of the sheep sufficient to explain their differences in fatness.

   (Calorimetry Experiment 1)

2. Fasting (25% of maintenance energy requirements) for 2-3 days showed that the long/lean phenotypes were capable of mobilising body lipids in response to fasting. Mean afternoon plasma FFA levels of fasted ewes were significantly higher than those in fed ewes. No significant differences in glucose or GH were detected.

   (Experiment I)

3. Experiments II, III and IV provided insufficient data to establish responses in plasma hormones or metabolites or to identify differences between sheep.

(Experiments II, III & IV)

4. Calorimetry confirmed that RQ's of fed sheep remained about 1 while values declined with time in fasted sheep. The fat and lean groups of ewes differed in their response to fasting as measured by RQ. The fat pair had steeper regressions of RQ on time which probably indicated a greater rate of lipolysis. The results of Sidhu et al (141) also showed greater lipolysis in fatter sheep (see Section 2.4.5).

   It is possible that fasting RQ's can be used to identify sheep with a greater propensity to be overfat. This aspect deserves further study as the relative simplicity and objectivity of the method has much to recommend it.

(Calorimetry Experiment 2)

5. The injection of ACTH produced some interesting but inconclusive results. All 4 sheep had undetectable GH levels at the last sample taken on treatment days (8-9 hours after ACTH injection), while this occurred in only 3 out of 16 cases in untreated sheep. In one ewe (Ewe 186) GH levels fell to undetectable levels within 30 minutes and remained that way throughout the following day. Following ACTH injection 2 ewes (Ewes 57 & 116) produced unusually high plasma FFA levels at the last sample taken corresponding to the low GH levels, but this response was not detected in the other 2 ewes. Four unusually high glucose concentrations were detected but could not be clearly attributed to ACTH treatment.

   Thus study has not resolved the questions regarding the role of ACTH in lipolysis in sheep. Very few studies have been carried out on this aspect (156) and at least one of them (102) did not study the time period suggested by the results of work with goats (130) and by the two unusual FFA levels reported in this study. Further study of plasma hormones and metabolites is required with more

frequent sampling throughout the 24 hour period following ACTH administration.

(Experiment V)

6. The catecholamine Bronkephrine did not produce any significant effects of the catecholamine on plasma GH, FFA or glucose levels. Several inexplicable results were observed including several very low plasma GH levels at various times, higher earlier glucose peaks in N.A. treated ewes than in control ewes, and peak FFA levels following N.A. injection.

(Experiment VI)

In a second study N.A. injections were followed by small transitory GH and large brief insulin peaks after which GH fell to low levels, but these effects also occurred following control injections in Ewe 142. Large peaks in FFA levels followed N.A. injections in both ewes while control injections had little effect on FFA. Plasma glucose levels increased to higher levels following N.A. than after control injections.

(Experiment IX)

All responses were confused with the response to feeding and unexpected peaks and nadirs during non-treatment periods complicated interpretation. Deficiencies in the experimental design and method may have been responsible for the lack of significant differences (see Section 4.7.3). In the light of evidence in the literature (see Section 2.4.3.3) a lipolytic response was certainly expected.

Future studies should examine the same parameters at times well removed from feeding and include other catecholamine preparations. An alternative approach worth consideration would involve examining metabolic responses to central sympathetic stimulation, but such work would require sophisticated skills and techniques.

7. Fasting (25% maintenance) cull Romney ewes resulted in higher ($P = 0.03$) mean afternoon GH levels than in fed sheep. Fasted sheep also had higher mean afternoon FFA levels although the statistical significance of this result ($P = 0.15$) limited its value. No significant differences were found in glucose or insulin levels.

   The increased GH levels, concurrent with elevated FFA levels support a lipolytic role for GH, but this response does not agree with reports in the literature (10, 11, 104, 158) (see Section 2.3.3.6). The increased frequency and amplitude of oscillations in plasma GH levels reported by Bassett (10, 11) and by Driver et al (47) were not observed, although sampling may not have been frequent enough to detect that effect. Alternatively the difference may be due to a breed effect, since Bassett used Merinos. In none of the experiments presented in this thesis can GH levels be accurately described as oscillating: plasma GH concentrations in the sheep studied appeared to have a relatively well defined diurnal variation centred around the once-daily feeding time.

   Bassett (10) recorded lower levels of insulin in fasted sheep than in fed, and although the fasted ewes in the present study had lower average insulin levels in samples taken after feeding during the last 4 experimental days, the difference was not significant. More frequent sampling in the afternoon may establish statistical significance, or perhaps complete fasting is necessary to reduced insulin levels significantly.

   (Experiment VII)

8. No changes in heat production or R could be attributed to N.A. or ACTH in the Romney ewes. Although three ewes produced their highest heat production following N.A. injection, no statistical significance could be established. Again any responses to hormones may have been masked by responses to feeding. Furthermore the design of the experiment was not suitable for reasons discussed in section 4.9.3. To be effective, studies of this nature would need to be divorced from feeding and from rumen fermentation, requirements which present technical difficulties.

9. Both PBS and BGH infusions in 2 Romney ewes produced marked declines in plasma insulin to levels lower than any previously recorded (in those sheep). However insulin levels did not remain depressed, but increased again during the BGH infusion then rapidly increased further following cessation of infusion. No clear pattern of glucose levels was found during infusions but they increased markedly following the end of infusions. FFA levels increased during BGH infusions but did so also during PBS infusions.

No satisfactory explanation for the action of PBS can be offered. Perhaps PBS ellicited the secretion of another hormone that mediated the effects. One is tempted to assume that changes were effected in the hormones regulating blood pressure and volume, but the known action of these hormones do not explain the effects observed. Strangely enough, the one hormone which would be expected to induce the responses observed is GH itself. Perhaps endogenous GH mediated the effects or perhaps PBS stimulated GH secretion. Could it be that the dilution of circulating metabolites induces mobilization of body tissues in defence of blood metabolite concentrations? GH might be expected to be released in response to lowered concentrations of plasma FFA's and to simultaneously protect tissue protein. Since the infusion volume was small in relation to total blood volume these suggestions are probably extending conjecture too far. Nevertheless it is generally assumed that plasma components are regulated by sensitive feedback control mechanisms and we know virtually nothing of the effects of changes in circulating blood volume or pressure on these mechanisms. What effects do post-prandial changes in blood volume have on these mechanisms? What is the significance of the release of VIP (vasoactive intestinal peptide) from peptidergic nerve endings in the GIP (12)? How do changes in blood flow through peripheral tissues such as adipose tissue affect plasma volume and hormone and metabolite concentrations?

According to Gregory (67) in most studies it is usually assumed or implied that the blood concentration of the hormone reflects the tissue exposure to the hormone. However, charges in blood flow through adipose tissue presumably alters the supply of

hormones and metabolites to cells. Gregory (67) reported that adipose tissue blood flow rate and the exposure of the fat to insulin were lower in fat (Clun) than in lean (Blackface) lambs. Further studies are needed on haemodynamics in relation to hormone action.

Another problem in interpreting plasma hormone levels relates to the receptors. Of what significance is a knowledge of the blood levels of a hormone if we do not know the state of its receptors. Ideally receptor affinity and concentration should be measured along with blood concentrations.

An unexpected aspect of interest in these studies was the response to feeding. Initially there was no intention to study such responses, but the observation of the trough in RQ (see Calorimetry Experiment 3, Section 4.9) and the report by Chase et al (35) that portal insulin increased within 2 min of meal initiation prompted more frequent blood sampling during the feeding and post-prandial period.

The changes in plasma hormones around feeding are thought to be involved in providing energy substrates for the immediate needs of digestion and in preparing the anabolic environment necessary for the efficient utilisation of absorbed nutrients. The roles of insulin and GH in this context were discussed in Experiments VII and IX. Overall control of the prandial and post-prandial environment is probably attributable to the autonomic nervous system and the GEP system described by Bassett (12).

(Experiment IX)

10. No significant differences between the responses of the ewes in the second group were detected in any of the experiments. However Ewe 142 occasionally exhibited unusual or different responses. No pattern or reason for these differences could be identified. While they may be only chance occurrences or assay errors it is thought that they may indicate genetic variation in metabolism between sheep. Screening of large numbers of sheep with intensive blood sampling may be necessary to identify possible genotypes.

5.3 CONCLUSIONS

These conclusions are based partly upon the results of this study but draw heavily on the literature which includes considerable information published since the experiments in this study were carried out.

1. The prime control of lipolysis is almost certainly the autonomic nervous system, through the release of N.A. at sympathetic nerve endings in adipose tissue. This action is aided by GH which potentiates lipolysis, defends tissue protein stores, and promotes the transfer to and utilisation of FFA in "productive" body tissues (muscle, mammary gland etc).

   GH secretion is neurally mediated as is insulin (via the GEP system). Insulin is primarily anabolic and antilipolytic in adipose tissue. It antagonises GH action in adipose tissue yet supports the anabolic role of GH in the productive tissues.

2. GH levels are high and insulin levels are low during fasting as adipose tissue is mobilised. Around the time of feeding both GH and insulin are centrally involved along with autonomic mechanisms in providing the immediate energy needs for digestion and in preparing the anabolic environment needed for the utilization of absorbed nutrients.

3. Declining RQ values initiated by feeding may indicate increased lipolysis and utilisation of long-chain fatty acids to provide the initial energy required for digestion. Subsequent increases in RQ reflect the return to oxidation of dietary acetate and glucose (derived from propionate) as the main energy sources, with concurrent anabolic activities, including lipogenesis if energy supply is surplus to immediate requirements. The increasing RQ values are confused by increasing production of CO_2 through rumen fermentation.

4. BGH probably promotes lipolysis in sheep but the effect of PBS which was very similar to that of BGH requires further study.

5. ACTH may be lipolytic in sheep, but it is not thought to be a prime factor in a day to day regulation of lipid metabolism.

6. Similarly the catecholamines are lipolytic, but the role of the adrenal gland is probably only an overriding mechanism involved in emergency situations.

7. Although not studied in these experiments it is thought that thyroid hormones and corticosteroids modulate the actions of lipolytic hormones. While their presence is essential, the mechanisms and reasons for their actions in adipose tissue remain unknown.

8. Fatter sheep exhibited higher rates of lipolysis upon fasting, but showed no differences in metabolic rate when fed. No differences in plasma metabolite profiles between fat and lean sheep were detected.

9. Variation in plasma metabolites and hormones was observed between individual sheep but could not be explained.

APPENDICES

APPENDIX 3.8.1A

Analysis of variance of factorial experiment to determine optimal antibody dilutions and incubation time for GH R.I.A. (see Table 3.5).

Source of Variance	F	d.f.	P
Between GH levels	392.88	1,43	$P < 0.001$
Between times	276.67	1,43	$P < 0.001$
GH levels X times	20.72	1,43	$P < 0.001$
Between 1st antibody levels	2.93	4,43	$P = 0.03$
Between 2nd antibody levels	0.55	3,43	$P = 0.65$
1st AB level X 2nd AB level	0.53	12,43	$P = 0.88$
2nd AB level X GH level	0.48	3,43	$P = 0.70$
2nd AB level X times	10.91	3,43	$P < 0.001$
1st AB level X GH level	0.92	4,43	$P = 0.46$
1st AB level X times	0.63	4,43	$P = 0.64$

APPENDIX 3.8.1B

Comparison of regression lines;
Professor Flux's BGH standards compared with authors BGH standards by analysis of covariance.

Author's		Prof. Flux's	
X	Y	X	Y
2.417	-0.602	2.597	-0.602
2.331	-0.301	2.508	-0.301
2.052	0	-	-
1.078	0.301	1.077	0.301
0.524	0.602	0.495	0.602
0.155	0.903	0.201	0.903
0.256	1.204	-0.059	1.204
0.358	1.505	0.013	1.505
ΣX 9.171	ΣY 3.612	ΣX 6.832	ΣY 3.612
ΣX^2 17.1405	ΣY^2 5.4361	ΣX^2 14.4835	ΣY^2 5.4361
$\bar{X}$ 1.1464	$\bar{Y}$ 0.4515	$\bar{X}$ 2.0662	$\bar{Y}$ 0.516

$\Sigma XY = -0.5298$ $\Sigma XY = -1.5661$

$n = 8$ $n = 7$

Calculation of total sums of squares for both sets of standards and their correction

$N = 15$

TOTAL $\Sigma X = 9.171 + 6.832 = 16.003$

TOTAL $\Sigma X^2 = 17.1405 + 14.4835 = 31.6240$

Correction $\Sigma x^2 = 31.6240 - \frac{(16.003)^2}{15} = \underline{14.55095}$

TOTAL $\Sigma XY = (-0.5298) + (-1.5661) = -2.0959$

CORRECTION $\Sigma xy = -2.095 - \frac{16.003 \times 7.224}{15} = \underline{-9.8029}$

TOTAL $\Sigma Y = 3.612 + 3.612 = 7.224$

TOTAL $\Sigma Y^2 = 5.4361 + 5.4361 = 10.8722$

Correction $\Sigma y^2 = 10.8722 - \frac{7.224^2}{15} = \underline{7.39304}$

Correcting sums of squares for each set of BGH standards.

Author's standards

X $\quad ss = 17.1405 - \frac{(9.171)^2}{8} = 6.6271$

XY $\quad ss = -0.5298 - \frac{(9.171 \times 3.612)}{8} = -4.6705$

Y $\quad ss = 5.4361 - \frac{(3.612)^2}{8} = 3.8052$

Prof. Flux's standards

X $\quad ss = 14.4835 - \frac{(6.832)^2}{7} = 7.8155$

XY $\quad ss = -1.5661 - \frac{(6.832 \times 3.612)}{7} = -5.0914$

Y $\quad ss = 5.4361 - \frac{(3.612)}{2} = 3.5727$

Comparison of BGH standard regression lines - Analysis of covariance

Line	Source of Variance	d.f.	Sums of Squares Σx^2	Σxy	Σy^2	Reg. Coeff. $\frac{\Sigma xy}{\Sigma x^2}$	Deviations from Regressions d.f.	ss	M.S.
1	Within author's standards	7	6.6271	-4.6705	3.8052	-0.70	6	0.51373	0.08562
2	Within Prof. Flux's stds.	6	7.8155	-5.0914	3.5727	-0.65	5	0.02554	0.05109
3							11	0.76917	0.06993
4	Pooled within (W)	13	14.4426	-9.7619	7.3775	-0.68	12	0.77936	0.06495
5			Difference between slopes				1	0.01019	0.01019
6	Between (B)	1	0.1084	-0.0410	0.0155				
7	W + B	14	14.5510	-9.8029	7.3930		13	0.07889	
8			Between adjusted means				1	0.00951	0.00951

Comparison of Slopes $F = \frac{0.01019}{0.069^{9}3} = 0.1457$ (d.f. = 1,11) P = 0.71

Comparison of elevations $F = \frac{0.00951}{0.06495} = 0.1464$ (d.f. = 1,12) P = 0.71

The curves do not differ significantly in either slope or elevation (P = 0.71)

APPENDIX 3.8.3

LOGITS programme to calculate hormone concentration from CPM given the regression line of the standard curve.

```
10   OPEN "SOUT" AS FILE 1 MODE 3
20   PRINT £1 , "logits 19.11.81"
30   PRINT "LOGITS PROGRAM TO CALCULATE HORMONE CONCENTRATION FROM CPM"
40   PRINT "GIVEN REGRESSION LINE OF THE STANDARD CURVE (IN LOGITS)"
50   DIM N1$(50)
60   PRINT "ENTER NAME OF ASSAY"
70   INPUT N1$
80   PRINT £1 , CHR$(14) ; N1$
90   PRINT "ENTER DATE"
100  INPUT D1$
110  PRINT £1 , D1$
120  PRINT £1
130  PRINT "ENTER TOTAL"
140  INPUT T
150  PRINT £1 , CHR$(15) ; "TOTAL =" ; CHR$(14) , T
160  PRINT "ENTER Y INTERCEPT  (ie ORIGIN)"
170  INPUT O
180  PRINT £1 , CHR$(15) ; "INTERCEPT" ; CHR$(14) , O
190  PRINT "ENTER SLOPE b"
200  INPUT S1
210  PRINT £1 , CHR$(15) ; "SLOPE =" ; CHR$(14) , S1
220  PRINT "ENTER CODE"
230  INPUT C1
240  PRINT £1 , CHR$(15) ; "CODE =" ; CHR$(14) , C1
250  PRINT "COUNTS LARGER THAN T RESULT IN CONCENTRATION OF ZERO"
260  PRINT "FOR MISSING DATA ENTER 0;FOR REJECTED DATA ENTER 1"
270  PRINT
280  PRINT "ENTER SAMPLE NUMBER"
290  INPUT S2$
300  PRINT £1 , CHR$(14) ; "SAMPLE" , S2$
310  PRINT £1 , CHR$(15) ; "50ul"
320  PRINT "ENTER COUNTS FOR 50ul SAMPLE"
330  INPUT B
340  IF B ) T THEN LET X2 = 0
350  IF B ) T THEN LET X1 = 0
360  IF B ) T THEN GOTO 440
370  IF B ( 0 THEN GOTO 1060
380  IF B = 0 THEN GOTO 460
390  IF B = 1 THEN GOTO 480
400  LET X = LN(B/(T-B))
410  LET X1 = (X+C1)*S1+O
420  LET X1 = X1*2.30258
430  LET X2 = EXP(X1)
440  PRINT £1 , CHR$(15) ; "COUNTS" ; B , "CONC" ; X2 , "pg/ml    Ln CONC =" ;
450  GOTO 490
460  PRINT £1 , "SAMPLE LOST"
470  GOTO 490
480  PRINT £1 , "SAMPLE REJECTED"
490  PRINT £1 , "100ul"
500  PRINT "ENTER COUNTS FOR 100ul SAMPLE"
510  INPUT B2
520  IF B2 ) T THEN LET X6 = 0
530  IF B2 ) T THEN LET X5 = 0
540  IF B2 ) T THEN GOTO 610
550  IF B2 = 0 THEN GOTO 630
560  IF B2 = 1 THEN GOTO 650
570  LET X3 = LN(B2/(T-B2))
580  LET X4 = (X3+C1)*S1+O
```

```
590  LET X5 = X4*2.30258
600  LET X6 = EXP(X5)
610  PRINT £1 , CHR$(15) ; "COUNTS" ; B2 , "CONC" ; X6 , "pg/ml    Ln CONC =" ; X

620  GOTO 660
630  PRINT £1 , "SAMPLE LOST"
640  GOTO 660
650  PRINT £1 , "SAMPLE REJECTED"
660  IF B = B2 THEN GOTO 1030
670  IF B = 0 THEN GOTO 870
680  IF B = 1 THEN GOTO 910
690  IF B ) T THEN GOTO 790
700  LET K = X1+LN(2)
710  IF B2 = 0 THEN GOTO 970
720  IF B2 = 1 THEN GOTO 990
730  IF B2 ) T THEN GOTO 840
740  LET L = EXP(K)
750  LET M = EXP(X5+LN(2))
760  LET L = (L+M)/3
770  PRINT £1 , "Weighted hormone conc =" ; L/1000 ; "ng/ml  LOG=" ; LOG(L/1000
780  GOTO 280
790  IF B2 ) T THEN PRINT £1 , "BOTH COUNTS )T"
800  IF B2 ) T THEN GOTO 280  ELSE GOTO 810
810  PRINT £1 , "WEIGHTED hormone conc =" ; X6/1500 ; "ng/ml  LOG =" ; LOG(X6/1

0)
820  PRINT £1 , "N.B. 50ul Count)Total i.e.CONC = 0"
830  GOTO 280
840  PRINT £1 , "WEIGHTED HORMONE CONC =" ; 2*X2/1500 ; "ng/ml  LOG=" ; LOG(2*X

1500)
850  PRINT £1 , "N.B.100ul COUNT)TOTAL i.e.CONC = 0"
860  GOTO 280
870  PRINT £1 , "50ul SAMPLE LOST.ANSWER BASED ONLY ON 100ul SAMPLE"
880  IF B2 = 1 THEN LET X6 = 0 ELSE GOTO 950
890  PRINT £1 , "50ul SAMPLE LOST,100ul SAMPLE REJECTED"
900  GOTO 280
910  PRINT £1 , "50ul SAMPLE REJECTED.ANSWER BASED ONLY ON 100 ul SAMPLE"
920  IF B2 = 0 THEN LET X6 = 0 ELSE GOTO 950
930  PRINT £1 , "50ul SAMPLE REJECTED,100ul SAMPLE LOST"
940  GOTO 280
950  PRINT £1 , "HORM. CONC.=" ; X6/1000 ; "ng/ml  LOG =" ; LOG(X6/1000)
960  GOTO 280
970  PRINT £1 , "100ul SAMPLE LOST.ANSWER BASED ONLY ON 50ul SAMPLE"
980  GOTO 1000
990  PRINT £1 , "100ul SAMPLE REJECTED.ANSWER BASED ONLY ON 50ul SAMPLE"
1000 IF B2 = 0 THEN LET X2 = 0
1010 PRINT £1 , "HORM CONC =" ; X2*2/1000 ; "ng/ml  LOG =" ; LOG(X2*2/1000)
1020 GOTO 280
1030 IF B = 0 THEN PRINT £1 , "BOTH SAMPLES LOST"
1040 IF B = 1 THEN PRINT £1 , "BOTH SAMPLES REJECTED"
1050 GOTO 280
1060 PRINT "HAVE A RIPPER DAY !"
1070 END
```

APPENDIX 3.8.4

Output of a microcomputer programme testing for parallelism errors in RIA's.

```
Insm 27.10.82
PARALLELISM TEST FOR RIA
INSULIN RUN VII WITH EXPANDED STD CURVE
27.10.82

Input data are the natural loss of hormone conc. in 50 & 100ul dilutions

50ul LEVEL   50ul DOUBLED   100ul LEVEL   SUM        SUM OF SQUARES
 5.564        6.25715        5.487        11.7441    69.259          1
 4.482        5.17515        5.415        10.5901    56.1044         2
 4.914        5.60715        5.342        10.9491    59.9771         3
 5.114        5.80715        5.307        11.1141    61.8872         4
 5.161        5.85415        5.858        11.7121    68.5872         5
 4.666        5.35915        5.671        11.0301    60.8807         6
 5.378        6.07115        5.954        12.0251    72.3089         7
 5.971        5.66415        6.145        11.8091    69.8436         8
 5.177        5.87015        5.977        11.8471    70.1831         9
 5.502        6.19515        7.082        13.2771    88.5346         10
 5.402        6.09515        5.666        11.7611    69.2543         11
 4.685        5.37815        5.737        11.1151    61.8376         12
 9.169        9.86215        9.079        18.9411    179.69          13
 5.411        6.10415        5.962        12.0661    72.806          14
 5.73         6.42315        6.212        12.6351    79.8457         15
 5.252        5.94515        6.248        12.1931    74.3823         16
 5.414        6.10715        6.178        12.2851    75.4649         17
 4.935        5.62815        5.774        11.4021    65.0151         18
 5.4          6.09315        6.404        12.4971    78.1376         19
 5.157        5.85015        6.193        12.0431    72.5775         20
 20           121.347        121.691      243.038    1506.58

                Correcting sums of squares
Correction factor C = 1476.69

TOTAL SS = 1506.58 - 1476.69 = 29.8914
LEVELS SS =  0.32835E-2
Samples raw SS = 3009.72
SAMPLES SS=  28.1741

                ANOVA

SOURCE      d.f.    SUM OF SQUARES    MEAN SQUARE
LEVELS      1        0.32835E-2       0.32835E-2
SAMPLES     19       28.1741          1.48285
ERROR       19       1.714            0.902106E-1
TOTAL       39       29.8914

SAMPLES F =     16.4376      d.f. 19  19
SAMPLES P=0.0000
LEVELS  F =     0.363982E-1  d.f. 1 , 19
LEVELS P= 0.85

NO SIGNIFICANT PARALLELISM ERROR
```

APPENDIX 4.1

a) Analysis of variance of heat production for sheep within breeds.

HEAT PRODUCTION KJ. $Kg^{-0.75}$

Day \ EWE	R323	R313	SD73	SD56
1	312.70	280.39	230.21	267.26
2	265.43	279.11	256.46	267.34
3	294.76	255.63	258.86	255.01
4			254.98	265.38
$\bar{X}$	290.96	271.71	250.08	263.75
ΣX	872.89	815.13	1000.31	1054.99
$\bar{X}$	290.96	271.71	250.08	263.75
ΣX^2	255117.83	221867.64	250688.17	278355.23
C	253978.98	221478.97	250155.02	278250.96
Σx^2	1138.85	388.67	533.15	104.25
df	2	2	3	3

ROMNEYS

ANOVA

$$\text{TOTAL ss} = 476985.47 - \frac{(1688.02)^2}{6}$$

$$= 2083.55 \text{ with } 5 \text{ d.f.}$$

Within Sheep

ss = 1527.52 with 4 d.f.

$$\text{Between Sheep} = \frac{(872.89)^2 + (815.13)^2}{3} - C$$

$$= 475457.96 - 474901.92$$

$$= 556.04 \text{ with } 1 \text{ d.f.}$$

Source	d.f.	s.s.	M.S.
Within	4	1527.52	381.88
Between	1	556.04	556.04
Total	5	2083.55	416.7

$$F = \frac{556.04}{381.88}$$

= 1.45 with 1,4 d.f. P = 0.29

No significant differences

SOUTH DOWNS

ANOVA

$$\text{TOTAL ss} = 529043.4 - \frac{(2055.3)^2}{8}$$

= 1011.14 with 7 d.f.

Between sheep

$$\text{ss} = \frac{(1000.31)^2 + (1054.99)^2}{4} - C$$

= 528405.999 - 528032.26

= 373 with 1 d.f.

Within sheep

ss = 533.15 + 104.25

= 637.4 with 3 + 3 = 6 d.f.

Source	d.f.	s.s.	M.S.
Within	6	637.4	106.23
Between	1	373	373
Total	7	1011.4	

$$F = \frac{373}{106} = 3.52 \text{ with } 1,6 \text{ d.f.}$$

P = 0.11

No significant differences

b) ANOVA of heat production within phenotypes

	FAT	LEAN	
1	280.39	312.70	
2	279.11	265.43	
3	255.63	294.76	
4	267.26	230.21	
5	267.34	256.46	
6	255.01	258.66	
7	265.38	254.98	
$\bar{X}$	267.16	267.61	
S	10.01	27.52	
ΣX	1870.12	1873.2	3743.32
$\bar{X}$	267.16	267.6	534.77
ΣX^2	500222.87	505806.00	1006028.87
-C	499621.26	501268.32	1000889.58
Σx^2	601.61	4537.68	5139.29
d.f.	6	6	12

ANOVA between fat and lean sheep heat production

$$\text{TOTAL SS} = 1006028.87 - \frac{(3743.32)^2}{14}$$

$$= 1006028.87 - 1000888.90$$

$$= 5139.97 \text{ with } 13 \text{ d.f.}$$

Within sheep

$$\text{ss} = 5139.29 \text{ with } 12 \text{ d.f.}$$

Between sheep

$$\text{ss} = \frac{(1870.12)^2 + (1873.2)^2}{7} - C$$

$$= 1000889.58 - 1000888.90$$

$$= 0.68 \text{ with } 1 \text{ d.f.}$$

Source	d.f.	ss	M.S.
Within	12	5139.29	428.27
Between	1	0.68	0.68
Total	13	5139.97	

$$F = \frac{0.68}{428.27} = 0.002 \text{ with } 1,12 \text{ d.f.}$$

No significant differences

$P = 0.97$

APPENDIX 4.4A

Regression of RQ on time (days) for each sheep under both treatments.

X = day Y = RQ

FASTED TREATMENT GROUP

Ewe 313

Yint = 1.0893	$\Sigma Y = 2.8778$	$\Sigma X = 7$
b = -0.0557	$\Sigma Y^2 = 2.7751$	$\Sigma X^2 = 21$
$r_{RQ,D}$ = -1.0000	n = 3	$\Sigma XY = 6.4549$

Ewe 323

Yint = 1.0025	$\Sigma Y = 3.7602$	$\Sigma X = 10$
b = -0.0250	$\Sigma Y^2 = 3.5380$	$\Sigma X^2 = 30$
$r_{RW,D}$ = -0.9872	n = 4	$\Sigma XY = 9.2756$

Ewe 73

Yint = 1.0374	$\Sigma Y = 4.7144$	$\Sigma X = 15$
b = -0.0315	$\Sigma Y^2 = 4.4577$	$\Sigma X^2 = 55$
$r_{RQ,D}$ = -0.8882	n = 5	$\Sigma XY = 13.8282$

Ewe 56

Yint = 1.209	$\Sigma Y = 3.8484$	$\Sigma X = 10$
b = -0.09876	$\Sigma Y^2 = 3.7522$	$\Sigma X^2 = 30$
$r_{RQ,D}$ = -0.9910	n = 4	$\Sigma XY = 9.1272$

Corrections of sums of squares and products.

Ewe 313

ΣX^2	= 21	ΣXY =	6.4549	ΣY^2 =	2.7751
$-\frac{(\Sigma X^2)}{n}$	16.3333	-C	6.7149	-C	2.7606
Σx^2	= 4.6667	Σxy =	-0.2500	Σy^2 =	0.0145

Ewe 323

ΣX^2	= 30	ΣXY =	9.2756	ΣY^2 =	3.5380
-C	25	-C	9.4005	-C	3.5348
Σx^2	= 5	Σxy =	-0.1249	Σy^2 =	0.0032

Ewe 73

ΣX^2	= 55	ΣXY =	13.8282	ΣY^2 =	4.4577
-C	45	-C	14.1432	-C	4.4451
Σx^2	= 10	Σxy =	-0.3150	Σy^2 =	0.0126

Ewe 56

ΣX^2	= 30	ΣXY =	9.1272	ΣY^2 =	3.7522
-C	25	-C	9.6210	-C	3.7025
Σx^2	= 5	Σxy =	-0.4938	Σy^2 =	0.0497

Total (n = 16)

ΣX_T = 42 $\bar{X}_T$ = 2.6250 ΣY_T = 15.2008 $\bar{Y}_T$ = 0.9501 ± 0.074

ΣX_T^2	= 136	ΣXY =	38.6859	ΣY_T^2 =	14.5230
-C	110.25	-C	39.9021	-C	14.4415
Σx_T^2	= 25.75	Σxy_T	-1.2162	Σy_T^2 =	0.0815

Yint = 1.074 b = -0.0472 r -0.8396 SD_Y = 0.074

ANALYSIS OF COVARIANCE

FASTED GROUP

Line	Source	df	Corrected sums of squares and products Σx^2	Σxy	Σy^2	Reg. Coeff.	Deviations from regression df	ss	M.S.	r
1	313	2	4.6667	-0.2600	0.0145	-0.0557	1	0.000014	0.000014	-1.00
2	323	3	5	-0.1249	0.0032	-0.0250	2	0.000080	0.000040	-0.99
3	73	4	10	-0.3150	0.0126	-0.0315	3	0.002678	0.000893	-0.89
4	156	3	5	-0.4938	0.0497	-0.0988	2	0.000932	0.000466	
5	Within sheep						8	0.003704	0.000463	
6	Reg. Coeff.						3	0.018529	0.006176	
7	Common	12	24.667	-1.1937	0.0800		11	0.022233	0.002021	
8	Adj means						3	0.001825	0.000608	
9	Total	15	25.75	-1.2162	0.0815		14	0.024058		

a) Test for differences in slope of regression lines

$Ho = b_{323} = b_{313} = b_{56} = b_{73}$

$$F = \frac{\text{MS for differences between slopes}}{\text{MS within sheep}} \qquad \frac{\text{(line 6)}}{\text{(line 5)}}$$

$$= \frac{0.006176}{0.000463} = 13.34 \quad 3,8 \text{ d.f.} \qquad P = 0.002$$

slope of regression lines differ significantly

b) Test for differences in elevations of lines

$$F = \frac{\text{M.S. for adj. means}}{\text{M.S. for common line}} \qquad \frac{\text{(line 8)}}{\text{(line 7)}}$$

$$= \frac{0.000608}{0.002021}$$

$= 0.30$ with 3, 11 d.f. N.S. $P = 0.82$

Elevation of lines are not significantly different

FED TREATMENT GROUP

Ewe 312

Yint =	0.9975	$\sum Y$ = 4.2307	$\sum X$ = 10
b =	0.0241	$\sum Y^2$ = 4.4780	$\sum X^2$ = 30
r =	0.9338	n = 4	$\sum XY$ = 10.6972

Ewe 323

Yint =	1.0227	$\sum Y$ = 4.1987	$\sum X$ = 10
b =	0.0108	$\sum Y^2$ = 4.4079	$\sum X^2$ = 30
r =	0.9407	n = 4	$\sum XY$ = 10.5508

Ewe 73

Yint =	1.0557	$\sum Y$ = 4.1902	$\sum X$ = 10
b =	-0.00326	$\sum Y^2$ = 4.3909	$\sum X^2$ = 30
r =	-0.19148	n = 4	$\sum XY$ = 10.4592

Ewe 56

Yint =	1.0773	$\sum Y$ = 5.4987	$\sum X$ = 15
b =	0.0075	$\sum Y^2$ = 6.0496	$\sum X^2$ = 55
r =	0.4747	n = 5	$\sum XY$ = 16.571

CORRECTION OF SUMS OF SQUARES AND PRODUCTS

Ewe 313

$\sum X^2$ =	30	$\sum XY$ =	10.6972	$\sum Y^2$ =	4.4780
-C	25	-C	10.5768	-C	4.4747
$\sum x^2$ =	5	$\sum xy$ =	0.1205	$\sum y^2$ =	0.0033

Ewe 323

$\sum X^2$ =	30	$\sum XY$ =	10.5508	$\sum Y^2$ =	4.4079
-C	25	-C	10.4968	-C	4.4073
$\sum x^2$ =	5	$\sum xy$	0.0540	$\sum y^2$ =	0.0006

Ewe 73

ΣX^2	= 30	ΣXY	= 10.4592	ΣY^2	4.3909
-C	25	-C	10.4755	-C	4.3894
Σx^2	= 5	Σxy	= -0.0163	Σy^2	0.0015

Ewe 56

ΣX^2	= 55	ΣXY	= 16.5710	ΣY^2	= 6.0496
-C	45	-C	16.4961	-C	6.0471
Σx^2	= 10	Σxy	= 0.0749	Σy^2	= 0.0025

Total (n = 17)

ΣX_T	= 45 $\bar{X}_T$ = 2.6470	ΣY_T	= 18.1183	$\Sigma \bar{Y}_T$	= 1.0658
ΣX_T^2	= 145	ΣXY	= 48.2782	ΣY^2	= 19.3264
-C	119.1176	-C	47.9602	-C	19.3102
Σx_T^2	25.8823	Σxy_T	= 0.3180	Σy_T^2	= 0.0162

Yint = 1.0332 b = 0.01229 r = 0.490 SD_y = 0.0319

ANALYSIS OF COVARIANCE

FED GROUP

Line	Source	df	Σx^2	Σxy	Σy^2	Reg. Coeff.	df	ss	MS	r
1	313	3	5	0.1205	0.0033	0.024	2	0.000396	0.000198	0.93
2	323	3	5	0.0540	0.0006	0.011	2	0.000017	0.000008	0.94
3	73	3	5	-0.0163	0.0015	0.003	2	0.001447	0.000723	-0.19
4	56	4	10	0.0749	0.0025	0.0075	3	0.001939	0.000646	0.47
5	within sheep						9	0.003799	0.000422	
6	Reg Coeff						3	0.001928	0.000643	
7	Common	13	25	0.2331	0.0079	0.00932	12	0.005727	0.000477	0.52
8	Adj Means						3	0.006566	0.00219	
9	Total	16	25.8823	0.3180	0.0162		15	0.012293		

Test for differences in slopes of regression lines

Ho: $b_{323} = b_{313} = b_{73} = b_{56}$

$$F = \frac{\text{M.S. for differences between slopes} \quad \text{(line 6)}}{\text{M.S. for differences within sheep} \quad \text{(line 5)}}$$

$$= \frac{0.000643}{0.000422} \text{ with 3, 9 d.f.}$$

= 1.52 with 3, 9 d.f. P = 0.27

Test for differences in elevation of regression lines

$$F = \frac{\text{M.S. for differences between adj. means (line 8)}}{\text{M.S. for differences from common line} \quad \text{(line 7)}}$$

$$= \frac{0.00219}{0.000477}$$

= 4.59 with 3, 12 d.f. P = 0.02

The slopes do not differ significantly but the elevations do

TOTAL (n = 33)

$\Sigma X = 87$ $\bar{X} = 2.6364$

$\Sigma Y = 33.3191$ $\bar{Y} = 1.0097$

$\Sigma X^2 = 281$	$\Sigma XY = 86.9641$	$\Sigma Y^2 = 33.8484$
$-C \quad 229.364$	$-C \quad 87.8413$	$-C \quad 33.6413$
$\Sigma x^2 = 51.6364$	$\Sigma xy = -0.8772$	$\Sigma y^2 = 0.2081$

The common regression for the 2 common regression lines are compared to see if they differ significantly

ANALYSIS OF COVARIANCE

Line	Source	df	Σx^2	Σxy	Σy^2	Reg Coeff	df	SS	MS	r
1	Fasted	12	24.6667	-1.1937	0.0800		11	0.000014	0.000014	-1.00
2	Fed	13	25	0.2331	0.0079	0.00932	12	0.005727	0.000477	0.52
3	Within treatments						23	0.005741	0.000250	
4	Reg Coeff						1	0.063580	0.063580	
5	Common	25	49.6667	-0.9606	0.0879	-0.0193	24	0.069321	0.002888	0.46
6	Adj Means		5.8485	0.0447	0.1202		7	0.123877	0.017697	
7	Total	32	51.6364	-0.8772	0.2081	-0.0165	31	0.193198		

Test for differences in variance between treatment groups

$$F = \frac{\text{M.S. Fed}}{\text{M.S. Fasted}} = \frac{0.000477 \quad \text{(line 2)}}{0.000014 \quad \text{(line 1)}}$$

$$= 34.07 \text{ with } 12, 11 \text{ d.f.}$$

Difference is significant $P < 0.001$

Test for differences in slope of treatment regressions

$$F = \frac{\text{M.S. reg. coeff} \quad \text{(line 4)}}{\text{M.S. within treatments} \quad \text{(line 3)}} = \frac{0.063580}{0.000250}$$

$$= 254.32 \text{ with } 1, 23 \text{ d.f.}$$

Difference is significant $P < 0.001$

Test for difference in elevation of treatment regressions

$$F = \frac{0.017697}{0.002888} = 6.128 \text{ with } 1, 24 \text{ d.f.}$$

Difference is significant $P = 0.02$

APPENDIX 4.4b

COMPARISON OF FAT AND LEAN "PHENOTYPES" WHEN FASTED

Note that the slopes of the regressions of Ewes 313 and 56 when fasted appear steeper than for the lean pair 323 and 73.

The individual regressions have been calculated elsewhere.

ANALYSIS OF COVARIANCE

FAT PAIR (313 + 56)

Total (n = 7)

$\Sigma X_T = 17$ $\bar{X} = 2.43$	$\Sigma Y_T = 6.7262$	$\bar{Y} = 0.9609$
$\Sigma X_T^2 = 51$	$\Sigma XY = 15.5821$	$\Sigma Y^2 = 6.5273$
$-C = 41.2857$	$-C = 16.3351$	$-C = 6.4631$
$\Sigma x_T^2 = 9.7143$	$\Sigma xy = -0.7530$	$\Sigma y^2 = 0.0642$

LEAN PAIR (323 + 73)

Total (n = 9)

$X_T = 25$ $\bar{X} = 2.7778$	$\Sigma Y = 8.4746$	$\bar{Y} = 0.9416$
$\Sigma X^2 = 85$	$\Sigma XY = 23.1038$	$\Sigma y^2 = 7.9957$
$-C$ 69.4444	$-C = 23.5406$	$-C = 7.9799$
$\Sigma x_T^2 = 15.5555$	$\Sigma xy_T = -0.4368$	$\Sigma y_T^2 = 0.0158$

Comparison of regression lines within each pair and derivation of common regression line for each "Genotype" Group

			FAT GROUP					
Line	Source	df	Σx^2	Σxy	Σy^2	df	SS	MS
1	313	2	4.6667	-0.2600	0.0145	1	0.000014	0.000014
2	56	3	5.0000	-0.4938	0.0497	2	0.000932	0.000466
3	within group					3	0.000946	0.000315
4	Reg Coeff					1	0.004473	0.004473
5	Common	5	9.6667	-0.7538	0.0642	4	0.005419	0.001355
6	Adj Means					1	0.000413	0.000413
7	Total	6	9.7143	-0.7530	0.0642	5	0.005832	

1. Comparison of residual variances $F = \frac{0.000014}{0.000466} = 0.030$ with 1,2 d.f.

   $P = 0.88$

2. Comparison of slopes

   $F = \frac{0.004473}{0.000315} = 14.2$ with 1,3 d.f.

   $P = 0.03$

3. Comparison of elevation of lines

   $F = \frac{0.000413}{0.001355} = 0.3$ with 1,4 d.f.

   $P = 0.61$

Slopes of regressions differ significantly between ewes within the fat group, but elevations do not.

LEAN GROUP

Line	Source	df	Σx^2	Σxy	Σy^2	df	SS	MS
1	323	3	5	-0.1249	0.0032	2	0.000080	0.000040
2	73	4	10	-0.3150	0.0126	3	0.002678	0.000893
3	within group					5	0.002758	0.000552
4	Reg coeff					1	0.000141	0.000141
5	Common	7	15	-0.4399	0.0158	6	0.002899	0.000483
6	Adj. means					1	0.000636	0.000636
7	Total	8	15.5556	-0.4368	0.0158	7	0.003535	

1. Comparison of residual variances for each sheep

   $F = \frac{0.000040}{0.000893} = 0.045$ with 2,3 d.f.

   $= 0.96$

2. Comparison of slopes

   $F = \frac{0.000141}{0.000552} = 0.26$ with 1,5 d.f.

   $P = 0.63$

3. Comparison of elevations

   $F = \frac{0.000636}{0.000483} = 1.32$ with 1,6 d.f.

   $P = 0.29$

   Regressions do not differ significantly between ewes in the lean group

COMPARISON OF REGRESSIONS FOR FAT AND LEAN GROUPS

Line	Source	df	Σx^2	Σxy	Σy^2	df	SS	MS
1	Fat	5	9.6667	-0.7538	0.0642	4	0.005419	0.001355
2	Lean	7	15	-0.4399	0.0158	6	0.002899	0.000483
3	Within					10	0.008318	0.000832
4	Reg Coeff					1	0.013915	0.013915
5	Common	12	24.6667	-1.1937	0.08	11	0.022233	0.002021
6	Adj means					3	0.003247	0.001082
7	Total	15	25.2698	-1.1898	0.0815	14	0.025480	

1. Comparison of residual variances

$$F = \frac{0.001355}{0.000483} = 2.81 \text{ with } 4, 6 \text{ d.f.}$$

$$P = 0.12$$

2. Comparison of slopes

$$F = \frac{0.013915}{0.000832} = 16.725 \text{ with } 1, 10 \text{ d.f.}$$

$$P = 0.002$$

3. Comparison of elevation

$$F = \frac{0.001082}{0.002021} = 0.54 \text{ with } 3, 11 \text{ d.f.}$$

$$P = 0.66$$

The slopes of regressions for fat and lean groups differ significantly
P = 0.002

APPENDIX 4.8.2

Experiment VII

Analysis of glucose, FFA and GH levels (average of afternoon samples)

By Paired T Test (using the method of Brandt, A.E. (27))

GH EXPERIMENT VII

Analysis of differences between means of last 3 afternoon samples of fasted and fed.

Sheep	Period	$\log_{10}$ GH $ngcm^{-3}$			Mean	1st difference.	2nd difference
57	I	0.176	1.211	1.283	0.890		
	II	1.217	0.933	1.363	1.171	-0.281	-0.666
	III	1.044	0.513	0.801	0.786	0.385	
116	I	0.970	1.030	1.483	1.161	0.246	
	II	0.561	0.979	1.206	0.915		0.737
	III	1.092	1.370	1.757	1.406	-0.491	
142	I	0.918	1.203	0.828	0.983	-0.077	
	II	1.136	0.912	1.134	1.060		0.199
	III	1.420	1.051	1.538	1.336	-0.276	
186	I	0.693	0.468	1.230	0.797	-0.476	
	II	1.228	1.172	1.418	1.273		-1.009
	III	0.988	0.610	0.621	0.740	0.533	

Sheep No	Period I	Period II	Period III	Differences
		GROUP A		
	X_1	Y_2	X_3	$X_1 - 2Y_2 + X_3$
57	0.890	1.171	0.786	-0.666
186	0.797	1.273	0.740	-1.009
				-1.675
		GROUP B		
	Y_1	X_2	Y_3	$Y_1 - 2X_2 + Y_3$
116	1.161	0.915	1.406	0.737
142	0.983	1.060	1.336	0.199
				0.936

Testing significance of difference between GH levels in fed and fasted ewes.

PAIRED T TEST

d = difference n = 2

Symbol	Group A	Group B
Σd	-1.675	0.936
$\bar{d}$	-0.8375	0.4680
Σd^2	1.4616	0.5828
$(\Sigma d)^2/n$	1.4028	0.4380
$\Sigma(d-\bar{d})^2$	0.0588	0.1448

Standard deviation $Sd = \sqrt{\frac{\Sigma(d - \bar{d}_A)^2 + \Sigma(d - \bar{d}_B)^2}{(n-1) + (n-1)}}$

$\Rightarrow Sd = \sqrt{\frac{0.0588 + 0.1448}{1 + 1}}$

$= 0.31906$

$$t = \frac{\bar{d}_B - \bar{d}_A}{sd} \times \sqrt{\frac{n \times n}{n + n}}$$

$$t = \frac{0.468 - (-0.8375)}{0.31906} \times \sqrt{\frac{2 \times 2}{2 + 2}}$$

$= 4.092$

$t_o = 4.092$ (d.f. = 2)

$P = 0.027$

The means are significantly different (P = 0.03)

GLUCOSE

Analysis of afternoon samples

Sheep No	Period I	Period II	Period III	Differences
		Group A		
	x	y_2	x_3	$x_1 - 2y_2 + x_3$
57	11.00	12.56	12.40	-1.72
186	10.15	9.72	11.01	+1.72
				0.00
		Group B		
	Y_1	x_2	y_3	$y_1 - 2x_2 + y_3$
116	8.71	8.93	8.77	-0.38
142	9.31	11.29	9.87	-3.40
				-3.78

Paired t Test

Symbol	Group A	Group B
Σd	0.00	3.78
$\bar{d}$	0	1.89
Σd^2	5.9168	11.7044
$(\Sigma d)^2/n$	0	7.1442
$\Sigma(d-\bar{d})^2$	5.9168	33.137

$$Sd = \sqrt{\frac{\Sigma(d-\bar{d}_A)^2 + \Sigma(d-\bar{d}_B)^2}{(n-1) + (n-1)}} \qquad t = \frac{\bar{d}_B - \bar{d}_A}{Sd} \cdot \sqrt{\frac{n.n}{n+n}}$$

$$\Rightarrow Sd = \sqrt{\frac{5.9168 + 33.137}{1 + 1}} \qquad \Rightarrow t = \frac{1.89-(0)}{4.4189} \cdot \sqrt{\frac{4}{4}}$$

$$= 4.4189 \qquad = 0.4277 \quad (d.f. = 2)$$

$$P = 0.355$$

= means are not different

= No sig. diff. betw. glucose levels after 2pm

See graph which shows fasted sheep have higher glucose on day 4
(i.e. animal x treatment interaction)

FFA

Analysis of afternoon samples. Last three each day

Sheep	Period	[FFA] mM			Σ[FFA]$_3$	$\bar{x}$	1st difference	2nd difference
57	I	0.195	0.183	0.180	0.558	0.186	-0.112	
	II	0.403	0.178	0.313	0.894	0.298	0.101	-0.213
	III	0.144	0.224	0.223	0.591	0.197		
116	I	0.180	0.544	0.358	1.082	0.361	0.179	
	II	0.180	0.133	0.234	0.547	0.182		0.337
	III	0.224	0.326	0.470	1.02	0.340	-0.158	
142	I	0.308	0.180	0.672	1.16	0.387	0.213	
	II	0.183	0.195	0.144	0.522	0.174		0.306
	III	0.266	0.268	0.268	0.802	0.267	-0.093	
186	I	0.180	0.217	0.223	0.620	0.207	-0.482	
	II	0.178	1.218	0.672	2.068	0.689		-1.035
	III	0.178	0.089	0.141	0.408	0.136	0.553	

Sheep No.	Period I	Period II	Period III	Differences
		Group A		
	X_1	Y_2	X_3	$X_1 - 2Y_2 + X_3$
57	0.186	0.298	0.197	-0.213
186	0.207	0.689	0.136	-1.035
				-1.248
		Group B		
	Y_1	x_2	Y_3	$Y_1 - 2x_2 + Y_3$
116	0.361	0.182	0.340	0.337
142	0.387	0.174	0.267	0.306
				0.643

SIGNIFICANCE TEST

Paired t Test

d = difference

Symbol	Group A	Group B
Σd	-1.248	0.643
$\bar{d}$	-0.624	0.322
Σd^2	1.1166	0.2072
$(\Sigma d)^2/n$	0.7788	0.2067
$\Sigma (d-\bar{d})^2$	0.3378	0.00048

Standard deviation $Sd = \sqrt{\frac{\Sigma(d - \bar{d}_A)^2 + \Sigma(d - \bar{d}_B)^2}{(n-1) + (n-1)}}$

$Sd = \sqrt{\frac{0.3378 + 0.00048}{1 + 1}}$

$= 0.41127$

$t = \frac{\bar{d}_B - \bar{d}_A}{Sd} = \sqrt{\frac{n\ n}{n+n}}$

$\Rightarrow t = \frac{0.322 - (-0.624)}{0.041127} = \sqrt{\frac{2 \times 2}{2 + 2}}$

$= \frac{0.946}{0.41127} = \sqrt{\frac{4}{4}}$

$= 2.300 \ . \ 1$

to $= 2.3$ (d.f. $= 2$)

P $= 0.074$

Mean FFA levels are significantly different at the 7% level

APPENDIX 4.8.3a

Regression of Log_{10} GH concentration on Log_{10} insulin concentration and analysis of covariance.

1. The regression of log_{10} GH on Log_{10} insulin was calculated for each sheep. After analysis of covariance the regression lines of sheep within treatments were not found to differ significantly so a common regression line was derived for each treatment group.

2. Upon similar analysis the regression lines for the treatment groups were found to be not significantly different; thus allowing a common regression for all 4 sheep to be derived. The line was almost flat with a very low correlation between log_{10} GH and log_{10} insulin

$$r < 0.03 \quad (P < 0.05)$$

This appendix contains only the calculations described in 1 above for treatment group A, as an example of the method used. The remainder of the analysis is essentially repetition of this basic method.

TREATMENT GROUP A

Regression of log_{10} GH ng cm^{-3} on log_{10} Insulin pg cm^{-3}

Ewe 57

	Insulin X	GH Y	
Mean	2.603	0.934	$\Sigma Y = 24.288$
S.D.	0.183	0.344	$\Sigma Y^2 = 25.772$
			$n = 26$
			$\Sigma X = 67.690$
Y intercept	= -0.503		$\Sigma X^2 = 177.096$
slope	= 0.552		$\Sigma XY = 63.712$
Correlation coeff. r	= 0.293		

Ewe 186

	Insulin X	GH Y	
			$\Sigma Y = 23.880$
			$\Sigma Y^2 = 25.888$
Mean	2.716	0.918	$n = 26$
S.D.	0.222	0.388	$\Sigma X = 70.620$
			$\Sigma X^2 = 193.097$
Yintercept	=	0.562	$\Sigma XY = 65.030$
Slope	=	0.131	
$r_{GH,I}$	=	0.075	

Correction of sums of squares and products

Ewe 57

ΣX^2	= 177.096	ΣXY	63.712	ΣY^2	= 25.772
$-\frac{(\Sigma X)^2}{n}$	= 176.228	$-C$	63.233	$-C$	22.689
Σx^2	= 0.868	Σxy	0.479	Σy^2	3.083

Ewe 186

ΣX^2	193.097	ΣXY	65.030	ΣY^2	25.838
$-C$	191.815	$-C$	64.862	$-C$	21.933
Σx^2	1.282	Σxy	0.168	Σy^2	3.905

Total (n = 52)

ΣX_T 138.310	$\bar{X}_T = 2.660$	ΣX_T^2	= 370.193	ΣXY	128.742	ΣY^2	51.610
ΣY_T 48.168	$\bar{Y}_T = 0.926$	$-C$	= 367.878	$-C$	128.118	$-C$	44.618
		ΣX^2	2.315	Σxy	0.624	Σy^2	6.992

TABLE A ANALYSIS OF COVARIANCE

Line	Source	df	Corrected sums of squares and products Σx^2	Σxy	Σy^2	Reg Coeff	Deviations from regression df	SS	MS	$r_{GH,}$ insulin
1	Ewe 57	25	0.868	0.479	3.083	0.55	24	2.819	0.117	0.293
2	Ewe 186	25	1.282	0.168	3.905	0.13	24	3.883	0.162	0.075
3	Within sheep						48	6.702	0.140	
4	Reg. Coeff.						1	0.091	0.091	
5	Common	50	2.150	0.647	6.988	0.301	49	6.793	0.139	0.167
6	Adj. Means						1	0.031	0.031	
7	Total	51	2.315	0.624	6.992	0.27	50	6.824		0.155

$SS = \Sigma y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2}$ $b = \frac{\Sigma xy}{\Sigma x^2}$ $r^2 = \frac{(\Sigma xy)^2}{(\Sigma x^2)(\Sigma y^2)}$

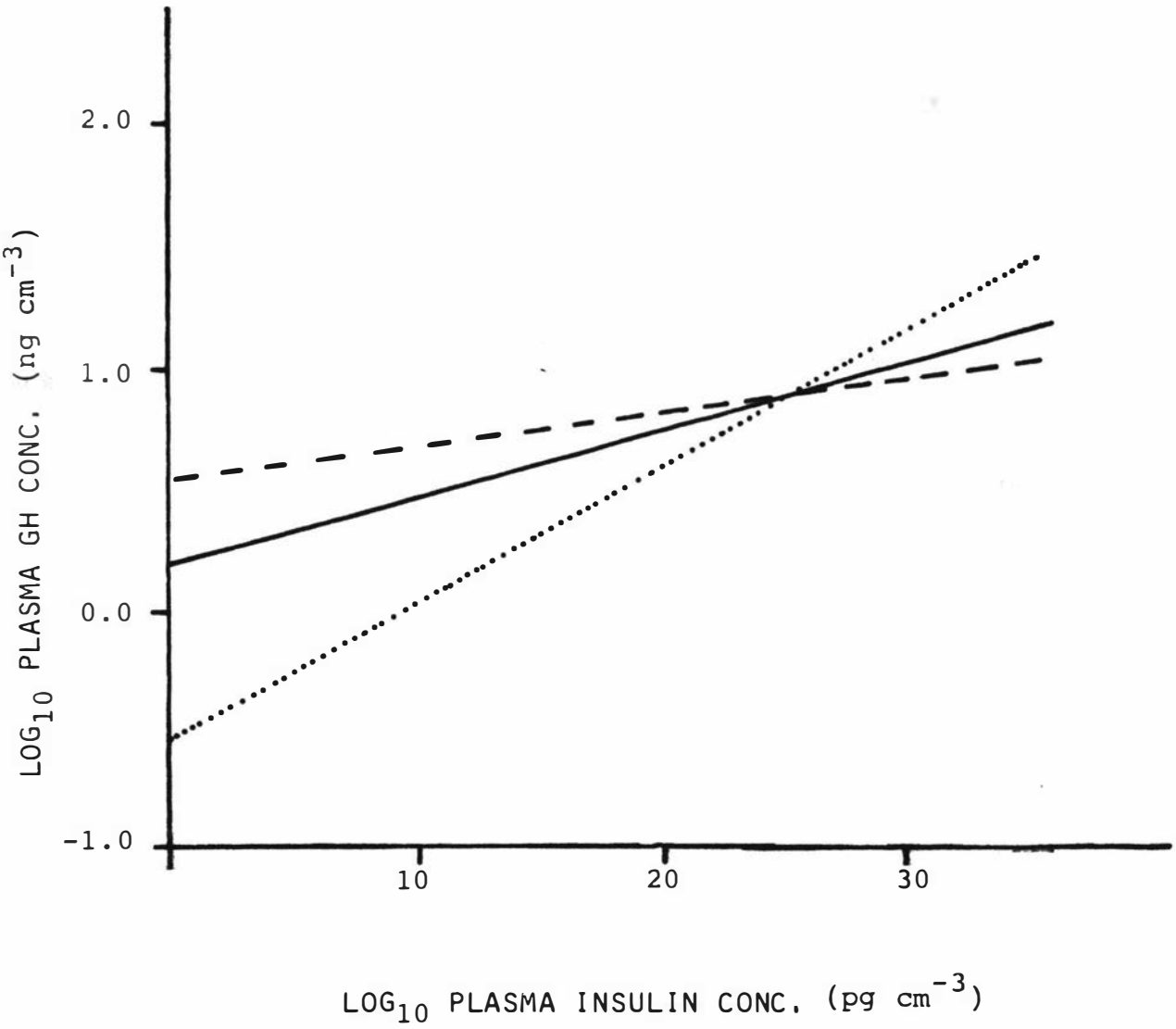

Figure A1 Slopes of regressions of Log_{10} plasma GH concentration on Log_{10} plasma insulin concentration over all sampling times for ewes 57 and 186 (group A) and of the common regression line

• • • • Ewe 57 Y = 0.552X - 0.503
– – – Ewe 186 Y = 0.131X + 0.562
——— Common line Y = 0.301X + 0.126

COMPARISON OF REGRESSION LINES

See figure A1

To determine whether the linear regression of Log_{10} GH conc. on Log_{10} insulin conc. are the same for both ewes using the method of Snedecor and Cochran (145) (section 18.9 page 385).

a) Test for differences in residual variance $\sigma_{y.x^2}$ between regressions

Ho: $\sigma^2_{57} = \sigma^2_{186}$

Test statistic $F = \frac{MS\ 186}{MS\ 57}$

From lines 1 & 2 $F = \frac{0.162}{0.117} = 1.385$ with 23, 24 d.f.

The difference is not significant (P = 0.216) so the null hypothesis is accepted i.e. the residual variance is not significantly different between the 2 ewes.

b) Test for difference in slope of regression lines

Ho: $b_{57} = b_{186}$

Test statistic $F = \frac{\text{MS for regression coefficients}}{\text{MS for within samples}}$

From lines 3 & 4 $F = \frac{0.091}{0.140} = 0.650$ with 1, 48 d.f.

The difference in slopes is not statistically significant (P = 0.42)

c) Test for differences in elevation of regression lines.

Ho: the adjusted means of the 2 sheep are the same.

$$F = \frac{\text{adjusted means MS}}{\text{Common MS}} = \frac{0.031}{0.139}$$

$= 0.223$ with 1, 49 d.f.

The adjusted means of the regressions are not significantly different (P = 0.64)

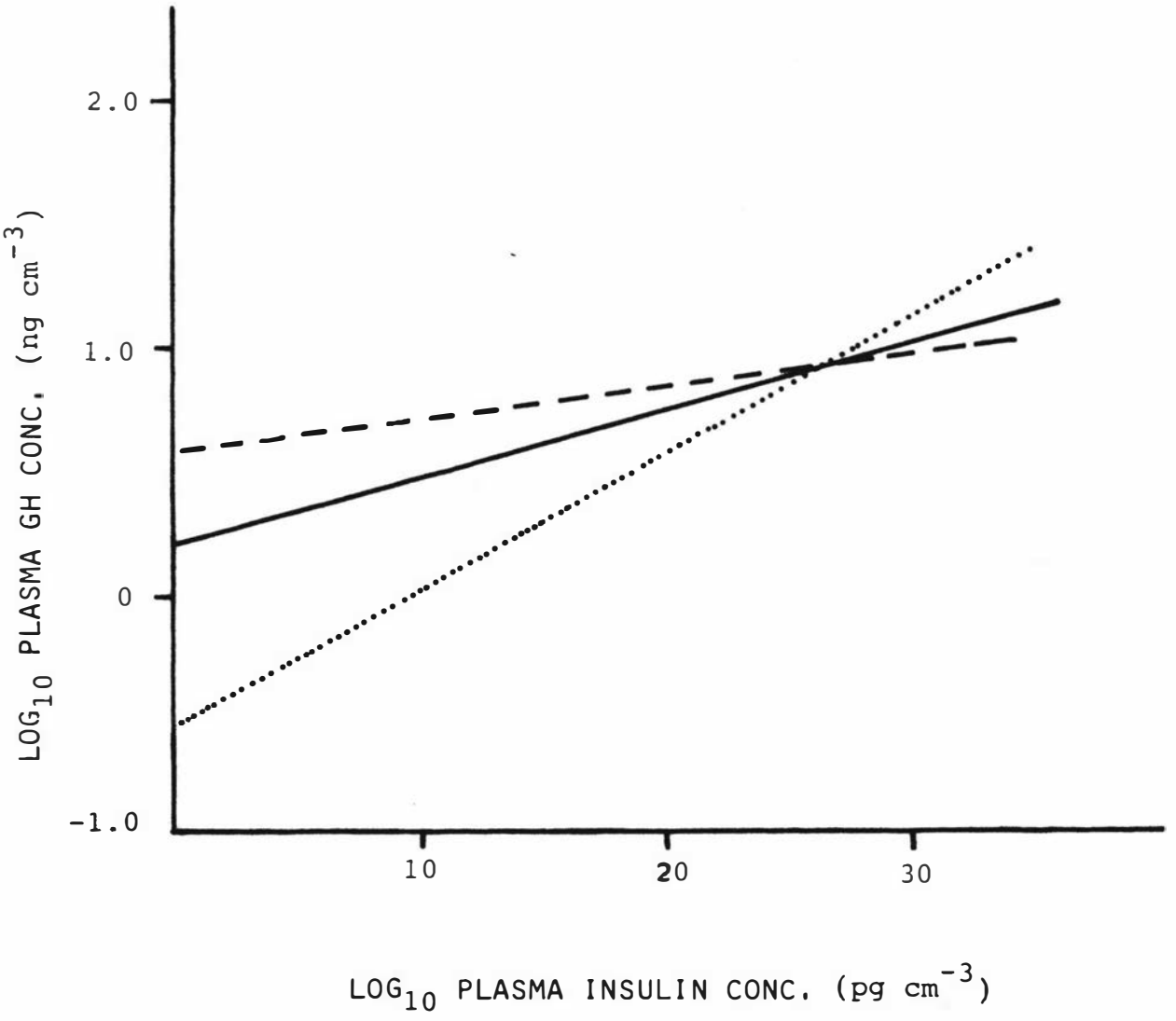

Figure A2 Slopes of regressions of Log_{10} plasma GH concentration on Log_{10} plasma insulin concentration over all sampling times for ewes 57 and 186 (group A) and of the common regression line all adjusted to a common mean.

• • • • Ewe 57 Y = 0.552X - 0.542
– – – Ewe 186 Y = 0.131X + 0.577
——— Common line

Since there is no significant difference between the regression lines of the 2 ewes in Group A it is acceptable to combine the data to form one common regression line designated regression A. The slopes of the 3 lines can be seen in Figure A2.

N.B. For comparative purposes the original regression lines have been moved to pass through the mean of the common regression. This was done by the following method. (Note that the line moved in 2 dimensions and it is not sufficient to adjust the Y intercept by the difference between the mean Y values of the 2 lines. The coordinates of the mean of the common line are $(\bar{X},\bar{Y})$ = (2.660, 0.926) (from Table A).

Ewe 57: Now when X = 2.660 the value of Y obtained from the regression equation for Ewe 57 is 0.965. When the line is adjusted to the mean Y will be 0.926. So when X = 2.660 the difference between the regression line for Ewe 57 and the common mean is 0.965 - 0.926 = 0.039. If the y intercept is decreased by 0.04 the regression line for Ewe 57 will pass through the common mean i.e.

The original regression equation for Ewe 57 was

$$Y = 0.552X - 0.503$$

The adjusted regression equation for Ewe 57 is

$$Y = 0.552X - 0.542$$

Ewe 186: When X = 2.660 Y = 0.911

Difference between lines when x = 2.66 is 0.926 - 0.911 = 0.015. Increase Yintercept by 0.015.

The original regression equation for Ewe 186 was

$$Y = 0.131X + 0.562$$

The adjusted regression equation is

$$Y = 0.131X + 0.577$$

Calculation of confidence limits of the mean.

$n = 52$ $\quad$ d.f. $= 51$

$t_{0.05} = 2.006$ $\quad$ s.d. $= \sqrt{MS} = \sqrt{0.139}$

$= 0.373 \log_{10} ngcm^{-3}$

tx sd = 2.006 x 0.373

$= 0.748 \log_{10} ngcm^{-3}$

95% confidence limits of the mean $\bar{Y} = 0.926$

0.926 ± 6.748

Limits are 0.178 to 1.674

APPENDIX 4.9.2

ANALYSIS OF VARIANCE OF THE POLYNOMIAL REGRESSION COEFFICIENT T_1

	Ewe 57	116	142	186	
Day 1	-0.0540	-0.0651	missing	-0.0744	-0.1935
2	-0.8687	0.5231	-1.4593	0.5661	-1.2388
3	0.0529	0.1204	-0.0273	-0.7541	-0.6081
Σx	-0.8698	0.5784	-1.4866	-0.2674	-2.0404
Σx^2	0.7604	0.2924	2.1303	0.8945	
$\bar{x}$	-0.2899	0.1928	-0.7433	-0.0875	

Now the missing data may be replaced by the method of Snedecor & Cochrane (145) (see p. 275).

$Xij = [3(-0.1935) + 4(-1.4866) - (-2.0404)] / [(2)(3)] = -0.7478$

So the table will now be

	Ewe 57	116	142	196	ΣX	ΣX^2
Day 1	-0.0540	-0.0651	-0.7478	-0.0744	-0.9413	0.5719
Day 2	-0.8687	0.5231	-1.4593	0.5661	-1.2388	3.4783
Day 3	0.0529	0.1204	-0.0273	-0.7541	-0.6081	0.5867
ΣX	-0.8698	0.5784	-2.2344	-0.2624	-2.7882	
ΣX^2	0.7604	0.2924	2.6895	0.8945		4.6369
$\bar{x}$	-0.2899	0.1928	-0.7448	-0.0875		

$$C = -2.7882^2\ 2/12 = 0.6478$$

Total SS = 4.6369 - C = 3.9891

Sheep SS = 6.1525/3 - C = 1.4030

Treat. SS = 2.7905/4 - C = 0.0498

Source	d.f.	SS	MS	F	d.f
Bet. sheep	3	1.4030	0.4677	0.92	3,5
Bet. treat	2	0.0498	0.0249	0.05	2,5
Residual	5*	2.5363	0.5073		
Total	10*	3.9891			

* reduced due to missing data value
see Snedecor & Cochrane (145) p 276

Correction of Treatment MS by method of Snedecor & Cochrane (145) p. 276

$$\text{Corrected MS} = \text{MS} - \frac{[-1.4866 - 2(-0.7478)]^2}{3 \times 2 \times 2}$$

$$= 0.0249 - 0.00000675$$

$$= 0.0249$$

MS remains unchanged

Between sheep F = 0.92 3,5 d.f. P = 0.50 N.S.
Between Treatments F = 0.05 2,5 d.f. P = 0.95 N.S.

APPENDIX 4.8.3b

Some examples of GH : insulin ratios calculated on afternoon samples (Experiment VII)

$$\text{Ratio of } \frac{\log_{10} \text{ GH conc.}}{\log_{10} \text{ insulin conc.}}$$

		TREATMENT	
DAY	SAMPLE	FASTED	FED
2	7	0.486	0.266
	8	0.571	0.365
	9	0.645	0.748
4	16	0.686	0.353
	17	0.534	0.442
	18	0.846	0.607

APPENDIX 4.9.3

Estimation of the possible extracellular HCO_3^- store in sheep

ECF (not including reticulo-rumen)

A 40kg ewe has about 8l of ECF. Pitts (122) gives an example of a dog of similar weight (30kg) that lost 94mEq of HCO_3^- from its extracellular store during forced hyperventilation, over a period of 60 minutes. Now venous blood of sheep has an HCO_3^- concentration of about 30mEq l^{-1} after eating and about 25mEq l^{-1} before eating (37). It would not seem unreasonable to assume a loss of about 10mEq l^{-1} in an hour of hyperventilation (respiratory compensation). i.e.8l x 10mEq/l = 80mEq in total might be lost from the ECF. Now 22.4cm³ is 1mM (1mEq) of CO_2 (STP) so 80mEq of CO_2 is 22.4 x 80 = 1792cm³ CO_2(STP). This would represent a loss of about 0.03 l. min^{-1} of CO_2. In the present experiment the sheep were producing 0.06 - 0.58 l. min^{-1} CO_2 with a mean value of about 0.3 l. min^{-1}. Thus the ECF could only alter the CO_2 production by about 10%. A 100% increase in CO_2 production is needed to explain the highest RQ value.

RETICULO-RUMEN FLUIDS

The fluids in the Reticulo-rumen provide an additional source of HCO_3^-. Turner and Hodgetts (153) recorded bicarbonate levels in ovine rumen fluid of 50mEq l^{-1} after 16 hours fasting, 4mEq l^- in fed sheep. Assuming a capacity of about 10l this would imply a loss of (50 - 4) x 10 = 460mEq (over an unknown period).

HCO_3^- will be added to the rumen fluid throughout the day in saliva. The total daily salivary secretion for the sheep will be in the range of 8-13 l and HCO_3^- will be in the range 80-140mEq l^{-1} (121). This could provide a maximum of 13 x 140 = 1820mEq of HCO_3 per day, with say 200mEq being produced in the hour after feed is given.

The sources of extracellular CO_2 then appear to be;

ECF	80 mEq	(1 hour)
Rumen	460 mEq	(unknown period)
Saliva	200 mEq	(1 hour)
	740	

If all were exhausted in the hour after feeding they would produce;

$740 \times 22.4 = 16.6\text{l } CO_2$ (STP)
or about 0.28 l min^{-1}

This would provide almost a 100% increase in CO_2 production over and above the intracellular CO_2 production and could explain the high RQ values. However it is unlikely that in a relatively normal daily feeding situation, a sheep would need to call upon its whole HCO_3^- reserve. This would only be expected in severe cases of acidosis and is unlikely to be a daily occurence. The ewes in the present experiment did not show any signs of acidosis or of any discomfort (apart from the indignity of the apparatus).
Note also that the salivary HCO_3^- would have to come from the ECF.

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