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The Hormonal Control of Lipid Metabolism in Sheep

A thesis
submitted in
partial fulfilment
of the requirements
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by

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Abstract

The hormonal control of lipid metabolism was investigated by the incubation of ovine adipose tissue slices in the presence of hormones *in vitro*. The effects of insulin, catecholamines, growth hormone (GH) and insulin-like growth factors (IGFs) on lipogenesis and lipid mobilization were studied to assess their likely individual contributions to overfatness *in vivo*.

From the various measurements of lipid metabolism made, the rates of fatty acid accumulation in the tissue during the incubation were calculated. Since most of the fatty acid released by lipolysis appeared to be re-esterified, observation of glycerol data alone may give a false impression of the extent of fatty acid mobilization or the overall energy balance of the tissue. About 60 - 100% of fatty acid released by lipolysis could be recycled within the tissue, a phenomenon which is under hormonal control and is possibly linked with maintenance of non-shivering thermogenesis.

Insulin stimulated the rate of lipogenesis (from acetate) by up to 6-fold, with the average stimulation about 70%, but it failed to affect the rate in tissue from one quarter of the animals tested. Also insulin stimulated the retention of fatty acid by the cell as well as fatty acid recycling, even in tissue in which it was not lipogenic. Thus observation of lipogenesis alone can give a false impression of the lipid-storing effects of a hormone.

IGFs, when present at high concentrations, were lipogenic. However, because the binding proteins may restrict the activity of IGFs *in vivo*, low concentrations of IGF were tested and shown to be lipolytic. It is suggested that whereas the high concentrations of IGFs have been thought to act via the insulin receptor, low concentrations may mediate the lipolytic response via the IGF receptor. Similar states of negative energy balance in the tissue were caused by the presence of either IGF I, IGF II or adrenalin in the medium.

GH sometimes stimulated a transient increase in the rate of lipolysis. It is suggested that GH may stimulate the local release of IGF *in vitro* which then mediates the observed lipolytic activity. GH was also sometimes observed to be lipogenic over the 6 hour incubation period.

To investigate the effects of a known physiological change in the animal *in vivo* on the subsequent effects on lipid metabolism *in vitro*, GH was administered to animals and their adipose tissue was compared with tissue from control animals. The basal rate of lipogenesis and fat accumulation *in vitro* was depressed in the bGH-dosed animals as was deposition of fat *in vivo*. The effectiveness of insulin in increasing lipogenesis *in vitro* was reduced in the bGH-dosed animals although the percentage response was unaffected. Thus the "lipolytic" effects of exogenous GH may in fact be due to a reduction in lipogenesis, caused by possible post-receptor desensitization of the tissue to the lipogenic effects of insulin as well as to the presence of increased IGF *in vivo*. Thus the physiological state of the animal can have profound and lasting effects on lipid metabolism *in vitro*.

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List of Abbreviations

ACP	acyl carrier protein
ADP	adenosine diphosphate
AMP	adenosine 3':5'-monophosphate
ATP	adenosine triphosphate
bGH	bovine growth hormone
BSA	bovine serum albumin
CoA	coenzyme A
COV	coefficient of variance
CPM	counts per minute
CR	channel ratio
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
FA	fatty acid
FFA	free fatty acid
GH	growth hormone
G-6-PDH	glucose-6-phosphate dehydrogenase
GR	a measure of fatness *in vivo* (see Section 3.2 for definition)
HEPES	N-2-hydroxy-ethylpiperazine-N^1-ethanesulphonic acid
hGH	human growth hormone
hIGF I	human insulin-like growth factor I
hIGF II	human insulin-like growth factor II
ICDH	isocitrate dehydrogenase (decarboxylating)
IGF I	insulin-like growth factor I (somatomedin C)
IGF II	insulin-like growth factor II
IMCC	International Minerals and Chemical Corporation

MDH	NADP-malate dehydrogenase (decarboxylating)
MSA	multiplication stimulating activity (rat IGF II)
MW	molecular weight
n	number of observations
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NEFA	non-esterified fatty acid
NS	not significant
oGH	ovine growth hormone
p	probability
PPO	2,5-diphenyloxazole
r	correlation coefficient
RIA	radioimmunoassay
sd	standard deviation
SDS	sodium dodecyl sulphate
S.E.D.	standard error of the difference
S.E.M.	standard error of the mean
TC	total count
TCA	tricarboxylic acid
TG	triacylgylcerol
v/v	volume to volume
w/v	weight to volume
w/w	weight to weight
6-PGDH	6-phosphogluconate dehydrogenase

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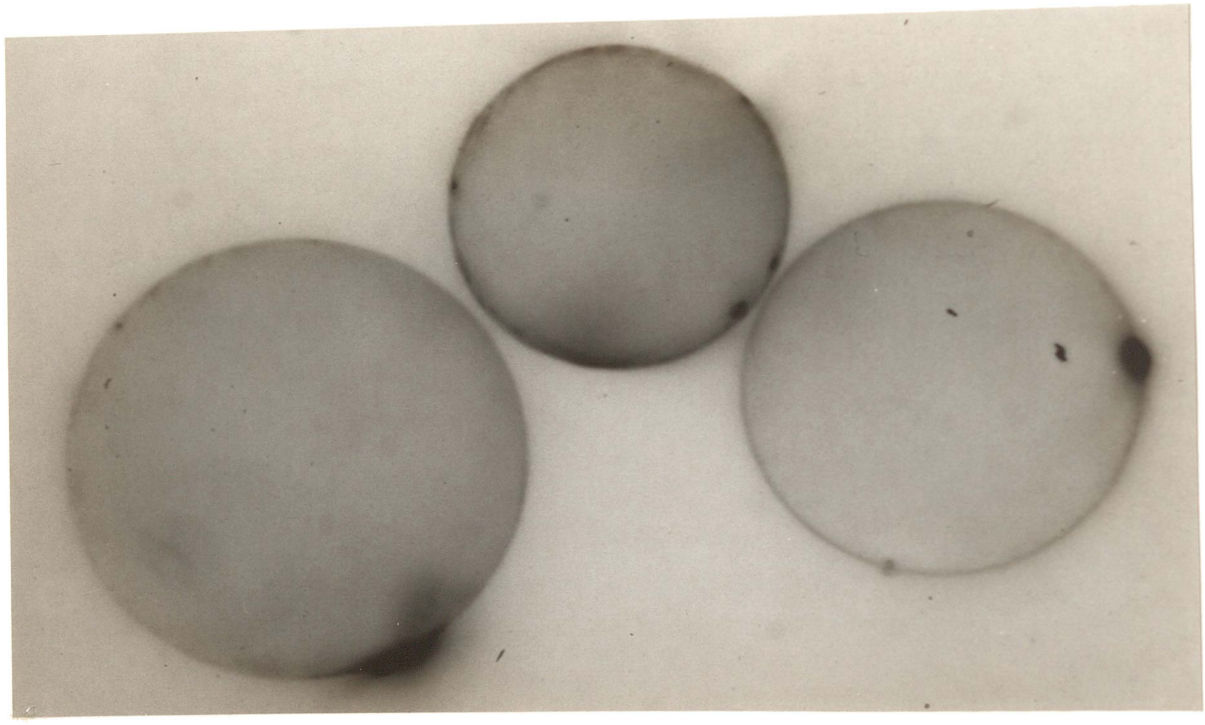


Plate 1.1 A photomicrograph of an adipocyte

A photomicrograph of an ovine adipocyte illustrating how the large globule of lipid in the cell compresses the cytoplasm and nucleus to the periphery of the cell. The nucleus can be seen to protrude out the side of the cell.

This photomicrograph was taken and kindly provided by Dr R.F. Thornton.

Chapter 1

Introduction

It is well known that excess food intake and lack of exercise lead to increased storage of lipids in the adipose tissue. However, there are some individuals within breeds (in domestic animals) and races (in humans) that appear to have a greater propensity to fatten than others.

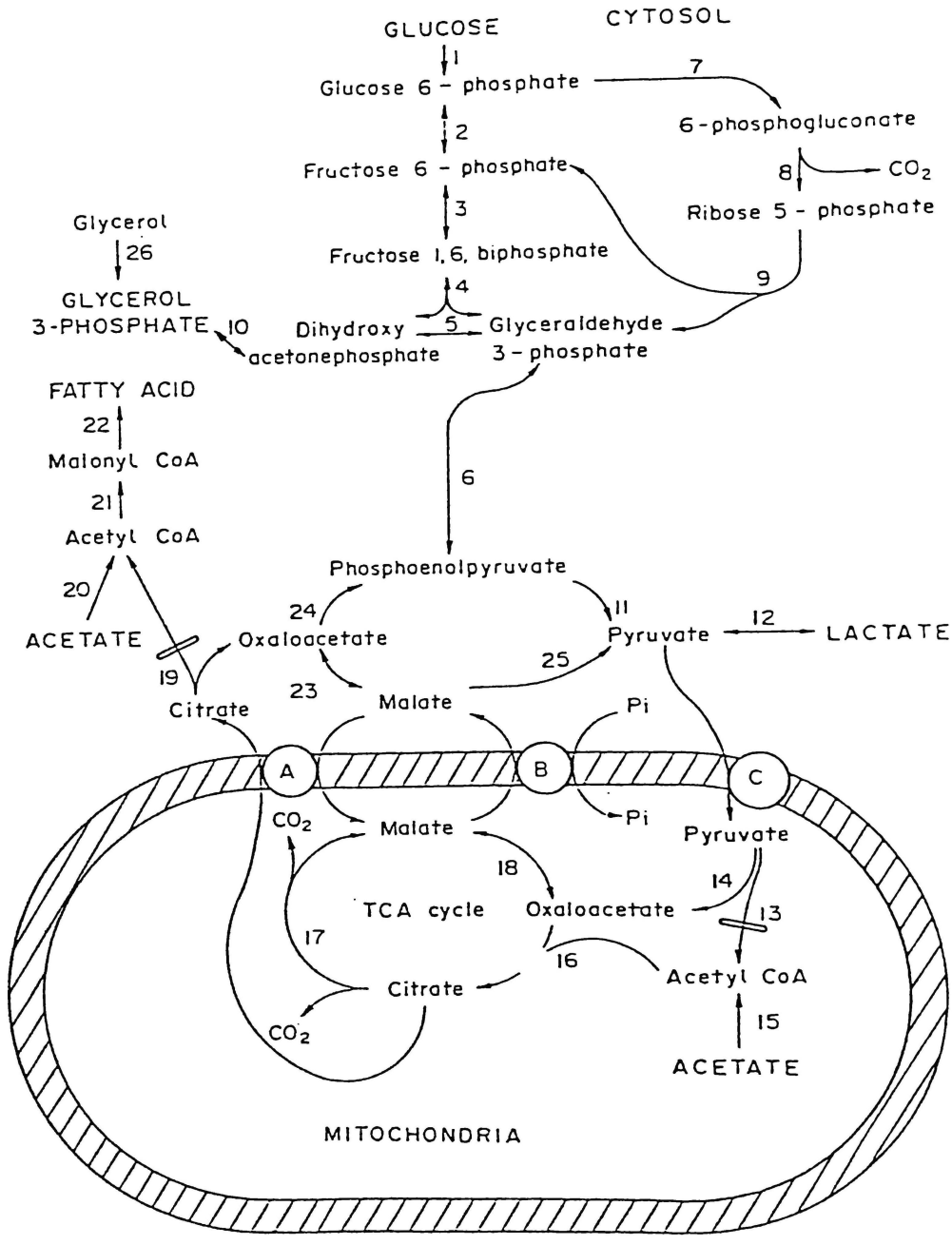
This characteristic of some adipose tissues to retain lipid more than others is influenced by hormones. Although the major hormones involved are known, their functions in controlling the rate of lipid accumulation are not as yet fully understood. There are also undoubtedly many other endocrine factors which play a role in the adipose tissue and determine the overall rate of metabolism there but which have not yet been fully characterised.

Once all the hormones involved in the process have been identified and their relative importance determined, it is possible that the knowledge gained could be exploited both in the area of obesity in humans and to control over-fatness in meat-producing animals.

Obesity and its contribution towards heart disease and other complications has become one of the major health concerns of the twentieth century and any reduction in the amount of fat we ingest with our meat will benefit the health of many. Increased knowledge of the control mechanisms of lipid accumulation in adipose tissue may lead to the development of drugs to safely control obesity. There is also the possibility that these could be used, via implants, to control overfatness in meat-producing animals.

Still further in the future is the possible exploitation of the technology of gene manipulation. If the genes controlling the production of appropriate hormones could be manipulated then the resulting animal would produce only meat with a very low fat content. Through cloning and interbreeding, the entire domestic flock could be producing only lean meat in a very short time. However, the successful utilization of this technology in the future will depend on the knowledge we gain now about the overall endocrine control of lipid metabolism in adipose tissue.

Figure 1.1 Lipid metabolism in adipose tissue of ruminants



from Vernon, 1980.

Figure 1.1 Lipid metabolism in adipose tissue of ruminants

Pathways for the synthesis of fatty acids and glycerol-3-phosphate in adipose tissue. Principle products and precursors are in block letters; arrows indicate the direction of flow through a pathway. Bars (reactions 13 and 19) indicate blocking by low enzyme activities.

ENZYMES:

1. hexokinase
2. hexose isomerase
3. phosphofructokinase
4. aldolase
5. triose phosphate isomerase
6. other enzymes of glycolysis
7. glucose-6-phosphate dehydrogenase
8. 6-phosphogluconate dehydrogenase
9. other enzymes of the pentose phosphate pathway
10. glycerol-3-phosphate dehydrogenase
11. pyruvate kinase
12. lactate dehydrogenase
13. pyruvate dehydrogenase
14. pyruvate carboxylase
15. acetyl CoA synthetase (mitochondrial)
16. citrate synthetase
17. other enzymes of the tricarboxylic acid cycle
18. NAD-malate dehydrogenase (mitochondrial)
19. ATP-citrate lyase
20. acetyl CoA synthetase (cytosolic)
21. acetyl CoA carboxylase
22. fatty acid synthetase
23. NAD-malate dehydrogenase (cytosolic)
24. phosphoeenolpyruvate carboxykinase
25. NADP-malate dehydrogenase (malic enzyme)
26. glycerol kinase. Pi phosphate

TRANSLOCASES:

A. tricarboxylic acid translocase
B. bicarboxylic acid translocase
C. pyruvate translocase

PATHWAYS:

Glycolytic pathway: reactions 1-6 and 11.
Gluconeogenesis: reactions 1-6,11 and 12.
Pentose phosphate pathway (for NADPH production): reactions 7 to 9.
Tricarboxylic acid (TCA) cycle: reactions 17 to 18.
Isocitate dehydrogenase cycle is not shown.

1.1 Lipid metabolism in ruminants

Ruminant Nutrition

The pattern of ruminant nutrition differs from that of monogastric animals owing to the metabolic activities of the rumen microorganisms which degrade essentially all the dietary carbohydrate to acetic, propionic and butyric acids. During passage through the gut wall, most of this is converted to acetate and some propionate and butyrate. As a result, acetate is the major precursor of lipid biogenesis (lipogenesis) in non-lactating ruminants, in contrast to non-ruminants that utilize glucose (Hood *et al.*, 1972; Mayfield *et al.*, 1966).

Lipogenesis

Adipose tissue is the major site of fatty acid synthesis in the non-lactating ruminant (Ingle *et al.*, 1972). During times of plentiful food intake, the adipose tissue acts as a store of excess energy which can be mobilized by lipolysis during times of famine.

Precursors of lipogenesis

Vernon (1979b) showed that, ovine adipose tissue *in vitro*, the incorporation of glucose into fatty acids was 2.2 nmoles/h/g tissue while that of acetate was 588 nmoles/h/g tissue. After three days of incubation, glucose had contributed only about 5% of carbon for lipogenesis, the rest being derived from acetate. However, under certain circumstances, glucose can be utilized by ruminant adipose tissue for the synthesis of fatty acid. Vernon (1979b) demonstrated that with adipose tissue *in vitro*, in the presence of insulin and excess glucose, fatty acid synthesis from glucose increased several fold. Abomasal and intravenous infusion of glucose (Ballard *et al.*, 1972) increased the rate of fatty acid synthesis from both glucose and acetate. These studies seem to indicate that the biochemical pathways for the utilization of glucose (see Figure 1.1), as seen in non-ruminants, are essentially in place in the ruminant but lack of glucose prevents them from being used.

Pathway of lipogenesis

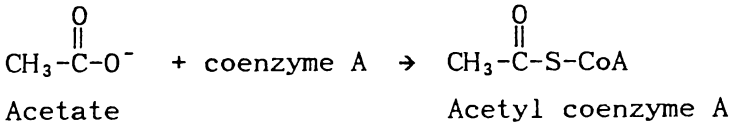
Outline

The process of lipogenesis involves the combination of seven molecules of malonyl CoA with one molecule of acetyl CoA to form a long chain fatty acid with 16 carbon atoms (palmitic acid). The cofactor NADPH is required to provide H atoms. Synthesis of fatty acid occurs in the cytosol of the cell. The overall equation for the process is:

$$1\ \text{Acetyl CoA} + 7\ \text{Malonyl CoA} + 14\ \text{NADPH} + 14\ H^+ \rightarrow$$
$$1\ \text{Palmitic acid} + 7\ CO_2 + 8\ \text{CoA} + 14\ NADP^+ + 14\ H_2O$$

Formation of acetyl CoA

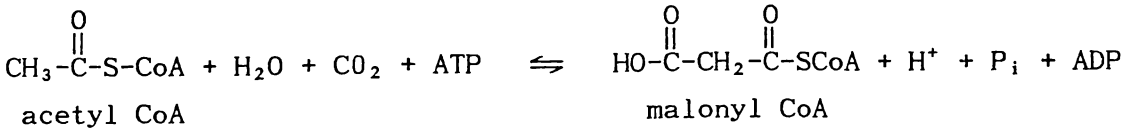
The enzyme acetyl CoA synthetase (Number 20 in Figure 1.1) catalyses the reaction where acetate combines with coenzyme A (CoA) to form acetyl coenzyme A:



Acetate — Acetyl coenzyme A

Formation of malonyl CoA

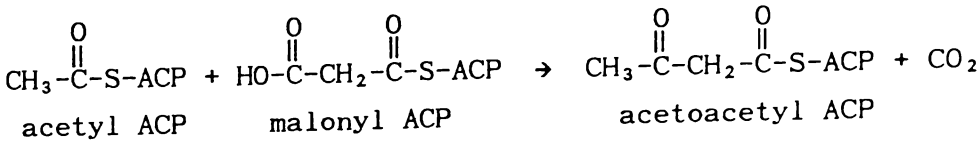
The enzyme acetyl CoA carboxylase in the presence of bicarbonate and Mn^{++}, catalyses the formation of malonyl CoA from acetyl CoA:



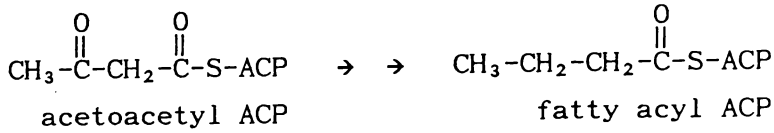
acetyl CoA — malonyl CoA

Lipogenesis and the acyl carrier protein

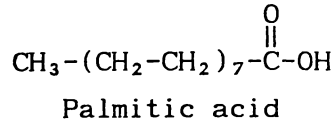
A single molecule of acetyl CoA is required to initiate the process. First acetyl CoA and then malonyl CoA are esterified to two sulphur-hydride end groups of the acyl carrier protein (ACP). ACP is a small protein located at the centre of the seven-enzyme fatty acid synthetase cluster where all subsequent steps of lipogenesis occur. The process which then takes place is essentially a cyclic carbon-chain elongation.



After the two carbon acetyl group has been added to the growing chain, hydrogenation takes place at the expense of NADPH to convert its carbonyl group into a $-CH_2-$ group.



This represents the first cycle of the chain lengthening process which occurs during lipogenesis. Malonyl CoA is added to the fatty acyl ACP chain, lengthening it by two carbon atoms during each cycle. Seven cycles normally occur, resulting in the 16-carbon chain of palmitic acid.



All other fatty acids are derived from palmitic acid, with stearic, oleic and palmitic acids being the most commonly found in ovine adipose tissue (Deeth and Christie, 1979).

Gluconeogenesis and glucose-sparing in ruminants

The predominant use of acetate as a precursor for fatty acid synthesis in the ruminant allows for the conservation of glucose. With the animal having no reliable exogenous source of glucose, most of the blood glucose is produced by gluconeogenesis (Ballard *et al.*, 1969). The majority of tissues, with the exception of the brain, have reduced capacities to utilize glucose so that gluconeogenesis can supply the animal's requirements for glucose. Glucose is needed by the adipose tissue for the production of NADPH and glycerol-3-phosphate, with the remainder being released as lactate. Vernon (1979b) showed that lactate

was the principle product of glucose metabolism by ovine adipose tissue *in vitro*. This is the major difference between ruminant and non-ruminant metabolism because in the non-ruminant most of the glucose is oxidized in the TCA cycle.

The difference would appear to lie in the comparative activities of the enzyme, pyruvate dehydrogenase (No.13 in Figure 1.1) which plays a critical role in non-ruminant glucose metabolism. It has been found (Vernon, 1980; Read *et al.*, 1977) that in ruminants, the activity of this enzyme is extremely low when compared with that of non-ruminants. This low activity could provide an effective blocking mechanism so that pyruvate derived from glucose would not be oxidized by the TCA cycle but would instead be converted to lactate.

Annison *et al.* (1963) demonstrated the recycling of the synthesized lactate back to glucose *in vivo*. This is a glucose-sparing mechanism in the ruminant and would explain the low rate of glucose oxidation via the TCA cycle. The ATP required for lipogenesis in ruminant adipose tissue is obtained by oxidation of acetate and fatty acids in the mitochondria (Vernon 1980).

The liver has a high rate of gluconeogenesis. Bergman (1973) demonstrated that, in non-pregnant fed sheep, there was a large glucose production (4.5 g/h) in the liver but only a small utilization (0.8 g/h). The liver uses propionate and to a lesser extent butyrate to manufacture glucose.

Fatty acid turnover within adipocytes

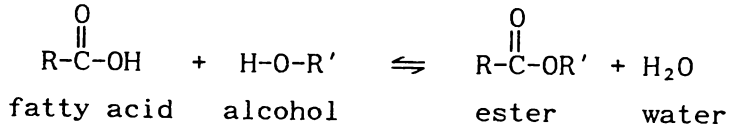
The fate of fatty acid within the adipocyte is outlined in Figure 3.8.

Glycerol and esterification

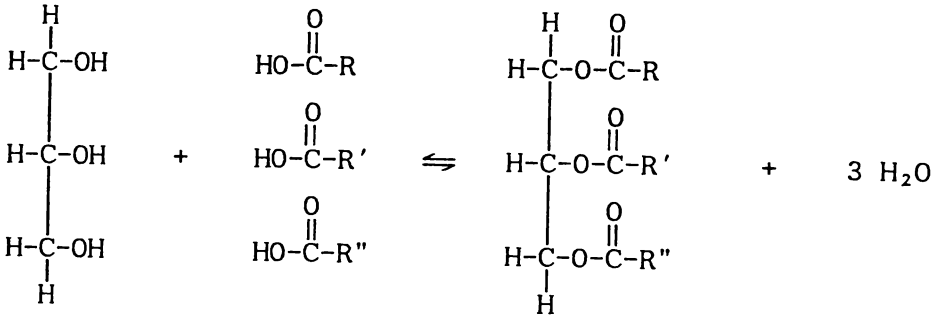
Glucose is used in adipose cells for the manufacture of dihydroxy-acetonephosphate, a specific precursor for glycerol-3-phosphate used in esterification.

It has been reported (Hood *et al.*, 1972; Metz *et al.*, 1973; Khadachurian *et al.*, 1967) that the activity of the enzyme glycerol kinase (No. 26 in Figure 1.1), which catalyses the conversion of free glycerol to glycerol-3-phosphate, is extremely low in ruminant adipose tissue. According to Vernon (1980), this means that essentially all the glycerol requirements of the tissue must be met by glucose metabolism. Glycerol released by lipolysis cannot be used by the adipose cells for esterification and instead passes into the bloodstream.

The process of esterification of fatty acids results in the formation of mono-, di- and triacylglycerol. The process involves the reaction of a fatty acid with an alcohol:



Glycerol has three alcohol groups and can thus form triacylglycerol, the form in which most fatty acids are stored in the adipose tissue.



1 glycerol + 3 fatty acid ⇋ 1 triacylglycerol + 3 water

The fatty acids for this process can come from any of three sources:

(i) *de novo* synthesis within the cell

(ii) as a result of lipolysis occurring either in the same cell or in other cells.

(iii) from lipids available in the diet. This source is especially important in the suckling animal since milk is a rich source of fatty acids (Vernon, 1977a).

The rate of esterification is influenced by fatty acid concentration (Benson and Emery, 1971) and glucose availability (Payne and Masters, 1971). The latter probably occurs because it is required for the production of glycerol-3-phosphate.

Lipolysis

The triacylglycerol stored in adipose tissue constitute the principle reservoir of substrate for oxidative metabolism in the entire body. Mobilization, distribution and oxidation of these fatty acids can occur rapidly enough to support as much as 50% of the metabolism of the body during periods of starvation, exposure to cold, exercise, reproduction and growth (known as times of "negative energy balance").

Fat is mobilized from the adipose tissue in the form of free fatty acids (FFA). The amount of free fatty acid within the cell is normally very small and any increase in the availability of free fatty acid is accomplished by the breakdown of the triacylglycerol molecules into their component fatty acid and glycerol molecules, a process known as lipolysis. It is possible that the processes of lipolysis and fatty acid release from the cells are under hormonal control. However, the rate is also determined by the rate of build-up of fatty acid inside the cell.

Free fatty acids have very limited solubility in aqueous solutions and they cannot pass out of the cell unless there is albumin available as an acceptor molecule. Non-esterified fatty acids (NEFA) are transported to other cells in the blood attached to proteins.

It is apparent that the fatty acids within the adipocyte are in a dynamic state of flux where triacylglycerol can be broken down to fatty acids which can be re-incorporated into triacylglycerol (re-esterified) without ever leaving the cell. The synthesis of new fatty acid and triacylglycerol and their subsequent breakdown by lipolysis continue to occur within the cell under virtually all conditions.

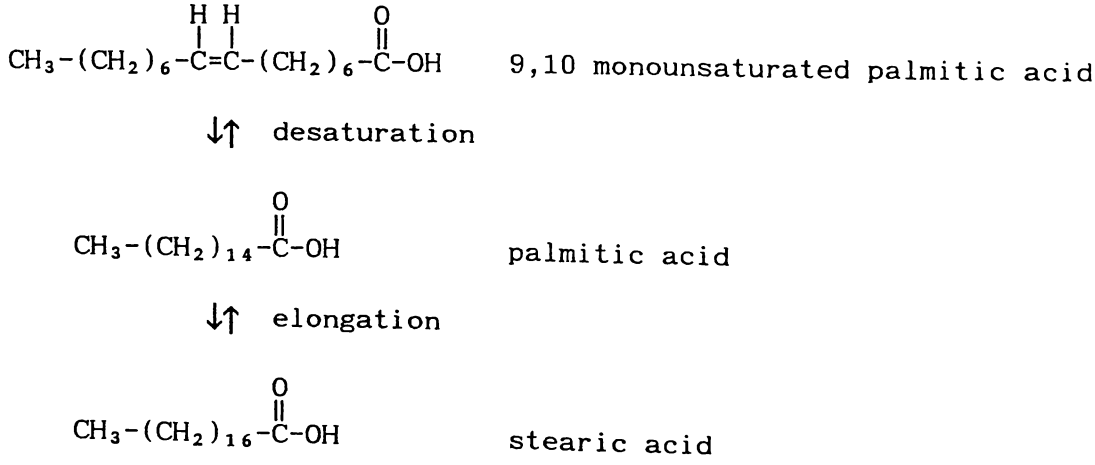
Fatty acid oxidation

Palmitic acid oxidation has been reported to occur in bovine adipose tissue *in vitro* at a rate of 0.13-0.15 nmol/min/g tissue, a rate markedly less than the rate of acetate oxidation observed during the same study *viz* 3 nmol/min/g tissue (Baldwin *et al.*, 1973). However, the oxidation of a mole of palmitic acid can theoretically yield 129 moles of ATP compared with 11 moles from the oxidation of a mole of acetate. This suggests that fatty acid oxidation in the above study would yield about 75% as much ATP as acetate oxidation under the same conditions (Vernon, 1980).

Chain elongation and desaturation

Fatty acid chain elongation activity has been demonstrated by the formation of stearic and oleic acids from ^{14}C-acetate in ovine (Deeth and Christie, 1979) and bovine (Pothoven *et al.*, 1974) adipose tissue *in vitro*. During the process of chain elongation, the fatty acid is first converted to acyl CoA and then reacts with malonyl CoA, followed by reduction with an NADPH-requiring enzyme. The intermediates are similar to those in palmitic acid synthesis except that they are not bound to the acyl carrier protein in the synthetase complex.

The subsequent specific desaturation of these long-chain saturated fatty acids to their 9,10,cis-monounsaturated derivatives has also been demonstrated in ruminant adipose tissue (Deeth and Christie, 1979; Payne and Masters, 1971; Pothoven *et al.*, 1974) eg:



The production of NADPH

Introduction

The hydrogen atoms required during the synthesis of fatty acid are provided via NADPH. Fourteen molecules of NADPH are required for the synthesis of one molecule of palmitic acid. From this it can be calculated that, in ruminant adipose tissue, an NADPH production rate of 5-160 nmol/min/g tissue is required (Vernon, 1980).

The principle enzymes for NADPH production, all of which have been shown to be present in ruminant adipose tissue, are glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-malate dehydrogenase (decarboxylating) and NADP-isocitrate dehydrogenase (decarboxylating) (Martin *et al.*, 1973).

Pentose phosphate cycle

It is generally thought that, as in the rat, the major source of NADPH for lipogenesis in the ruminant is the pentose phosphate cycle.

Bartos and Skarda (1970) estimated that, in goat adipose tissue, in the presence of acetate and insulin, 29% of glucose utilized was via the pentose phosphate cycle. Baldwin *et al.* (1973) calculated that, even if all glucose oxidation to CO_2 occurred via the pentose phosphate cycle in bovine adipose tissue slices, it could provide only 75-80% of the NADPH required for lipogenesis, although other studies in sheep (Vernon, 1976) suggest that the pentose phosphate cycle would provide more than enough NADPH for lipogenesis. Although the precise proportions are still uncertain, it is now assumed that the pentose phosphate pathway is the major source of NADPH for lipogenesis in ruminant adipose tissue.

Isocitrate dehydrogenase cycle

This cycle appears to be responsible for providing the NADPH deficit left by the pentose phosphate cycle (Baldwin *et al.*, 1973; Ingle *et al.*, 1972). The isocitrate dehydrogenase cycle involves the translocation of citrate (or isocitrate) from the mitochondria into the cytosol where it is oxidized with the production of NADPH. It is returned to the mitochondria in the form of 2-oxoglutarate. Once back in the mitochondria, the 2-oxoglutarate can be directly reduced to citrate or it can be metabolized normally via the TCA cycle. Gumma *et al.* (1973) has suggested that, if 50% of the 2-oxoglutarate were metabolized by each route, then oxidation of one molecule of acetate in the mitochondria would produce two molecules of NADPH in the cytosol. However citrate, isocitrate and 2-oxoglutarate do not diffuse through the mitochondrial membranes but are transported by specific translocases. Both of these translocases require that malate is simultaneously transported in the opposite direction.

The use of the isocitrate dehydrogenase cycle for the production of NADPH in ruminant adipose tissue accounts for the observed ability of those cells to synthesise fatty acids from acetate in the absence of glucose (Ballard *et al.*, 1972; Hanson and Ballard, 1967; Ingle *et al.*, 1972; Yang and Baldwin, 1973a).

Enzymes involved in lipid metabolism

Lipogenesis

The activity of the enzyme acetyl CoA carboxylase, a key lipogenic enzyme involved in the production of malonyl CoA from acetyl CoA, is dependent on the presence of citrate or isocitrate. The inactive form of the enzyme consists of protomers of 400 000 to 500 000 daltons. When activated by citrate or isocitrate, the enzyme polymerizes to a filamentous form of 5 to 10 million daltons. The polymerized form will in turn depolymerize to its inactive form in the presence of malonyl CoA and long-chain fatty acyl CoA. Since malonyl CoA seems to have no other role besides that which it plays in lipogenesis, the control of the activity of acetyl CoA carboxylase will effectively control the rate of lipogenesis without interfering with any other pathway.

When lipogenesis occurs there will be an increase in the concentrations of long-chain fatty acyl CoA. If the concentrations of these molecules becomes excessive, and esterification and fat deposition are not proceeding, then lipogenesis could be slowed by the decrease in activity of the enzymes involved. This is an example of product control of lipogenesis.

The enzymes involved in the conversion of acetate and glucose to fatty acids are thought to be the same as those present in non-ruminants (Vernon, 1980), although the proportions differ.

(i) Acetyl CoA synthetase (No.20 in Figure 1.1):
This enzyme, which is involved in the conversion of acetate to acetyl CoA, has been found in the mitochondria and cytosol of ruminant cells (Ballard, 1972), and is thought to be involved in lipogenesis. Activities of 40-150 nmoles converted/min/g tissue have been reported for ovine adipose tissue (Ballard, 1972; Hanson and Ballard, 1967; Ingle *et al.*, 1973; Vernon, 1976).

(ii) Acetyl CoA carboxylase (No.21 in Figure 1.1):
Most studies suggest that this is the least active of the three enzymes directly involved in fatty acid synthesis from acetate and thus it plays a crucial role in determining the rate of lipogenesis in the adipose cell. The maximal rate of lipogenesis has in fact been shown to correspond to the activity of acetyl CoA carboxylase. In ovine adipose tissue slices *in vitro*, rates of acetate incorporation in the range 15-74 nmoles/min/g tissue have been reported (Ballard *et al.*, 1972; Ingle *et al.*, 1972; Vernon, 1976; Ingle *et al.*, 1973). The corresponding activity of acetyl CoA carboxylase in the same tissue was 12-89 nmoles converted/min/g tissue at 37°C (Ingle *et al.*, 1973; Vernon, 1976).

(iii) Fatty acid synthetase:
Fatty acid synthetase (or palmitate synthetase) is actually a complex of seven enzymes which are involved in the conversion of acetate to fatty acids. Activities of between 70 and 400 nmoles converted/min/g tissue at 37°C have been reported for this enzyme complex (Ingle *et al.*, 1973; Vernon, 1976).

Lipolysis

The regulation of the degradation of adipose tissue lipid, the process of lipolysis, is achieved by control of the activity of the rate-limiting enzyme involved in lipolysis: the hormone-sensitive lipase. Hormone-sensitive lipase catalyses the first step of lipolysis involving the degradation of the triacylglycerol molecule to diacylglycerol, releasing a free fatty acid, as well as the second step when diacylglycerol is degraded to monoacylglycerol, releasing a further fatty acid molecule. The monoacylglycerol is then broken down into glycerol and a third fatty acid molecule by the action of the enzyme, monoacylglycerol lipase.

Factors which regulate the mode of action of lipogenic enzymes

The rate of lipogenesis is altered by a number of factors which include the concentrations of substrate, product, cofactors and hormones. All these regulate the rate of lipogenesis by altering either the amounts or the activities of the rate-determining enzymes.

Substrate concentration

Acetate

It is probable that changes in the blood acetate concentration will lead directly to changes in the rate of acetyl CoA production and hence the rate of lipogenesis (Vernon, 1980).

Although in non-ruminants (rat), the presence of acetate stimulates fatty acid synthesis from glucose (Saggerson, 1972), it appears that, with bovine adipose tissue *in vitro*, the presence of acetate inhibits the rate of fatty acid synthesis from glucose (Whitehurst *et al.*, 1978).

Glucose

Addition of glucose increased the rate of fatty acid synthesis from acetate 3 to 10-fold in ruminant adipose tissue *in vitro* (Ballard *et al.*,1972; Ballard *et al.*, 1969; Hood *et al.*, 1972; Vernon, 1977b; Whitehurst *et al.*, 1978; Yang and Baldwin, 1973a). Insulin was necessary

for this effect to be observed, although it was subsequently confirmed that it was glucose and not insulin that was the prime stimulant for an increased rate of lipogenesis (Vernon, 1977b, 1980). This effect was thought to be due to increased NADPH and glycerol-3-phosphate production.

Lactate and pyruvate

The incorporation of lactate into tissue fatty acids in sheep *in vivo* (Prior *et al.*, 1978) and in ovine adipose tissue slices *in vitro* (Vernon, 1980) have been demonstrated. Pyruvate was also incorporated into fatty acids in adipose tissue slices from steers (Whitehurst *et al.*, 1978).

Product concentration

Fatty acids originating from a high fat diet can lead to decreased rates of lipogenesis. This has been demonstrated with ovine adipose tissue (Vernon, 1977b; Deeth and Christie, 1979), with exogenous stearic acid being the most potent inhibitor, while palmitic, oleic, linoleic and linolenic acids also affected the rate. This effect could be achieved by conversion of the enzyme, acetyl CoA carboxylase, into the inactive form. During these studies *in vitro*, a fatty acid concentration over 400 μmol/l was required before lipogenesis was inhibited, although normal plasma concentrations of NEFA in fed sheep are 100-400 μmol/l. However, as a result of increased lipolysis or lipogenesis, or alternatively decreased production of NADPH and glycerol-3-phosphate from glucose, it is quite probable that cytosolic fatty acids in the adipose cells could reach higher concentrations than this and would thus inhibit further production of fatty acid. It is in this way that inhibition of acetyl CoA carboxylase activity by fatty acids is thought to play such a critical role in the regulation of lipogenesis in ruminant adipose tissue.

Cofactor availability

ATP

ATP is required for several steps in the process of lipogenesis. It is produced in the mitochondria by the oxidation of acetate and fatty acids, and in the cytoplasm by glycolysis.

In the non-ruminant, where glucose is the source of acetyl CoA for lipogenesis, fatty acid synthesis results in a net production of ATP. As a result, the cell's ability to utilize the excess ATP could limit the rate of lipogenesis (Flatt, 1970a, 1970b). On the other hand, in the ruminant, where acetate is used to form acetyl CoA, and if NADPH is produced by the pentose phosphate cycle, there will be a net utilization of ATP (Bauman and Davis, 1975). If NADPH is produced by acetate oxidation via isocitrate dehydrogenase and the TCA cycle, net production of ATP would occur. It is not known whether the cell's ability to utilize ATP ever becomes rate-limiting but there is a continuing requirement for ATP for esterification of fatty acids (Vernon, 1980).

NADPH

Although the stimulatory effect of glucose on ruminant lipogenesis *in vitro* is thought to be partly due to increased NADPH production, it is still not clear whether NADPH limits lipogenesis *in vivo*. Since NADPH can be produced by any of several pathways, a deficiency from one source can be made up from another. During periods of reduced glucose availability (such as poor diet or diabetes), other regulatory mechanisms will probably limit lipogenesis before NADPH production becomes limiting (Vernon, 1980).

Summary

The rate of lipogenesis must vary with the changing physiological state of the animal and this is brought about by a variety of methods. Concentrations of plasma acetate would appear to be a critical factor in the short-term regulation of lipogenesis in ruminants. Glucose availability for NADPH or glycerol-3-phosphate production may become rate-limiting while concentrations of fatty acids (or their CoA esters) are potential inhibitors of acetyl CoA carboxylase.

Acetate is the most important precursor for lipogenesis in ruminant adipose tissue. Although the low rate of fatty acid synthesis from glucose in ruminants can be explained in terms of glucose-sparing, all the regulatory mechanisms which result in the exclusion of this pathway are not as yet fully understood.

In many respects, glucose metabolism in adipose tissue of the fed ruminant resembles that of the fasted rat. There is in each:

(i) a low rate of fatty acid synthesis from glucose.
(ii) a high lactate output
(iii) probably a low pyruvate dehydrogenase activity
(iv) a low ATP citrate lyase activity
(v) a low insulin:glucagon ratio

(Vernon, 1980)

However, the main difference is that, in the fed ruminant, there is an active pathway for fatty acid synthesis from acetate. From this it could be concluded that it is the utilization of acetate for lipogenesis, rather than the restricted use of glucose which is the peculiarity of ruminant adipose tissue.

1.2 Adipose tissue

Nearly all lipid metabolism in ruminants occurs in the adipose tissue (Ingle *et al.*, 1972). Adipose tissue can take either of two forms: white or brown. Only white adipose tissue is used for storage of lipid for later mobilization. Brown adipose tissue, which is present in new-born lambs but tends to disappear after 2 to 3 weeks, is thought to be involved in heat production.

White adipose tissue has large cells, each of which consists almost entirely of a single vacuole of lipid with no surrounding membrane. There is a flattened nucleus on one side of the cell which causes the membrane to protrude in a bulge (Plate 1.1). Ocassionally, golgi apparatus, endoplasmic reticulum and ribonucleoprotein have been seen in the flattened periphery (Lindsay, 1983). Brown adipose tissue cells have more cytoplasm and mitochondria, less lipid as well as a richer vascular and nervous supply than white adipose tissue (Ricquier *et al.*, 1985).

Adipose tissue cells are called "pre-adipocytes" before they begin to store lipid. There is little to distinguish pre-adipocytes from other cells in the connective tissue and thus little is known about their development. Although brown adipose tissue can convert to white adipose

tissue, this is not thought to be the normal source of white adipose tissue cells. Once fat cells begin to store lipid, they no longer divide (Kirtland and Gurr, 1979). Pre-adipocytes, on the other hand, are able to divide but it is not known for how long or if their increase in number is dependent on the amount of lipid stored in neighbouring adipocytes or by the level of nutrition. There is an increase in cell number (hyperplasia) in sheep until they are about 11 months old (Hood and Thornton, 1979, Hood, 1982). Further development of the adipose tissue, after hyperplasia ends, must only be by hypertrophy (increase in cell size). Following nutritional restriction in sheep, the resulting decrease in adipose tissue mass was found to be due to a decrease in cell size and not cell numbers (Hood and Thornton, 1979).

It was suggested by Knittle and Hirsch (1968) that, in rats, the level of nutrition soon after birth determined the number of fat cells which lasted throughout the life of the animal. Since lipogenic activity was similar in large and small cells, they concluded that a high level of nutrition early in life, which led to greater number of fat cells, resulted in a permanent propensity to fatten. Kirtland and Gurr (1979) suggest that the observations of increasing fat cell numbers in the Knittle and Hirsch study may have been merely an increase in size of existing pre-adipocytes.

Thus it is generally thought that, in the mature animal, there are a fixed number of adipocytes which are unaffected by long-term or extreme changes in the size of the adipose tissue depot. Irrespective of whether the numbers of cells in the depot are determined by the time of birth or some time later, once formed, the adipose tissue cells can increase in size but not number. It is thought that some metabolic events in adipose tissue are a function of cell number and appear to be unaffected by cell size. This is true of processes that are controlled by the nucleus, the functioning of which occurs independently of the amount of triacylglycerol stored in the cell (*i.e.* independently of cell size).

However, there are some processes where it can be envisaged that increased cell size could result in increased metabolic rates. An increased cell size means that there is an increased cell surface. For instance, a greater surface area may result in increased flow rates of substances in and out of the cell. It could also determine the number of

membrane receptors if such receptors appear at a fixed density rather than at a fixed number. Thus the cellularity of adipose tissue is an important aspect of the measurement of the lipid metabolism occurring therein.

1.3 Factors influencing the rate of lipid metabolism *in vivo*

The rate of fatty acid synthesis is influenced by:

(i) the quantity and quality of the diet
(ii) the age of the animal and the site of the adipose tissue
(iii) genetic differences between breeds and individuals
(iv) the sex of the animal
(v) environmental conditions
(vi) the physiological state of the animal.

Diet

Of the above factors, the most important is the energy balance of the animal which is determined by the composition of the diet. A diet which is rich in high energy-yielding substances will lead to a high rate of lipogenesis and fat deposition. There are, however, mechanisms to ensure that fat deposition does not occur to the detriment of normal tissue metabolism.

Age of the animal and site of the adipose tissue.

Adipose tissue development is thought to occur in the order: abdominal, intermuscular, subcutaneous, and finally intramuscular. Therefore the rates of lipid deposition in the various sites change with the age of the animal (Vernon, 1980). In sheep, the rate of increase in volume of subcutaneous adipose cells (expressed as pl/day/cell) is 1.8 at 170-220 days, 3.1 at 290-350 days and rising to a maximum of 5.8 at 350-400 days (Hood and Thornton, 1979). These changes imply that there are changes occurring in the rates of lipogenesis and lipolysis in the adipose tissue which are dependent on the age of the animal. There are, however, other physiological factors (such as rate of growth) which depend on age and which may also influence the rate of lipid accumulation in adipose tissue (see Section 1.11).

Breed of the animal

Some breeds of sheep mature earlier than others so that, at a given age, they will be at different stages of development. Studies of subcutaneous adipose tissue from Southdown (early maturing) and Suffolk (late maturing) lambs of the same age showed that the Southdown animals had a higher basal rate of both lipolysis and lipogenesis although there were no differences in the rate of catecholamine-stimulated lipolysis or lipoprotein lipase activity (Sidhu *et al.*, 1973). Beef cattle mature and fatten at an earlier age than dairy cattle which tend to have more internal fat and less subcutaneous fat than beef cattle (Vernon, 1980). It might be expected therefore that, in samples of subcutaneous fat taken at the same age from beef and dairy cattle, there would be a higher rate of lipogenesis and a lower rate of lipolysis in beef cattle than in dairy cattle, especially in young animals. The different breeds of animal can also show differences in adipocyte number and volume (Vernon, 1980) which may reflect the different propensity of some breeds to fatten.

Sex

It is thought that differences in the total amount of adipose tissue, its distribution among the various sites in the body and the rate of growth is influenced by the sex of the animal. Castration stimulates fat deposition but at the same time reduces muscle growth. It is possible that increased fat deposition results because less energy is required for muscle growth and is thus stored as fat (Vernon, 1980).

Environmental Conditions

During exposure to cold, animals must increase their rate of metabolic heat production in order to maintain normal body temperature. Lipids, in the form of plasma NEFA, are vital as a readily available and easily oxidizable source of energy during cold exposure. Concentrations of plasma NEFA quickly rise in sheep exposed to cold (Halliday *et al.*, 1969). There is also a considerable increase in blood flow to adipose tissue (Bell *et al.*, 1976) and this would assist release of free fatty acid from these sites by increasing the rate of presentation of albumin binding sites for free fatty acids. It appears that this mobilization of free fatty acid from adipose tissue during cold stress is under the control of the sympathetic nervous system since the normal increase in

plasma NEFA can be prevented by the application of a number of different adrenergic blocking agents (Alexander *et al.*, 1968). There is also evidence of seasonal changes in plasma NEFA in sheep, with lowest levels in winter and spring and highest levels in summer and autumn (Leat, 1974). However, age and nutritional changes with the seasons could also have influenced these results.

1.4 The measurement of lipid metabolism *in vitro*

Most of the current knowledge of the process of lipid metabolism in adipose tissue and its regulation has been obtained by studies *in vitro*. There are a number of factors necessarily missing from an *in vitro* system which would normally control lipid metabolism *in vivo*, often making *in vitro* studies difficult to interpret. On the other hand, direct measurements of lipid metabolism *in vivo* also have a number of disadvantages:

(i) The adipose tissue is widely distributed, with up to 16 distinct depots in mammals.

(ii) There are such a large number of factors which could influence the rate of metabolism *in vivo* that interpretation of results becomes extremely difficult. The presence of feedback mechanisms in the endocrine system means that any manipulation of the animal's hormone balance causes a sequence of events which may be extremely difficult to monitor and control. Thus, the direct cause of experimental observations may not be known at the end of an experiment.

The measurement of lipogenesis *in vitro*

The rate of lipogenesis in adipose tissue, both *in vivo* and *in vitro* has traditionally been measured by the rate of incorporation of radioactive substrate molecules into long-chain fatty acids. In studies of non-ruminant metabolism, ^{14}C-glucose is normally used, while in ruminant adipose tissue, where acetate is the major precursor for lipogenesis, ^{14}C-acetate gives the highest rates of incorporation of ^{14}C. The use of ^{14}C-glucose with ruminant adipose tissue results in incorporation of ^{14}C into the glycerol moiety of the triacylglycerol molecules. The incorporation of ^{3}H from $^{3}H_2O$ into the fatty acid chains

in both ruminants and non-ruminants gives a measure of lipogenesis from all precursors.

Unless the fatty acid in the cell is being esterified, the buildup of free fatty acid will inhibit the rate of lipogenesis. Thus, in ruminants, the rate of ^{14}C-acetate incorporation into triacylglycerol probably depends also on the rate of glucose incorporation into glycerol. The same applies to the production of glycerol which could be inhibited if esterification were not proceeding owing to a lack of free fatty acid. Thus the rate of incorporation of one depends on the other and any changes in observed rates could reflect changes in either or both processes.

Adipose tissue has a well defined, although relatively sparse, network of sympathetic nerves. It is known that these nerves play an important role in the storage of lipids in adipose tissue. For example, patients with damaged nerve systems can show large increases in mass of adipose tissue in the affected regions (Fredholm, 1985). This means that any measurement of lipogenesis is necessarily made without this added control mechanism.

The measurement of lipolysis *in vitro*

The measurement of free fatty acids appearing in the incubation medium is one indication of the rate of lipolysis occurring within the cell. It is quite likely that there are factors operative *in vivo* which influence the rate at which free fatty acids leave the cells and which are not present *in vitro*. For example, blood flow may influence the rate either by increasing the availability of albumin molecules or by providing other factors which influence the flux in and out of the cells. It may also be influenced by hormones which are not present *in vitro*. Thus *in vitro* measurements of fatty acid release must be interpreted with reference to these factors.

Glycerol release from the adipose tissue is also used as a measurement of the rate of lipolysis. Free glycerol is metabolized only very slowly in adipose tissue and is seldom used for re-esterification of fatty acids. Instead glucose, in the form of glucose-6-phosphate, is used. Thus the glycerol released by lipolysis accumulates as an end product in adipose tissue and probably offers a more reliable means to

estimate the extent of lipolysis occurring in the cell (Vernon, 1980).

It has been shown that stimulation of the nervous supply to adipose tissue can increase the rate of release of both free fatty acid and glycerol (Fredholm, 1985). Thus measurements of lipolysis *in vitro* are made in the absence of this effect.

1.5 The nature of hormonal control of cellular mechanisms

Hormones function as chemical messengers delivering their information to target sites. They can operate only when the target site possesses a highly specific receptor to which the hormone can bind. As a result of this binding, a sequence of cellular events is initiated. The nature of this sequence is determined by both the chemical nature of the hormone and the type of cell in which the receptor is located.

Hormones are transported by the blood from the sites of production in the endocrine glands to target organs, although some may be produced and exert their effect in the same organ or even the same cell. Hormones act by accelerating or retarding the rates of biochemical reactions.

Most hormones have short half-lives, usually less than one hour. Since they function as regulators in the maintenance of the normal state, hormones must be continually synthesized and secreted, must exert their effects rapidly and then must be rapidly deactivated.

The chemical structures of hormones

Hormones generally have one of three types of chemical structure. One group is polypeptide in nature and includes insulin, glucagon, somatostatin and growth hormone while others, such as thyroxine, triiodothreonine, adrenalin and noradrenalin, are modified amino acids. The third common group are the steroids which include testosterone, progesterone, estrogen and corticosteroids. They are derivatives of cholesterol which has a fused cyclohexane ring structure.

Action on the cell membrane

The hormones travel in the blood stream to the target tissue where each hormone has a high affinity for its own receptor site. The receptors are located in the membranes and are generally large glycoproteins.

The binding of the hormone will depend on:

(i) the concentration of the hormone
(ii) the concentration of the receptor
(iii) the binding characteristics of the receptor

When small amounts of a polypeptide hormone are present, only receptor sites with a high affinity for the hormone will bind in significant quantities whereas higher concentrations of the hormone may saturate other sites of lower affinity to elicit an entirely different response. It is thought that different types of receptors probably differ in structure (Levey and Robinson, 1982) and would thus have different affinities for the hormone.

However, each hormone has its own peculiar mode of interaction with the cells and these will be dealt with in the following sections for each of the hormones included in the present study.

1.6 Insulin

Chemical structure and mechanism of synthesis

The insulin molecule consists of two polypeptide chains (chain A with 21 amino acids and chain B with 30 amino acids) which are joined together by two disulphide bridges between cysteine residues. It occurs in all vertebrates with minor variations in the sequence of amino acids.

Although the gene for the synthesis of insulin is present in all nuclii, it is synthesized only by the β cells in the islet of Langerhans of the pancreas. Insulin is derived from a single-chain precursor called proinsulin which is synthesized in the rough endoplasmic reticulum. Proinsulin has 78 - 86 amino acids, depending on the species. Tryptic digestion of proinsulin, which occurs in or near the golgi apparatus, produces 2 main products: insulin and the C peptide.

The action of insulin on target cells

Insulin acts on cells in the liver, muscle and adipose tissue as well as many other cell types to produce a wide range of biological responses. These actions include activation of transport systems for glucose, amino acids and ions, the activation of enzyme systems and alteration of the transcription of specific mRNA species (Czech, 1985). The responses to insulin interaction can be very rapid (for transport systems) or slow (for growth stimulation).

In non-ruminants, insulin is required for glucose entry into most cells. It affects the cell membrane by promoting carrier-mediated transport. In the ruminant, the situation is different because of the decreased role of glucose as a precursor for lipogenesis. However, it would appear that insulin still has the effect of increasing the flow of carbon atoms to adipose tissue in ruminants (Trenkle, 1981).

In the tail fat-pad of the Syrian sheep *in vivo* (Khadachurian *et al.*, 1966) and *in vitro* (Khadachurian *et al.*, 1967), insulin stimulates glucose uptake. As in non-ruminants, it would appear that insulin is required for glucose entry into the adipocyte. Because glucose is normally phosphorylated as it enters the cell, there is virtually no glucose flux out of the cell. For this reason, insulin, as well as controlling the influx of glucose, also controls the rate of metabolism of glucose. This was observed in cattle (Baldwin *et al.*, 1973; Yang and Baldwin, 1973a), goats (Skarda and Bartos, 1969) and sheep (Mears and Mendel, 1974) where insulin increased glucose oxidation in adipose tissue *in vitro*. Addition of acetate to the medium increased the effect of insulin on glucose oxidation in adipose tissue from sheep and cattle (Yang and Baldwin, 1973a; Mears and Mendel, 1974) but the effects were small.

Numerous reports have shown that incorporation of acetate and glucose into fatty acids in ruminant adipose tissue is not markedly affected by insulin in short-term incubations *in vitro* (Baldwin *et al.*, 1973; Vernon 1977b, 1978, 1979b; Vernon *et al.*, 1985a; Prior and Jacobson, 1979; Prior and Smith, 1982). In some experiments, there was no significant effect at all (Baldwin and Smith, 1971; Yang and Baldwin, 1973a; Thornton *et al.*, 1982; Vasilatos *et al.*, 1983). It was stated by Prior and Smith (1982) that the role of insulin in regulating the rate of

lipogenesis in bovine adipose tissue is negligible. Vernon (1980) suggests that the effects of insulin on acetate incorporation into fatty acids in ruminants is usually no more than 10-50%.

Conversely, in similar studies with rat adipose tissue, there was a 5 to 10-fold increase in the rate of glucose metabolism in the presence of insulin, the response decreasing with age (Di Girolamo *et al.*, 1974; Holm *et al.*, 1975; Salans and Dougherty, 1971). Bovine adipocytes have been shown to possess insulin receptors which appear to be similar in number and binding characteristics to those of the rat (Vernon *et al.*, 1985a).

From the evidence of these studies, it could be concluded that, although insulin stimulates lipogenesis and glucose utilization in ruminant adipose tissue, the effect appears to be smaller than that in non-ruminants. There are several possible reasons why this limited response to insulin in ruminants could have evolved. The rumen ensures a steady flow of nutrients into the animal and thus insulates it against sudden changes in nutrient availability (Bauman, 1976), thus making the insulin response less crucial to cellular maintenance. Also, since ruminant adipose tissue utilizes acetate for fatty acid synthesis, the poor response to insulin could be due to the low capacity for glucose metabolism seen in ruminants. In any case, the concentration of insulin in the blood of ruminants is not as closely correlated with blood glucose as it is in non-ruminants (Trenkle, 1972).

Etherton and Evock (1986) attributed this lack of responsiveness in ruminant adipose tissue *in vitro* to an inhibitory factor present in the BSA. They obtained a marked stimulation of acetate incorporation into fatty acids in bovine adipose tissue slices incubated with an appropriately chosen batch of BSA. However, several of the studies where very little stimulatory effect of insulin was observed in ruminant adipose tissue do not appear to have included BSA in the medium (Vernon, 1978, 1979b). The effect of insulin on the rate of lipogenesis may be greater in isolated adipocytes than in tissue slices (Yang and Baldwin, 1973a), possibly because of increased accessibility of the isolated cells to the hormone.

Insulin has been shown to stimulate both glucose utilization and pyruvate kinase activity in ovine adipose tissue slices in long-term incubations (Vernon and Taylor, 1984). It was postulated by the authors that adipose tissue from ruminants may become temporarily insensitive to insulin on removal from the animal and that restoration of full insulin responsiveness of the tissue is achieved in long-term incubations.

The uptake of acetate by peripheral tissue is restored to normal by insulin treatment of alloxan-induced diabetic sheep (Jarrett *et al.*, 1974). There is also a decrease in the concentration of plasma acetate following insulin administration to normal sheep (Call *et al.*, 1972; Muller and Colenbrander, 1970). It would therefore appear that the effects of insulin on acetate uptake is more clearly defined in ruminants *in vivo* than it is with ruminant adipose tissue *in vitro*.

Insulin has been shown to be an anti-lipolytic hormone in ruminants, as in non-ruminants. Insulin administration stimulates a rapid (within 5 minutes) decrease in plasma NEFA (Bergman, 1968; Bauman, 1976; Hertelendy, 1973; Trenkle and Kuhlmeier, 1966; West and Passey, 1967) and glycerol (Bergman, 1968). There was also a measurable decrease in the net output of glycerol (Brockman *et al.*, 1975) and NEFA (Khadachurian *et al.*, 1966) from adipose tissue following insulin administration to sheep. However, studies *in vitro* have shown that insulin alone or in combination with glucose does not affect the basal rate of lipolysis (Metz and Van den Bergh, 1972, 1977; Yang and Baldwin, 1973b). It was concluded that insulin cannot act alone in inhibiting lipolysis by any measurable amount.

The action of insulin on the enzymes of lipid metabolism

There are three key enzymes in the lipogenesis pathway that have been shown to be regulated by insulin (Denton *et al.*, 1979; McCormack, 1982). These are pyruvate kinase (No. 11 in Figure 1.1), the pyruvate dehydrogenase complex (No. 13 in Figure 1.1) and acetyl CoA carboxylase (No. 21 in Figure 1.1). Acetyl CoA carboxylase is thought to be rate-limiting for lipogenesis in ruminant adipose tissue since it has been shown to be the least active of the enzymes. On the other hand, the pyruvate dehydrogenase complex is thought to have a minor role in ruminant adipose tissue (Vernon, 1980).

When insulin interacts with the cell, many of the cellular enzymes undergo phosphorylation or dephosphorylation. Pyruvate dehydrogenase has been shown to be dephosphorylated in response to insulin (Hughes *et al.*, 1980). On the other hand, insulin action appears to result in the phosphorylation of acetyl CoA carboxylase (Brownsey and Denton, 1982; Witters, 1981). However, Prior and Smith (1982) reported that acetyl CoA carboxylase activity was unchanged in adipose tissue from alloxan-induced diabetic steers. This implies that insulin is not required to maintain the activity of this enzyme.

Insulin activity in the adipose tissue seems to result also in the phosphorylation of ATP citrate lyase (Alexander *et al.*, 1982; Pucci *et al.*, 1983). ATP citrate lyase (No. 19 in Figure 1.1) activity is very low in sheep adipose tissue and was shown to be less than 20% that of rat adipose tissue measured under identical conditions (Hanson and Ballard, 1967). The reduced activity of ATP citrate lyase is thought to be the cause of the low rate of glucose utilization in ruminant adipose tissue compared with that from the rat. However, the phosphorylation of these enzymes have not yet been shown to alter their biological activities and it is not known what role the phophorylations and dephosphorylations play in the cellular responses to insulin.

It is also thought that insulin may activate some of the enzymes of the fatty acid esterification pathway, such as fatty acyl CoA synthetase and glycerol phosphate acyl-transferase (Jason *et al.*, 1976; Sooranna and Saggerson, 1976).

The insulin receptor

The insulin receptor is made up of 2 glycoprotein subunits with apparent masses of 125 kDa (α subunit) and 90 kDa (β subunit) which are linked together with disulphide bonds. The α and β receptor subunits may have separate functions with the α unit binding with the hormone and the β unit possessing tyrosine kinase activity (Czech, 1985). The receptors appear to be dispersed over the entire cell surface (Bergeron *et al.*, 1985).

The interaction of the receptor with insulin initiates the biological actions of the hormone. The concave, curvilinear Scatchard plots obtained when radio-iodinated insulin binds with the receptors in

intact cells suggest that there are multiple insulin receptor sites with different affinities or that the interactions of the hormone with the receptor exhibits negative co-operativity (Czech, 1985). It has also been suggested that each insulin receptor unit binds with two insulin molecules and that these two binding sites have markedly different affinities for insulin (Pang and Shafer, 1984).

It has been discovered that some parts of the receptors for insulin (Kasuga *et al.*, 1982) and IGF I (Jacobs *et al.*, 1983) are in fact enzymes which catalyse tyrosine phosphorylation and are thus tyrosine kinases. These phosphorylations are thought to play a part in the sequence of molecular events occurring during the biological actions of the receptor (Czech, 1985). They have been observed to occur in isolated adipocytes (Haring *et al.*, 1982).

Regulation of insulin binding

There are a large number of agents and altered physiological conditions known to interfere with insulin's interaction with its receptor. These include the presence of catecholamines and glucagon (Pessin *et al.*, 1983), glucocorticoids (Olefsky, 1975) and conditions such as diabetes (Olefsky, 1976a; Schoenle *et al.*, 1977; Jochen *et al.*, 1986)) and obesity (Kahn *et al.*, 1973; Olefsky, 1976b) which all effect insulin binding to cells.

Insulin itself can decrease the amount of insulin receptor binding in intact cells. This phenomenon is thought to be the result of insulin-induced internalization of the insulin-receptor complex (Gavin *et al.*, 1974; Marshall *et al.*, 1981; Standaert and Pollet, 1984). It has been shown that bovine adipocytes have similar characteristics of both internalization and down-regulation as those of rat adipocytes under the same conditions (Walker and Flint, 1983; Vernon *et al.*, 1985a).

The process of internalization involves the movement of the insulin-receptor complex from the cell surface into the cell following the binding process. The hormone is then degraded intracellularly, probably in the lysosomes, and the receptor is recycled back to the cell surface (Marshall *et al.*, 1981). The loss of the cell surface receptors and their appearance inside the cell have been shown to be linearly dependent on time and insulin dose (Green and Olefsky, 1982). The

biological functions of internalization are not clear although it is possible that the process is involved in the long-term effects of insulin (Etherton, 1982).

However, insulin may not enter the cell to mediate at least some of its rapid biological effects such as glucose transport (Cheng and Larner, 1985). The actions of insulin could instead involve the formation of a mediator which is thought to be an oligopeptide or glycopeptide (Larner *et al.*, 1979). Insulin mediator, which has been isolated from rat adipocytes (Kiechle *et al.*, 1980; Kikuchi *et al.*, 1981), has been shown to stimulate lipogenesis and inhibit lipolysis in these cells (Zhang *et al.*, 1983). The actions of the mediator were not inhibited by insulin antibody and are therefore unlikely to be the result of contamination of the mediator preparation with insulin.

1.7 Glucagon

Chemical structure and source

Glucagon is a polypeptide hormone consisting of a single chain of 29 amino acids, with a molecular weight of 3485. It is manufactured in the α cells in the islets of Langerhans of the pancreas.

The action of glucagon on target cells.

The most important actions of glucagon are:

(i) inhibition of glycogen synthesis in the liver
(ii) stimulation of glycogenolysis in the liver
(iii) stimulation of gluconeogenesis in the liver
(iv) stimulation of lipolysis in adipose tissue
(v) stimulation of catecholamine release

Although the actions of glucagon were not included in the present study, its functions are noted because it is derived from the same source (the pancreas) as the biologically derived insulin used in this study and small quantities of contaminants could well have been present during incubation.

1.8 Catecholamines

Chemical structure and source

There are three main catecholamines known:

(i) dopamine — a neurotransmitter in the brain and a precursor of nor-adrenalin.

(ii) nor-adrenalin (nor-epinephrine) is released by the post-ganglionic sympathetic nerve terminals by certain cells in the brain and by the adrenal medulla.

(iii) adrenalin (epinephrine) is released only by the adrenal medulla, and possibly certain brain cells. It is synthesized from nor-adrenalin.

The mechanism which links the arrival of nerve impulses in the splanchnic nerves to the actual release of catecholamines into the blood is called "stimulus-secretion coupling". This mechanism is facilitated by the release of acetylcholine when the nerve impulse arrives. The resultant depolarization of the membrane and rush of Ca^{++} into the cell in some way affects the release of catecholamines. The half-life of catecholamines in the blood is extremely short and their effects are extremely potent.

The action of catecholamines on target cells

The release of catecholamines is invariably associated with activity of the sympathetic nervous system as a whole and their action on target organs cannot be considered in isolation from the effects of direct neural stimulus. The so-called "fright, flight or fight" stimulation of catecholamine release is an essential component of the emergency response of the animal.

The effects of catecholamines include:

(i) breakdown of hepatic glycogen leading to an increase in blood glucose

(ii) increase in the rate of lipolysis

(iii) increased heat production and oxygen consumption

(iv) inhibition of insulin release.

Many studies have shown that, in ruminants, injected catecholamines stimulate an almost instantaneous increase in concentration of plasma NEFA (Kronfeld, 1965; Alexander *et al.*, 1968; Bergman, 1968; Bassett, 1970; Hertelendy and Kipnis, 1973; Bauman, 1976). There was also a corresponding increase in plasma glycerol in some studies (Bergman, 1968; Hertelendy and Kipnis, 1973). These observations suggest that catecholamines do increase the rate of lipolysis in ruminants and this has been confirmed in studies *in vitro* (Khadachurian *et al.*, 1967; Metz and Van den Bergh, 1972,1977; Metz *et al.*, 1973; Pothoven *et al.*, 1975; Bartos *et al.*, 1975a,1975b; Etherton *et al.*, 1977; Yang *et al.*, 1978). Concentrations of adrenalin and nor-adrenalin of 50-60 μmol/l stimulated lipolysis by 2 to 10-fold in these studies.

Injection of adrenalin and nor-adrenalin was shown to stimulate the uptake of glucose and the release of lactate by the tail fat pad of Syrian fat-tailed sheep (Khadachurian *et al.*, 1966). *In vitro* studies of bovine adipose tissue, on the other hand, have shown that adrenalin inhibits fatty acid synthesis from acetate and decreases the rate of glucose oxidation (Yang and Baldwin, 1973a). In the latter study, there appeared to be reduced oxidation via the pentose phosphate pathway, increased glucose oxidation via the glycolytic pathway and increased conversion glucose to glyceride glycerol. These effects of adrenalin were not prevented by the presence of insulin.

The above effects could be explained by an increased rate of lipolysis resulting in an increased concentration of long-chain fatty acyl CoA in the tissue. This would inhibit the activity of acetyl CoA carboxylase and hence decrease glucose oxidation while at the same time stimulating glyceride glycerol synthesis and possibly prohibiting the oxidation of glucose via the pentose phosphate cycle (Vernon, 1980). The increased rate of glucose conversion to glyceride glycerol and lactate could be due to the increase in phosphofructokinase stimulated by cyclic AMP as demonstrated in rat adipose tissue (Denton and Randle, 1966).

Catecholamine receptors

Specific adrenergic receptor sites on or perhaps occasionally inside the target cells, are of two types: α and β. The α receptors respond preferentially to nor-adrenalin, with α actions generally involving the contraction of smooth muscle, while β receptors respond principally to adrenalin and involve relaxation of smooth muscles or metabolic effects. However, both α and β adrenergic blocking agents have been shown to reduce the effect of adrenalin on the level of plasma NEFA in sheep (Bassett, 1970).

The release of fatty acids is a unique function of adipose tissue cells. The process of fatty acid mobilization, which is essential to fulfil the energy requirements of the rest of the body, is under the control of the body's endocrine and nervous system. When a lipolytic hormone such as adrenalin interacts with a cell in the adipose tissue, a sequence of events is triggered which leads to the reversible phosphorylation of the hormone-sensitive lipase. The events following the initial hormone interaction with the receptor are known as the "lipolytic activation cascade" (Steinberg and Huttunen, 1972).

The sequence of events appears to be initially an enhanced level of intracellular cyclic AMP followed by increased concentrations of cyclic AMP-dependent protein kinase. It is the protein kinase which is thought to be involved in the phosphorylation of the hormone-sensitive lipase which then results in increased rates of lipolysis in the cell. Little is known of the mechanisms by which phosphorylation and subsequent dephosphorylation of the hormone-sensitive lipase alters the activity of the enzyme. However, it is thought that insulin is involved in the dephosphorylation process and this may account for its observed antilipolytic activity (Nilsson *et al.*, 1980).

1.9 Growth hormone

Chemical structure of growth hormone

Growth hormone (GH) is a linear peptide of 191 amino acids with two disulphide bridges and a molecular weight of about 22 kDa.

Using electrophoresis, it was found that preparations of GH are not homogeneous. The hormone can be contaminated with a proteinase which hydrolyses the molecule (Lewis *et al.*, 1980) and it was found that the cleaved parts of the molecule exhibited the normal biological activity of GH (Singh *et al.*, 1974). The cleavage is thought to occur in the area of the large disulphide-linked loop which converts the GH into a two-chain structure. This increases the effective radius of the molecule and results in retardation of the migration during SDS electrophoresis (Lewis *et al.*, 1980). The physiological importance of the cleaved forms is yet to be elucidated. GH can also form a disulphide-linked dimer which is physiologically inactive until cleaved. In addition, an active 20 kDa variant of GH has been demonstrated (Lewis *et al.*, 1978, 1980).

Secretion of growth hormone

GH is secreted by the adenohypophysis of the pituitary gland. The secretion is controlled by two hypothalamic hormones: growth hormone releasing factor (GRF) which stimulates the release of GH and somatostatin which inhibits the release of GH.

The secretion of GH in ruminants is episodic in nature, with release of hormone occurring throughout the 24 hour period. In cattle, there was no consistent pattern of release during the 24 period, either related to clock-time or time at which the animal was fed (Gluckman *et al.*, 1986).

The actions of growth hormone

Studies of the actions of GH on target cells have been hampered by several factors:

A *in vivo* studies

(i) The production of a specific GH deficiency. The adenohypothesis secretes a variety of hormones, including prolactin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and adrenocorticotrophic hormone (ACTH). Thus hypophysectomy will result in the loss of other hormones beside GH and any observed responses must be interpreted with caution.

(ii) There are a large number of factors which influence the secretion of GH in experimental situations. These include nutritional status, diurnal patterns, species and individual differences, sex of the animal, emotional condition and stress. Thus any observations of changes in the animal are often difficult to interpret.

(iii) Antibodies to GH can be used to block the action of GH *in vivo*. This work has been hampered by the difficulties associated with obtaining purified GH with which to raise antibodies. Recombinant DNA-derived hormone now available may resolve this problem.

B *in vitro* studies

(i) Studies *in vitro* have also been hampered by the difficulties associated with the production of purified preparations of GH.

(ii) Many of the actions of GH *in vivo* are dependent on, or modified by, the presence of other pituitary hormones and will not be observed in isolation *in vitro*.

(iii) Many of the actions of GH are delayed and long-acting, making them difficult to study *in vitro*.

The observed actions of GH, which may be either direct or mediated by another hormone are:

(i) stimulation of anabolic processes such as protein synthesis, cell division and growth

(ii) stimulation of lipolysis

(iii) inhibition of the transport of glucose into body tissues (diabetogenic effect)

It is thought that the effects of GH on growth are indirect and mediated through IGF I (see Section 1.10).

Administration of exogenous GH has long been known to cause short-term lipolytic, diabetogenic and anabolic effects on ruminants (Wheatley *et al.*, 1966; Davis *et al.*, 1970a, 1970b) although the results of these early studies could have been influenced by contaminating hormones present in the pituitary-extracted GH used. A more recent study (Muir *et al.*, 1983), using purified pituitary-derived hormone, found that oGH administration to growing lambs decreased the deposition of fat in the adipose tissue by 8.9% ($p < 0.05$). There was no change in average daily weight gain in these animals compared with controls receiving no hormone. In a similar experiment (Johnsson and Hart, 1985), where purified pituitary-extracted bGH was administered to lambs, there was a significant increase in the weight of protein depots in the body and only a moderate decrease in total fat deposition.

There have been inconsistencies in the lipolytic responses to GH *in vitro* obtained in various laboratories. Growth hormone was shown to increase lipolysis in rat adipocytes (Katosc *et al.*, 1973; Wieser *et al.*, 1974; Sengupta *et al.*, 1981). Frigeri (1980) and Frigeri *et al.* (1983) found that highly purified hGH and bGH failed to stimulate lipolysis in adipose tissue from starved rats *in vitro* and that chromatographic purification of the hormone had removed an acidic fraction apparently associated with the lipolytic activity. However, when this experiment was repeated in another laboratory (Goodman and Grichting, 1983), both the highly purified hGH from the same source and the acidic contaminant increased lipolysis in the presence of 0.1 μg/ml dexamethasone after a lag period of one hour.

Recombinant DNA-derived bGH and pituitary-extracted bGH have been shown to be immunologically identical (Hart *et al.*, 1984b). Hart *et al.* showed that neither recombinant DNA-derived bGH nor recombinant DNA-derived chicken GH were lipolytic in rat adipose tissue *in vitro*. On the other hand, it was shown that pituitary-extracted bGH did stimulate lipolysis. Hormones from both sources increased the concentration of plasma NEFA and reduced the ability of insulin to lower the concentration of plasma glucose in sheep *in vivo*. It was therefore concluded that, although both the lipolytic and diabetogenic activity of GH are intrinsic properties of the hormone, the lipolytic activity may only become apparent following modification of the hormone *in vivo* or if the hormone is able to activate lipolytic or diabetogenic intermediates.

Goodman (1984), however, demonstrated a lipolytic response in adipose tissue from hypophysectomized rat using recombinant DNA-derived GH (from Genentec and acknowledged to contain bacterial protein contaminants, see Plate 6.1). He concluded that both the insulin-like and lipolytic action of GH was an intrinsic characteristic of the GH and not due to contaminants in the pituitary extracts.

A lipolytic response to purified hGH in isolated adipocytes from normal rats was demonstrated following 3 hours of incubation (Grichting *et al.*, 1983). On the other hand, a recent study with highly purified hGH failed to demonstrate lipolytic activity either *in vivo* or *in vitro* in rabbits (Bowden *et al.*, 1985). There was no demonstrable lipolytic response to purified oGH in ovine adipose tissue *in vitro* (Duquette *et al.*, 1984) although, in the same study, oGH stimulated lipolysis in rat adipose tissue *in vitro*. Barenton *et al.*, (1984) also did not find any lipolytic activity of rabbit, porcine, ovine or human GH in rabbit isolated adipocytes *in vitro*. It is thought (Hart *et al.*, 1984a) that pituitary-extracted bGH is a mixture of different molecular forms, each of which appears to have different physiological effects. This could account for some of the discrepancies in GH activity between laboratories.

GH has been shown to cause insulin-like effects in rat isolated adipocytes. These effects include enhanced transport of glucose into the cell, accelerated conversion of glucose to fatty acid, glycogen and carbon dioxide, and antagonism of the lipolytic effects of adrenalin (Goodman and Schwartz, 1974).

Rat GH has been demonstrated to produce an initial insulin-like effect in epididymal fat slices from hypophysectomized rats (Goodman, 1981). The response followed rapidly after the initial exposure of the tissue to the hormone and had disappeared by 180 minutes. A second exposure to GH will not give the same response for some hours, indicating that GH induces a refractoriness to the insulin-like effects of the hormone. GH is probably not interacting with the insulin receptors to elicit this response (Grichting *et al.*, 1983). The insulin-like effect, once initiated by GH, does not appear to require the continued presence of the hormone (Goodman, 1981). The response continues following the addition of specific antisera ten or more minutes after incubation with GH.

Eden *et al.*, (1982) have reported that freshly isolated rat adipocytes did not increase glucose metabolism in response to hGH but that the cells recovered responsiveness when pre-incubated for three hours without hormone. This appears to indicate that the cells can be refractory to GH before incubation begins, perhaps by recent exposure to endogenous GH *in vivo*. It is not known whether this refractoriness is due to down-regulation of GH receptors or to a post-receptor phenomenon, although there is some evidence to support the latter (Grichting *et al.*, 1983). However, it is possible that varying degrees of refractoriness between species, individuals and laboratories could account for the varying responses to GH *in vitro*.

Growth hormone receptors

Since there are both short and long-term responses to GH, it is not known whether the different effects are mediated through a single type of receptor or whether a different hormone-receptor interaction is responsible for each response or group of responses (Isaksson *et al.*, 1985). Part of the difficulty in studying GH receptors has been that some of the responses to GH are indirect and mediated by GH-dependent factors. In addition, it has been shown that the various metabolic responses to GH

in rat adipocytes *in vitro* occur at different levels of receptor occupancy (Grichting *et al.*, 1983).

The results of a number of binding studies (reviewed by Hughes and Friesen, 1985) appear to indicate that GH interacts with multiple receptors, or at least multiple forms of one receptor. Thus the different forms of GH described earlier in this section may in fact possess different binding affinities to the receptor or receptors involved. Specific binding of GH to adipocytes has been shown in rats (Fagin *et al.*, 1980) and sheep (Chan *et al.*, 1978).

The mechanism by which GH receptors mediate hormone action is yet to be elucidated. This work has been hindered by the lack of a definable response in the tissues and by the absence of specific antagonists to the receptors (Hughes *et al.*, 1985). It is usually assumed that increased binding of GH involves increased synthesis of the receptors. It is also postulated by Hughes *et al.*, (1985) that this process could involve, in addition, the activation and subsequent inactivation of pre-existing GH receptors which may be transformed from a non-binding to a binding state by some, possibly independent, trigger mechanism. Hormone receptor levels could also be controlled by an internalization process although it is unclear to what extent and whether the receptors are recycled.

Little is known about the post-receptor mechanisms following GH interaction with the receptor in adipose tissue. Attempts to measure the intracellular concentrations of adenylate cyclase and cyclic AMP following GH administration have been inconclusive (Hughes *et al.*, 1985).

1.10 Insulin-like growth factors

Structures of the IGF molecules

Insulin-like growth factor (IGF) I and II are polypeptide hormones with chemical structures similar to that of pro-insulin (Rinderknecht and Humbel, 1978a, 1978b). IGF I consists of a single chain of 70 amino acids and has a molecular weight of 7649 (Rinderknecht and Humbel, 1978a). IGF II has 67 amino acids and a molecular weight of 7471 with 62% of the amino acid residues homologous to those of IGF I (Rinderknecht and Humbel, 1978b). Almost 50% of the amino acids in the A and B regions of

IGF II, IGF I and human pro-insulin are similar. Both IGF and insulin molecules have a three-dimensional structure determined by disulphide bridges. There appear to be structural similarities between the receptor binding regions of insulin and IGF but the antigenic regions do not resemble one another (Froesch *et al.*, 1985). This may relate to the observation that insulin and IGFs have similar biological actions but lack immunological cross-reactivity.

Release of the hormone

Although the liver is the major source of IGFs found in the blood (Schwander *et al.*, 1983), all tissues appear to have the capacity to synthesize IGFs (D'Ercole *et al.*, 1984). It is postulated therefore that IGFs can interact locally, close to their site of synthesis, by binding either with the cell of origin (as an autocrine factor) or diffusing across the interstitial spaces to bind with an adjacent cell (as a paracrine factor). The relationship between tissue-originated and hepatic IGFs, and whether both sources contribute to the IGF in circulation, have not as yet been established.

Plasma concentrations of IGFs have been shown to be growth hormone-dependent. IGF I levels are lowered in hypopituitary states and elevated in conditions of growth hormone excess. IGF II may be less dependent on GH since, although lower levels are associated with the hypopituitary state, IGF II levels are not elevated when the GH concentration is higher than normal (Clemmons and van Wyk, 1984). Following administration of GH *in vivo*, there was a marked rise in plasma levels of IGF I in humans (Copeland *et al.*, 1980), sheep (Wien *et al.*, 1983), pigs (Chung *et al.*,1985) and cows where there is some evidence that IGF II levels also rise (Davis *et al.*, 1984).

It has been proposed that GH stimulates growth directly through the actions of IGFs (Daughaday *et al.*, 1972). The infusion of human IGF I and IGF II into hypophysectomized rats produced similar effects on growth as GH infusion did. IGF I was clearly more potent than IGF II (Schoenle *et al.*, 1985). Thus a rise in circulating IGF I, whether produced indirectly by GH, or by IGF I administration in the absence of GH, leads to a comparable increase in growth.

There are two examples in nature which seem to indicate that it is IGF rather than GH that is responsible for the stimulation of growth:

(i) The pygmies of Central Africa, who do not appear to suffer from malnutrition, are small in stature but have normal GH levels. Their IGF I levels are very low however and, unlike GH-deficient dwarfs, the levels of IGF I do not rise when GH is administered. It therefore appears that pygmies are small because they do not synthesize normal levels of IGF I (Merimee *et al.*, 1972, 1981).

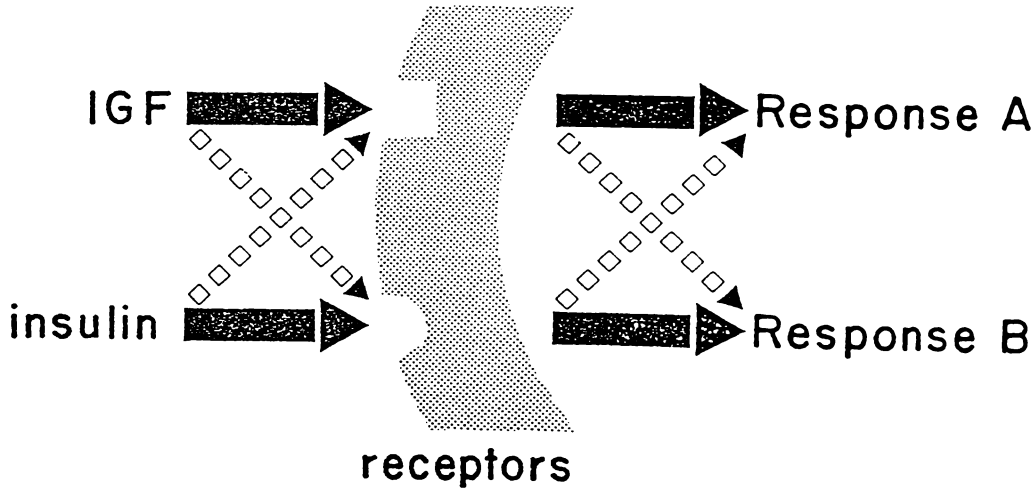
(ii) Inbreeding of poodles has led to three types: standard, miniature and toy which nevertheless all belong to the same race of dog. Although GH levels in all three groups are normal, the standard poodle has high IGF I, the miniature intermediate levels and the toy has very low levels (Eigenmann *et al.*, 1984). Thus, size and the level of IGF I appear to be directly related.

IGF receptors and their biological action

Like other peptide hormones, IGFs exert their biological effects by reacting with receptors located on the cell membrane. There are at least three receptors involved in their action: the insulin receptor which has a high affinity for insulin and low affinity for IGFs; the type I receptor which appears to bind IGF I preferentially over IGF II and has a low affinity for insulin; and the type II receptor which shows a high affinity for IGF II, a low affinity for IGF I and no affinity for insulin (Kasuga *et al.*, 1981; Massague and Czech, 1982; Rechler and Nissley, 1985). The type I receptor has been shown to have similar structural and immunological characteritics to the insulin receptor while the type II receptor differs in structure, peptide binding specificity and antigenic properties from the other two receptors (Massague and Czech, 1982).

Although it was originally considered (van Wyk and Underwood, 1978) that IGF receptors mediated the growth-promoting actions of both insulin and IGFs and that the insulin receptor mediated acute metabolic effects of both insulin and IGFs, this is now known to be an oversimplification (Rechler and Nissley, 1985). Since insulin and IGFs can produce the same biological responses at appropriate concentrations and since most cells

Figure 1.2 Possible interactions between hormones and receptors for IGF and insulin



from Nissley and Rechler, 1984

possess all three types of receptor, it is often difficult to determine which receptor is mediating a particular response. Indeed it is possible that a single response could be mediated by all three receptors.

The situation where two hormones elicit the same biological response can be achieved either by different receptors giving the same response or by activation of similar post-receptor pathways which, although mediated by different receptors, results in the same response. This is shown in Figure 1.2 for insulin and IGF where either receptor can mediate either response A (*e.g.* growth) or response B (*e.g.* glucose transport).

Which mechanism is utilized appears to depend more on the tissue than on the particular biological response. For instance, it has been shown that rat adipocytes possess insulin and type II receptors but lack type I receptors (King *et al.*, 1980; Massague and Czech, 1982). But rat adipocytes respond to IGF I (Zapf *et al.*, 1978), indicating that these metabolic responses could not have occurred via the type I receptor. In fact, it has been shown, as will be described later in this section, that these responses are mediated via the insulin receptor. It is also known that, since insulin binds to the insulin and type I receptors but not to the type II receptor, the type II receptor cannot be responsible for the actions of insulin (Rechler and Nissley, 1985).

Experiments have been carried out to assess the relative contributions of the three receptors. These have included the following techniques:

(i) Identification of the cell receptors and measurement of the relative binding affinities for ^{125}I-labelled insulin and IGFs.

(ii) Assessment of the relative biological dose responses.

(iii) Selective blockade of the insulin receptors with Fab fragments of antireceptor antibodies. Similar antibodies to block the IGF receptor have not yet been isolated.

As a result of this work, the relative roles of insulin and IGFs in mediating biological responses is gradually emerging. Two main areas of function have been identified for the IGFs: firstly their ability to

stimulate growth in hypophysectomized animals (Schoenle *et al.*, 1982, 1985) and mitosis in cell cultures (Rothstein, 1982) and secondly their insulin-like actions.

The acute metabolic effects of IGFs have been studied *in vitro* in adipose tissue, striated muscle and heart muscle. In rat adipocytes (Zapf *et al.*, 1978), IGF I and IGF II stimulated glucose transport and oxidation as well as lipid synthesis from glucose. When compared with insulin, the IGFs are only 1/50th to 1/100th as potent in stimulating glucose metabolism. It is thought that these effects of IGFs are mediated by the insulin receptor. This was demonstrated by King *et al.* (1980) when blocking of the insulin receptor in rat adipocytes by Fab fragments of insulin receptor antibodies inhibited the activity of MSA (otherwise known as rat IGF II), without grossly affecting MSA binding. Also Zapf *et al.* (1981a) destroyed the insulin receptor by short trypsinization. This procedure abolished the effects of both insulin and IGF on glucose metabolism but although the specific binding of insulin was no longer observed, that of IGF remained largely unaltered.

IGF I and IGF II, at concentrations in excess of 10 ng/ml have been shown to be antilipolytic, (by the inhibition of adrenalin-stimulating glycerol release) but again they are less potent than insulin in this activity (Zapf *et al.*, 1981b).

The stimulation of glucose uptake and lactate production by IGFs also occurred in perfused rat heart (Meuli and Froesch, 1975). In this case, the potency of IGFs was 20 to 40 times higher than in adipose tissue but still only 1/2 to 1/5th that of insulin. Poggi *et al.* (1979) showed that the effects of IGF on striated muscle (mouse soleus) *in vitro* were also similar to those of insulin: they stimulated 2-deoxyglucose uptake, glycolysis and glycogen synthesis and activated glycogen synthase. IGF I was 1/10th to 1/20th as potent as insulin. This is consistent with an earlier observation that, after injection *in vivo*, IGF stimulates glucose incorporation into rat or mouse diaphragm muscle more than into adipose tissue lipids (Oelz *et al.*, 1970). It appears that IGFs act on the rat heart through their own rather than through insulin receptors (Meuli and Froesch, 1976; Zapf *et al.*, 1981b) whereas in striated muscle the biological action may be mediated by both insulin and IGF receptors (Zapf *et al.*, 1984). In both cases, IGF stimulated the same glucose transport system as insulin.

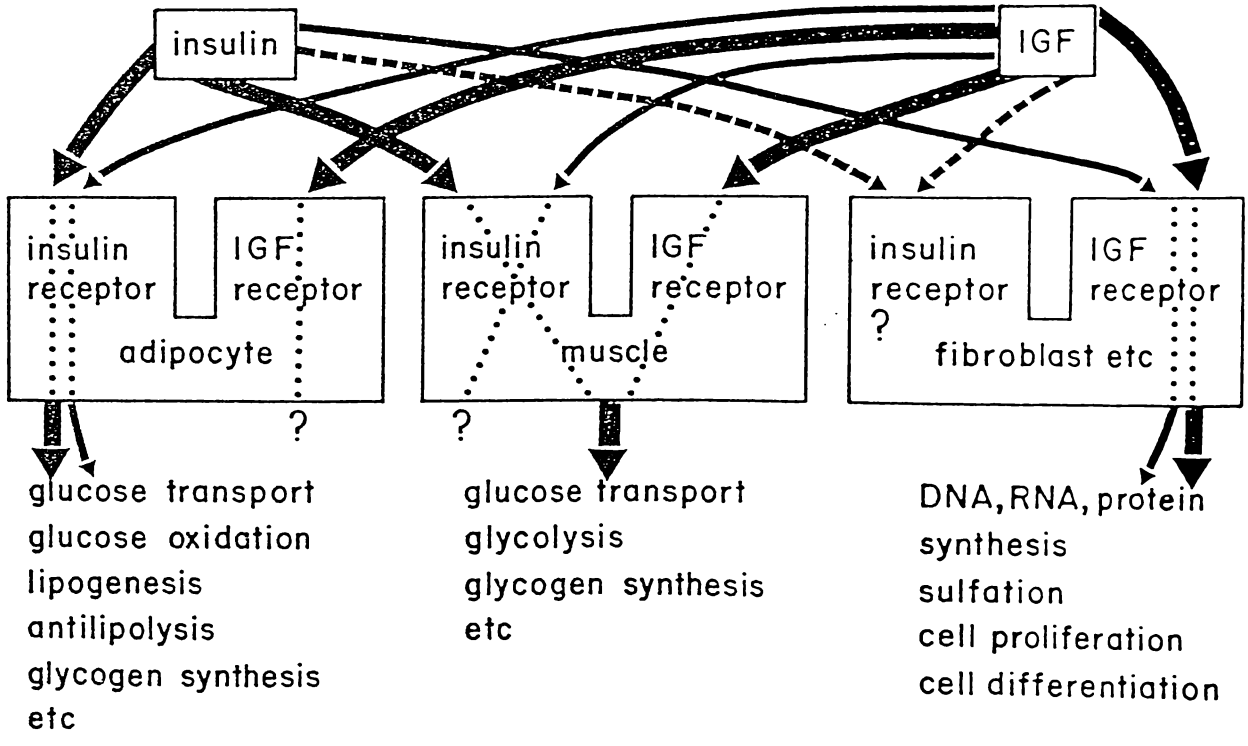
When IGFs were injected into rats, blood glucose levels fell (Zapf *et al.*, 1984) with IGF I being slightly more effective than IGF II. At the same time, IGF I was more potent than IGF II in the stimulation of ^{14}C-glucose incorporation into diaphragm glycogen, while in the adipose tissue, insulin caused a 10-fold stimulation of ^{14}C-glucose incorporation into the lipids but neither IGF I nor IGF II had any significant effect. These experiments demonstrate that IGFs are orchestrating a preferred utilization of glucose in the striated muscle and are consistent with the findings *in vitro*, that the potency differences between insulin and IGFs are much greater in adipose tissue than in heart or striated muscle (Meuli and Froesch, 1975; Poggi *et al.*, 1979). However it must be noted that the experiments *in vivo* involved a marked increase in the amount of free IGFs in circulation, a situation which may not normally occur, and so it is not known whether these results have any physiological relevance.

IGFs have been shown to stimulate mitosis in many cell lines in culture (Rothstein, 1982) as well as RNA, DNA and protein synthesis in rat organ cultures (Canalis, 1980), chick embryo fibroblasts (Zapf *et al.*, 1981b) and mouse soleus muscle (Monier and Le Marchand-Brustel, 1984). IGF I is 5 to 7 times more potent than IGF II and 50 times more potent than insulin, indicating that the effects may be mediated through specific IGF receptors (Zapf *et al.*, 1984). It has been shown in a number of cell lines that the mitogenic effects of insulin are mediated by the type I receptor (King *et al.*, 1980).

One of the main criteria by which IGFs have been characterized is their effects on sulphate incorporation into cartilage. The potency of IGF I is 3 times that of IGF II and 25 times that of insulin in chick embryo cartilage (Froesch *et al.*, 1976).

Although the acute metabolic actions do appear to occur mainly via the insulin receptor, and growth responses via the IGF receptors, insulin has been shown to stimulate growth by acting through the insulin receptor in rat hepatoma cells (Koontz and Iwahashi, 1981; Massague *et al.*, 1982) while in other tissues, evidence exists that IGFs are able to stimulate glucose transport and metabolism via the IGF receptors (Poggi *et al.*, 1979). A summary of all the known actions of insulin and IGF and their interactions with the insulin and IGF receptors in adipocytes, muscle and fibroblast cells is given in Figure 1.3.

Figure 1.3 The complementary anabolic action of insulin and IGF



from Froesch, 1985

The regulation of IGF receptors has also been investigated. Insulin has been observed to cause a marked increase in IGF II binding to type II receptors in isolated rat adipocytes (King *et al.*, 1982; Schoenle *et al.*, 1977; Oppenheimer *et al.*, 1983). This up-regulation requires only a brief (2-3 minute) pre-incubation with insulin at a concentration similar to that required to stimulate glucose transport. The effect on the type II receptors was abolished by the presence of Fab fragments of antibodies which block the insulin receptor. This suggests that the up-regulation of type II receptors is mediated by the insulin receptor.

It is uncertain whether the type II receptors increase in number or binding affinity. Wardzala *et al.* (1984) have studied this problem in adipocytes using KCN which prevents receptor cycling between the membrane and the intracellular space. This demonstrated that the cells have a large intracellular pool of unoccupied type II receptors which normally recycle and accumulate IGF II intracellularly. In the presence of KCN, bound IGF II is confined to the cell surface. It was found that the increased binding of IGF II to adipose cells treated with insulin appears to involve a redistribution of type II receptors to the cell surface. In addition, it has been observed that pre-incubation of IM-9 lymphocytes with IGF I resulted in a time- and concentration-dependent reduction of ^{125}I-IGF I binding (Rosenfeld *et al.*, 1982).

IGF binding proteins

The IGFs in the blood are bound to specific binding proteins (Zapf *et al.*, 1975) and it is probable that less than 1% of total IGFs in blood circulate as unbound hormone. Two types of carrier protein have been identified: one with a molecular weight of 150 kDa and the other of 40 - 50 kDa. They are specific for IGF I and IGF II and will not interact with insulin (Hintz, 1984).

The physiological significance of the presence of binding proteins is still being investigated. They appear to extend the half-life of circulating IGFs, with the 150 kDa form having the longest half-life. It is possible that the binding proteins allow a large pool of circulating IGFs to be kept relatively constant. This would be necessary, for instance, if the functioning of IGFs requires that the hormones are present at constant concentrations. This would be in contrast to other peptide hormones, such as insulin and GH, which circulate in a free state

and are required to change concentration rapidly to regulate urgent metabolic processes.

There are two models proposed for the functioning of the IGF-binding protein complex. The first is that IGFs circulate in the bound form but interact with the receptor only in the free state and the second is that both circulation of IGF and interaction with the receptor involve bound forms of IGFs. Experiments have been carried out to study the validity of each model. Meuli *et al.* (1978), during experiments *in vivo*, found that bound forms of IGFs do not diffuse readily across the capillary walls and concluded that the amount of IGF in plasma may not be the same as the amount at the extracellular surface of the target cell. Zapf *et al.* (1979) showed that addition of purified IGF binding protein to IGFs *in vitro* inhibits not only receptor binding but also the biological action of the IGFs. However, the IGF-binding protein complex formed in these experiments was not that commonly found in blood and it is unclear whether these results have any physiological relevance.

In favour of the second model, where the complexed form of IGF interacts with the receptor, it has been observed that, in some tissues, the IGF complex can compete equally for the receptor site with a similar concentration of free IGF (Hintz, 1984). Hintz also states that the estimated concentration of unbound IGF is an order of magnitude less than the apparent equilibrium constant, indicating that the kinetics of the binding between IGF and the binding protein may not be as simple as first envisaged. In addition, it is known that there are an excess of free binding sites in plasma which suggests that any free IGF would be quickly complexed.

However, IGFs have been shown to possess many insulin-like characteristics which, if expressed at the rate indicated by the concentration of IGFs in plasma, would result in hypoglycemia in the animal. To prevent this, the receptors could be protected from the IGFs by the presence of an inactive bound form. Thus the necessity to protect the insulin receptor from IGFs would dictate the amount of active IGF that could be present at the cell membrane. It is thought that this may be one of the physiological roles of the binding protein (Zapf *et al.*, 1984).

Measurement of the concentration of IGF at the cell surface has yet to be achieved and insufficient information is available to allow extrapolation from the (measurable) amount of bound and unbound hormone in the plasma to the amount of active hormone at the cell surface.

Thus the presence of binding proteins for IGFs leads to the situation where the concentrations of hormone used for incubation of cells *in vitro* is often arbitrarily chosen. Also, since it is not known whether the hormones act at the receptor in a complexed or free state, a decision to either include or exclude binding proteins in the incubation medium will obviously affect the results. However, since it has been shown that the addition of purified binding protein led to a suppression of activity (Zapf *et al.*, 1979) and since it is most widely believed that it is the free form which is active, most studies of biological action of IGFs do not include binding proteins in the medium.

It is possible that the IGF-binding protein complex plays a role in tissue selectivity of hormone action. The diffusion of IGF into the interstitial space and its interaction with the membrane receptors is thought to be quite different according to whether it is bound to the large (150 kDa) or small (45 kDa) binding protein (Froesch *et al.*, 1985). Thus an understanding of the nature of binding proteins and their interaction with the IGF molecule will be required before it will be possible to clearly define the physiological role of the hormone.

1.11 The regulation of energy utilization in the ruminant

Although each hormone has an independent role to play in the control of lipid metabolsm in the adipose tissue, each is a part of an overall pattern of regulation which involves the combined effects of many factors. The functioning of adipose tissue itself is not independent but must be co-ordinated with the activities occurring in other tissues so that all the processes of the body can operate in unison. For instance, both anabolic and catabolic processes take place simultaneously throughout the body. An anabolic process, such as growth or lactation, may require a catabolic process, such as lipolysis in the adipose tissue, to provide the energy required.

The overall control of the metabolic processes of the body has two main functions. Firstly, there is a requirement to maintain homeostasis so that nutrients within the body tissues and fluids are maintained at optimal levels to allow the essential functions to proceed. Secondly, there is a need for the diversion of nutrients required for the anabolic processes (such as growth, reproduction or fattening) to occur when required.

During these control processes, some organs or metabolic functions take precedence over others, resulting in a partitioning of the substrate supply. The precedence of any one metabolic process over others is not always constant and the partitioning is continually modified. An exception to this is that the maintenance of brain, heart and lung cell metabolism is almost certainly always of prime concern. Following this in precedence would be the normal cellular metabolism of other essential organs and tissues of the body.

The different organs and tissue of the body can be divided according to three main classifications:

(i) a supplier of nutrients
(ii) a user of nutrients
(iii) an exchanger of nutrients

(Young and Veenhuizen, 1984)

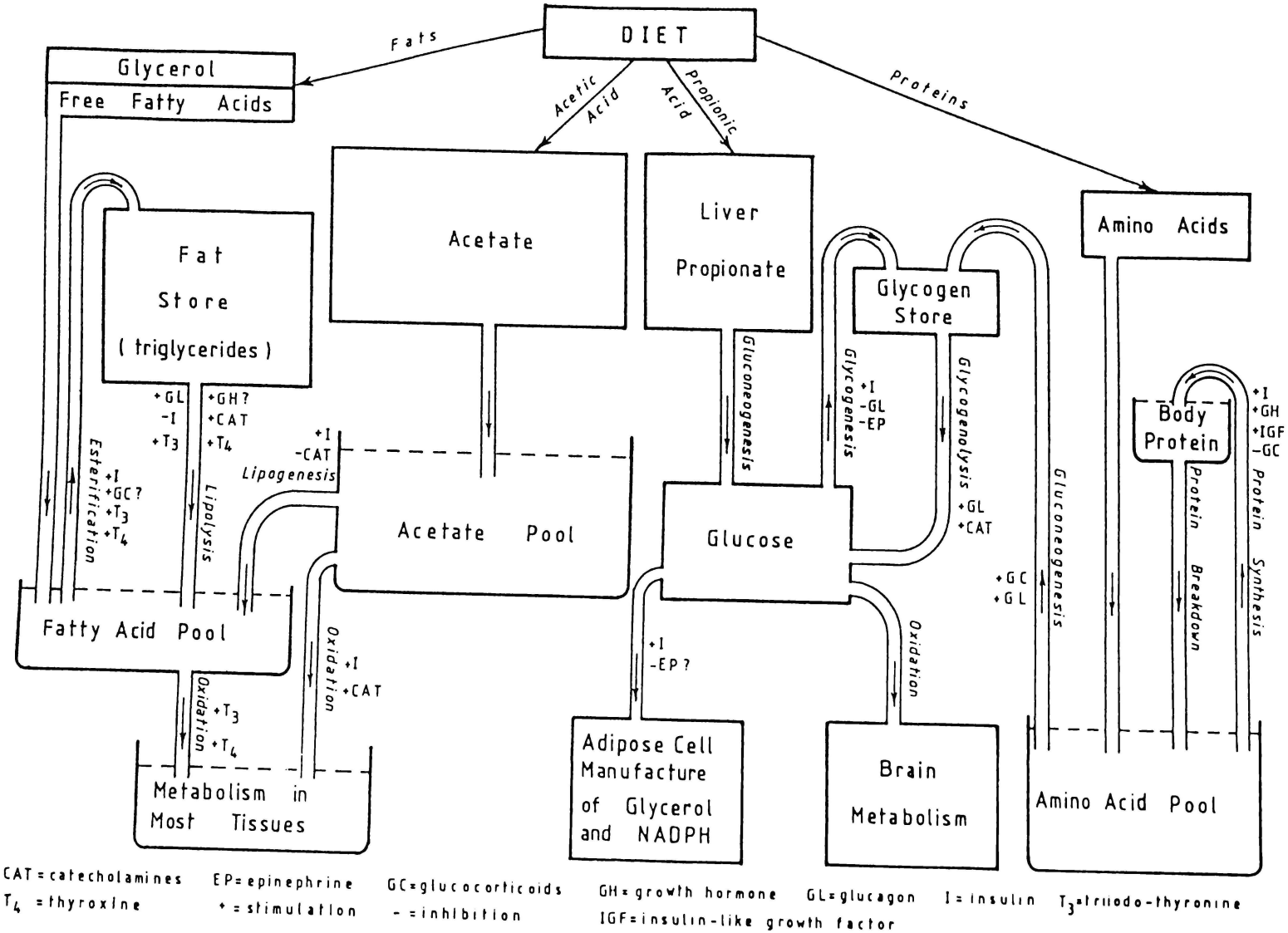
The gut is primarily a supplier of nutrients although, during maintenance of its cellular structure, it will be a periodic user of nutrients. The liver is an exchanger of nutrients as in the case of its role in gluconeogenesis in ruminants. The liver is involved in exchanging a large variety of different nutrient molecules and may account for as much as 20% of the oxygen consumed by the body during normal metabolism. Muscle is a major user of nutrients, especially during growth and movement of the animal. The foetus and mammary gland become important users of energy in the mother during reproduction. Adipose tissue is a user of nutrients during periods of normal nutrient input. However, in the lactating animal or during periods of prolonged exercise in all animals, the adipose tissue becomes a supplier of nutrients which were stored previously and are then required as an energy source by other tissues. When an animal is starved, the adipose tissue also becomes a supplier of nutrient. Thus, it is not possible to consider the regulation of lipid metabolism in isolation as it is intimately involved with the metabolism of both carbohydrate and protein.

The biochemical processes involved in these three classes of tissue are the synthesis and degradation of proteins, carbohydrates and lipids (see Figure 1.4). For proteins, there is synthesis of new molecules for growth and lactation, or replacement molecules of enzymes or structural proteins during normal cellular maintenance. Degradation may involve exchange of body protein between tissues or the oxidation of amino acids. Lipid synthetic processes involve lipogenesis and esterification while lipolysis and fatty acid oxidation are the degradation processes. With carbohydrates, synthesis involves gluconeogenesis and glycogen synthesis. Degradation includes breaking down of glycogen and oxidation of glucose in muscle or liver.

Changes in partitioning can occur rapidly following alterations in the external environment of the animal so that homeostasis will be maintained. For instance, in a lactating animal in a cold environment, the energy supplies are re-directed towards providing energy for heat production and the maintenance of body temperature while milk production decreases. The same partitioning process occurs if the lactating animal is starved; milk production fails and the available resources are used to ensure that the processes vital to the mother's own life are maintained. Thus emergency signals resulting from a stressful external environment can override those signals controlling the normal pattern of metabolism so that the internal environment of the animal remains as constant as possible throughout.The process of reproduction is one exception, however. Reproduction and lactation severely strain the mother's energy resources but, provided that the losses are not so great as to endanger her life (and thus endangering the life of her offspring as well), reproduction and lactation will take precedence over maintenance of the maternal good health.

During the process of growth, the production of lipid in the adipose tissue and glycogen synthesis in all tissues play only supportive roles, with the main energy flow being directed towards muscle and bone development. The partitioning of energy between protein and fat is a function of two main factors: the stage of development of the animal and the level of nutrition relative to maintenance requirements. For instance, a normal mouse fed *ad lib* will convert 40% of its energy supply to protein at a live-weight of about 100 g, but at 400 g less than 20% of the energy is utilized for protein manufacture, the rest being stored as fat (Webster, 1983). This means that the more mature the animal, the

Figure 1.4 The endocrine control of ruminant metabolism



greater the proportion of nutrient that is stored as fat. If the growing animal has a restricted calorie intake, it will tend to maintain protein manufacture at the expense of fat. In terms of animal production, this means that the slower the growth, the greater the ratio of protein to fat deposited. However, if proteins were restricted during growth, this would not apply and the ratio of protein to fat would decrease.

One of the mechanisms used for the partitioning of nutrients involves controlling the flow of blood to the organ or organs involved. This controls the amount of nutrient they receive. Another method is to alter the permeability of the cell membrane which will then regulate the amount of nutrient taken up by the cell. Thereafter, once the nutrient is inside the cell, a variety of mechanisms are in place so that the body can control the eventual fate of a nutrient in a cell. There are likely to be a wide range of metabolic processes occurring in each cell. The intracellular control mechanisms include the degree of activity of the specific enzymes involved and the concentrations of substrate, product or cofactors.

In summary then, the partitioning of nutrients within the body can be affected by the quality and quantity of food intake, the nature of the external environment and the stage of growth and reproductive processes.

The regulation of nutrient partitioning is controlled by the brain using a complex array of hormones and nerves which are sensitive to the overall metabolic status and to the external environment of the animal. The hormones and nerves maintain the physiological integrity of the animal by partitioning the nutrients to meet ever-changing demands. The functioning of the endocrine and nervous systems are closely inter-connected: nervous activity is modified by hormones and the secretion of hormones is influenced by the nervous system. Some substances are both hormones and neuro-transmitters.

The nervous system is sensitive to the animal's environment and can influence body mechanisms to meet the demands made by such conditions as cold and fear. Physiological states, such as pregnancy or growth, are controlled by the endocrine and nervous system which alter the body's metabolism to meet the new demand. The action of eating and the presence of new nutrients in the blood can influence both the endocrine and nervous system and cause them to change the metabolic status of the body

to utilize them fully.

Apart from their individual direct effects, hormones can influence metabolism by altering the responsiveness of a tissue to another hormone. These synergistic effects are thought to be caused by alteration of the numbers of hormone receptors present and the amounts of post-receptor intermediates, such as adenylate cyclase, cyclic AMP or hormone-sensitive lipase. Hormones, in this case, would presumably exert a concerted effect on the process of lipolysis. Some hormones, such as insulin and possibly GH, control the numbers of their own receptors and thus ensure that the response to even high concentrations of the hormone occurs only within the bounds of predetermined limitations.

Examples of co-ordinated hormones which control body metabolism are insulin and glucagon. When blood glucose is high, the concentrations of both glucagon and insulin increase (Trenkle, 1978), with insulin stimulating the utilization of glucose as a source of energy in such tissues as the brain, muscle and adipose tissue and to accumulate reserves of triacylglycerol and glycogen. With only a limited amount of glucose coming from the digestive tract in the ruminant, it is essential that there is a high rate of gluconeogenesis immediately after feeding. This appears to be the role of glucagon in the ruminant, with the rates of both gluconeogenesis and catabolism of glucose being greater in a fed animal then during fasting (Bergman, 1973).

This balance of hormones, with the actions of insulin dominating, has the effect of increasing utilization of nutrients. Insulin also acts by diverting excess amino acids to protein synthesis while adequate energy is available. The insulin-dominated hormone balance also favours the utilization of acetate for lipogenesis in adipose tissue. During fasting, the hormone balance shifts from domination by insulin, and glucagon levels rise. The greater glucagon:insulin ratio during fasting also acts to increase lipolysis as well as catabolism of muscle protein. The fasting hormone balance would promote a more rapid uptake of mobilized nutrients by the liver.

In the lactating cow, there is not so much insulin secreted after feeding, thus directing the flow of metabolites to the mammary gland rather than to the adipose tissue (Trenkle, 1981). Insulin administration *in vivo* decreases serum glycerol and NEFA concentrations (Bergman, 1968). If insulin is also diverting excess amino acids to protein synthesis, this implies that there will be a decrease in both glycerol and amino acids as precursors for gluconeogenesis following insulin release. There would therefore be a decrease in output of glucose from the liver (Prior and Smith, 1982). Insulin also decreases the amount of glucose in circulation by stimulating its uptake into muscle and adipose tissue. Milk yield in dairy cows has been shown to decrease following insulin administration (Kronfeld *et al.*, 1963) and it has been suggested that the availability of glucose is one of the main factors limiting milk production in high-yielding cows (Kronfeld, 1976). Thus insulin's combined effects of decreasing glucose output from the liver and increasing uptake by other cells could account for the decrease in milk production in these animals. The effects of exogenous insulin in decreasing milk yield can be reversed on administration of glucose (Kronfeld *et al.*, 1963; Linzell, 1967).

Conversely, in the presence of anabolic hormones such as GH, it would be expected that increased glycerol release from the adipose tissue in ruminants would result in increased gluconeogenesis and consequently increased blood glucose levels. Since it was shown that concentrations of GH were higher in dairy cows with a high yield of milk compared to those with a low yield (Hart *et al.*, 1978), it is possible that GH's effects on the adipose tissue may have precipitated a sequence of events to increase milk yield. In this case, the increased serum levels of NEFA shown to occur in these animals would also give increased substrate for milk production.

During the growth phase, insulin and GH are thought to antagonize each other's action, with insulin promoting the storage of fatty acid in the adipose tissue and GH appearing to have the effect of diverting energy from the adipose tissue to bone and muscle. However, since the animal requires reserves of fatty acids, the interactions of the hormones will be extremely complex. This is illustrated in the experiments described earlier in this chapter, where slow growth in rats was shown to result in an increase in the protein to fat deposition (Webster, 1983). At the same time, when GH is administered to growing lambs, an increase

in growth rate was also accompanied by an increased ratio of protein to fat (Johnsson *et al.*, 1985). It is possible that insulin may play a role in altering these ratios.

Although catecholamines appear to be involved in short-term responses similar to the fasting hormone balance, their role in determining the long-term energy balance remains to be established (Trenkle, 1981).

It appears that hormones which have been shown to regulate and co-ordinate adipose tissue metabolism in the ruminant have similar, though not identical, roles to those in the non-ruminant.

1.12 Aims of the present study

Lipid metabolism in adipose tissue is controlled by many different hormones, the individual functions of which have not been fully elucidated. To gain a better understanding of the individual action of some of these hormones, investigations were carried out with the following aims:

(i) To develop and test an *in vitro* system that could be used to observe the processes of lipid metabolism in the adipose tissue of growing lambs. It was essential that the system *in vitro* simulated as closely as possible the situation *in vivo*. The kinetics of lipid metabolism were of special interest since these would determine the rate at which the animals would normally fatten.

(ii) To measure the changes occurring in the tissue in the presence of several hormones of interest. Insulin was to be used as a lipogenic standard to ensure that the tissue *in vitro* reacted in the accepted way by increasing the rate of cellular lipogenesis when insulin was present. Conversely, adrenalin was used as a lipolytic standard to test that the process of lipolysis in the cells was functioning normally.

(iii) To investigate the functions of GH, IGF I and IGF II to compare their effects on lipid metabolism with that of insulin and adrenalin and with each other in order to establish the relative importance of all five hormones in the functioning of ovine adipose tissue. Special reference was to be made to the effects the hormones were having on the fattening processes of the animals *in vivo*.

(iv) After a number of measurements were made, it was apparent that variation between animals in the rate of lipid metabolism occurring in the adipose tissue *in vitro* and also in the response to the hormones was a major obstacle to achieving the aims specified in (iii). Thus a further aim developed: to search for a method by which lipid metabolism and the responses to the hormones could be measured to minimize variation. When it was found that most of the variation was real, and not the result of experimental error, investigations into the causes of the variations were undertaken.

Chapter 2

Methods and Materials

2.1 *In vitro* incubation of ovine adipose tissue

Chapter 2 gives a summary of the method used for the incubation of adipose tissue. For a full description of the method, together with a description of the development and validation processes, refer to Chapter 3.

Animals

Neutered, male Coopworth-cross lambs aged 3 to 11 months were used. They were allowed unrestricted access to pasture and water up to and immediately prior to being slaughtered for use in this study. Unless otherwise stated, the animals were not given any treatment with hormones or drugs prior to slaughter.

Preparation and incubation of tissue

Immediately following slaughter, subcutaneous adipose tissue from the lower dorsal region near the base of the tail was removed and sliced thinly with a razor blade. Approximately 0.1 g of tissue (5 - 6 randomly selected slices, each 2 - 3 cells thick) was incubated in 5 ml of Medium 199 with Earle's salts, containing 1.1 mmol/l sodium acetate, 25 mmol/l Hepes buffer at pH 7.4. BSA at a concentration of 1% w/v was added where specified. Hormones and radioactively labelled precursors were added where indicated at the appropriate concentrations (with the hormones adjusted if possible in accordance with measurements of the hormone purity). Incubation was carried out in polypropylene vials on a metabolic shaker at 39°C.

In experiments where growth hormone was being tested, a three hour pre-incubation was carried out as suggested by Eden *et al.* (1982). In experiments where insulin, IGFs or adrenalin were being tested, no pre-incubation was used unless their action was being compared to that of growth hormone. The basal rate of lipid metabolism was measured in medium

containing no hormone, with other incubation conditions being identical to the hormone studies. Blank incubations of tissue slices were carried out in medium containing 500 μl of 1 mol/l sulphuric acid for each 5 ml quantity of medium with the normal addition of isotope.

Incubation was terminated by immersing the tissue in ice-cold normal saline. The number of cells in each sample of tissue slices was estimated using the technique described by Sjostrom *et al.* (1971), with freshly prepared, hand-cut sections of adipose tissue.

2.2 Measurement of lipogenesis

Isotope Incorporation

To measure the rate of lipogenesis, radioactively labelled precursor molecules were added to the incubation medium. The substrate most frequently used was [1-^{14}C]-acetate, where the concentration of acetate in the medium was 1.1 mmol/l and the specific activity 90 mCi/mol. As a result of the process of lipogenesis, the labelled acetate molecules were incorporated into the long-chain fatty acids.

$^{3}H_2O$ (2.8 mCi/ml in the incubation medium) was used to give an estimate of the rate of lipogenesis from all precursors. ^{14}C-glucose, where the medium concentration was 5.5 mmol/l and the specific activity 7.3 mCi/mol, gave an indication of the fate of glucose in ovine adipose tissue.

Fatty acid extraction

Following termination of the incubation, the tissue was removed from the ice-cold saline, blotted dry on filter paper and placed in a glass test-tube. Then 5 ml of 10% w/v KOH in alcohol was added and the tissue lipid saponified at 60°C for one hour. After saponification was complete, the extract was acidified with 5 ml of 25% v/v concentrated HCl in distilled water which had been saturated with NaCl. Ten ml of n-hexane were added and the solution thoroughly mixed. Provided that the aqueous layer had a pH of 1, as checked with universal pH indicator paper, the upper hexane layer then contained the fatty acids. The hexane layer was removed, washed with three 5 ml portions of a mixed solution of acetic

acid and sulphuric acid, both at 0.1 mmol/l, (for ^{14}C-acetate analysis) or three portions of 0.01 mol/l sulphuric acid (for $^{3}H_2O$ and ^{14}C-glucose analysis).

The hexane layer, containing the purified fatty acids, was then transferred to a test-tube with approximately 0.5 g anhydrous sodium sulphate added to remove any water which could contain residual unincorporated substrate. The hexane was transferred to a pre-weighed scintillation vial and evaporated to dryness. After the determination of its total dry weight, the fatty acid residue was dissolved in toluol-PPO scintillation fluid (0.4% w/v PPO in toluol).The amount of ^{14}C-fatty acid was determined by scintillation counting.

Total lipid extraction

When the analysis of tissue metabolism required the extraction of total lipid without saponification (*i.e.* with the triacylglycerol molecules intact), the method described by Dole (1956) was used. The adipose tissue was minced finely with scissors and left to stand for 48 hours in 5 ml of Dole's extraction medium (isopropyl alcohol:heptane:0.5 mol/l sulphuric acid in the proportion 40:10:1). Once extraction was complete, 3 ml of heptane and 2 ml of distilled water were added and the lipids obtained by purification of the heptane layer. The amount of ^{14}C-labelled triacylglycerol was determined by scintillation counting.

2.3 Measurement of lipolysis

Glycerol release

The rate of lipolysis was estimated by measurement of the release of glycerol into the incubation medium. 250 μl samples of medium were removed from the incubation vessel at stipulated intervals throughout the incubation period. Glycerol was measured using the method described by Galletti (1967).

Free fatty acid release

Total lipid was extracted from 3 ml of medium or from tissue by Dole's extraction. The concentration of free fatty acid in the upper heptane layer was determined using the method described by Crane and Lane (1977), with an auto-analyser, using 2.5% w/v sodium barbitone and 1% w/v phenol red in sodium barbitone. Spectrophotometric readings were taken at 550 nm.

2.4 Estimation of hormone concentrations in the incubation medium

Insulin

Insulin was measured by radioimmunoassay based on the method of Wilson and Miles (1974) with the following modifications. Antisera to ovine insulin (Sigma) was raised in guinea pigs. Iodination (^{125}I) of ovine insulin was carried out using the Iodogen method (Salacinski *et al.*, 1981) which gave a single mono-iodinated peak after purification on Sephadex G50 (44 x 1 cm).

Unknown insulin levels were determined in 50 μl samples when compared against ovine insulin standards following a 24 hour incubation at room temperature and polyethylene glycol-separation of bound and free fractions. Serial dilutions of high insulin samples exhibited parallellism to the standard curve and recoveries of pure insulin added to samples was in the range of 92 to 104%. Assay sensitivity as determined by least detectable dose was 0.1 ng/ml. Within-assay and between-assay variation were 9.3 and 13.0 respectively (at 1.6 ng/ml).

Growth hormone

The concentration of ovine growth hormone (oGH) was measured by radioimmunoassay (Gluckman, 1980) using a rabbit anti-(oGH) serum against oGH (GH-S-10) from the National Institute of Health, USA. OGH, prepared by C.H. Li, (University of California, San Franscisco, USA) was used as a standard and tracer. It was iodinated by the lactoperoxidase method (Thorell and Johansson 1971) and purified on a column of Sephadex G100 (1 x 120 cm) before use. The assay was performed by pre-incubating standard or sample with antibody for 24 hours at 4°C before addition of

[^{125}I]-iodo-oGH (10000 CPM). The incubation at 4°C was then continued for a further 48 hours before the addition of sheep anti-rabbit immuno-γ-globulin serum (0.5% v/v) and 1 ml 10% polyethylene glycol 6000 and a final incubation at room temperature for 15 minutes to precipitate the bound fraction. The bound and free hormone were separated by centrifugation and then decantation of the supernatant. Serial dilutions of bovine plasma demonstrated parallelism with the standard curve. The relative potency of purified bGH (B-1, AFP-5200; United States Department of Agriculture Reproduction Laboratory, Beltsville, USA) to that of the oGH standard was 1:0.7 ± 0.065 (sd). The sensitivity of the assay was 0.1 ng, the intra-assay variation 8% and the inter-assay variation 11% .

IGF I

The measurement of IGF I in plasma samples by radioimmunoassay requires an initial acid-ethanol extraction to remove interference from the binding proteins. Since there were no binding proteins present in the incubation medium, the hormone could be assayed directly.

IGF I concentrations were measured by radioimmunoassay (Gluckman *et al.*, 1983) using IGF I (supplied by R. Humbel, Zurich, Switzerland and later by D. Burleigh, International Minerals and Chemical Corp., USA.) and rabbit anti-human IGF I antiserum (no. 2) provided by D. Underwood and D. Van Wyk (University of North Carolina, USA) and distributed by the National Hormone and Pituitary Agency of the National Institutes of Health. To perform the assay, samples and standards were incubated at room temperature for one hour with IGF I antibody. ^{125}I-IGF I tracer was added and incubated a further 18 hours at 4°C. Bound and free ^{125}I-IGF I were separated, following precipitation of the bound fraction with polyethylene glycol, by centrifugation and aspiration of the supernatant. The bound ^{125}I-IGF I in the pellet was measured by gamma counting for one minute. Cross-reactivity with IGF II (R. Humbel, Switzerland) was 1.4%, the means of inter-assay variation 11.5% and intra-assay variation was 5%.

2.5 Estimation of hormone purity using electrophoresis

All samples for electrophoresis were dissolved in distilled water to give a protein concentration of 200 - 400 μg/ml, with 10 - 20 μl applied to the gel.

Isolelectric focusing was perfomed on polyacrylamide gel using the LKB Multiphore apparatus (from LKB-Producter AB, S-161 25 Bromma, Sweden). The prepared polyacrylamide gel plates, obtained from the same company, had a pH gradient range of 3.5 to 9.5. The separation was terminated after 1600 volt-hours. Isoelectric points were estimated by a grid system and the pH determined by elution of the gel at each grid point.

The sodium dodecyl sulphate polyacrylamide gel electrophoresis system used was described by Laemmli (1970). The separating gel was 12% with a pH of 8.8 and had a 4% stacking gel with pH 6.8. The electrophoresis was carried out on the Biorad Protean II system (supplied by Biorad, Richmond, California, USA) using a 160 mm slab gel. Some of the samples were reduced with 0.2 mmol/l 2-mercaptoethanol prior to application to the gel.

Pharmacia Low Molecular weight Calibration Kit (Catalogue no. 17-0446-01, Pharmacia Fine Chemicals AB, Uppsala I, Sweden) was used to determine protein molecular weights in the SDS polyacrylamide gel electrophoresis. A standard curve was constructed using the relative migration of proteins through the SDS polyacrylamide gels against the log of the molecular weight. There was an inverse relationship ($r = -0.9960$) and the equation for the line, determined by linear regression analysis, was $Y = 5.127 - 0.011X$, where Y is the log of molecular weight and X is the relative migration..With these conditions of electrophoresis, the relationship was non-linear for protein subunits with a molecular weight less than 10 kDa.

2.6 Analysis of carcass composition

An entire half carcass was minced finely. After thorough mixing of the minced meat, 20 g samples were analysed for water, fat, protein and ash content using the method described by Kirton (1962). Water content was determined by the weight difference after freeze-drying and fat content by weight difference after extraction to constant weight with petroleum ether. Ash content was determined by the percentage of ground sample remaining after ashing in a muffle furnace. The protein content is calculated from the difference after water, fat and ash content has been subtracted from the original weight of ground sample.

2.7 NADPH-generating enzymes

Samples of subcutaneous adipose tissue were removed from the sheep at slaughter and assayed to determine the activity (expressed per gm fat) of several NADPH-generating enzymes. The adipose tissue was homogenized in a solution of 0.75 mol/l KCL in distilled water which contained 1.7% Triton X100. The Triton X100-induced interference was overcome by SDS treatment and ethyl acetate extraction (Dr G. Upreti, unpublished work). Total protein was then estimated using the method described by Lowry *et al.* (1951).

Standard methods were used to determine the total activity in the adipose tissue homogenates of the following enzymes:
glucose-6-phosphate dehydrogenase 1.1.1.49 (Lohr and Waller, 1974);
6-phosphogluconate dehydrogenase 1.1.1.44 (King, 1974);
NADP-malate dehydrogenase (decarboxylating) 1.1.1.40 (Ochoa, 1955) and
isocitrate dehydrogenase (decarboxylating) 1.1.1.42
(Bernt and Bergmeyer, 1974).

2.8 Materials.

Adrenalin, ovine insulin, BSA (Fraction V, essentially fatty acid free, catalogue no. A6003) and Hepes buffer were obtained from Sigma Chemical Co., Poole, Dorset, UK. Medium 199 used in the earlier part of the study was from Gibco Laboratories, Grand Island, New York, USA and contained Earle's salts, L-glutamine and no sodium bicarbonate (catalogue no. 400-1100). Subsequently, Medium 199 (catalogue no. 5915-37-D) without phenol red or sodium bicarbonate was purchased from Difco Laboratories, Detroit, USA. Sodium [1-^{14}C]-acetate, ^{14}C-glucose, glycerol-^{3}H-trioleate, $^{3}H_2O$ and IGF I (Somatomedin C, catalogue no. ARN 4010 and manufactured by Amgen Biologicals) were purchased from Amersham International, Amersham, Buckinghamshire, UK. [1-^{14}C]-acetic acid, sodium salt (code no. CFA.13 batch numbers 233 and 234), supplied as a solid, had a specific activity of 57 mCi/mmol, 679 μCi/mg. ^{14}C-glucose (code no. CFB35, batch no 41), a solid, had a specific activity of 307 mCi/mmol. $^{3}H_2O$ (catalogue no. TRS.1 had a specific activity of 2.5 Ci/ml. The Sterivex-GS filters (0.22 μm) used to sterilize the medium were from Millipore Corporation, Bedford, USA. All other hormones used in this study were obtained from non-commercial sources:

IGF I — recombinant DNA-derived human IGF I prepared and kindly donated by Dr D. Burleigh, International Minerals and Chemical Corp. (IMCC), Northbrook, Illinois, USA. Reconstitution was carried out according to the supplier's instructions with 50 mmol/l ammonium acetate at pH 5.5.

IGF II (i) — chemically synthesized IGF II prepared and kindly donated by Professor C.H. Li, University of California, San Fransisco, USA. Reconstitution was with 10 mmol/l HCl.

(ii) Multiplication stimulating activity (MSA) was biologically derived rat IGF II purified and kindly donated by Professor J.R. Florini, Syracuse University, New York, USA. Reconstitution was with 10 mmol/l HCl.

GH (i) ovine growth hormone: purified biologically derived hormone obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases, USA (reference no NIAMDDO-GH-12).

(ii) bovine growth hormone: (reference no. ICH/bGH/13) biologically derived bGH which was purified and kindly donated by Dr I. Hart, National Institute in Dairying, Shinfield, UK.

(iii) recombinant DNA-derived bGH: purified and kindly donated by Genentec inc., South San Fransisco, USA

(iv) recombinant DNA-derived bGH: purified and kindly donated by Cyanamid Corp., Wayne, New Jersey, USA.

2.9 Expression of results and statistical significance.

Results are expressed as means and S.E.M. with the number of observations in parentheses. All results given are obtained using replicate samples of adipose tissue slices from one animal unless otherwise stated.

The standard errors of incorporation rates in replicate samples showed a distinct tendency to increase with increasing values of the mean. Thus all metabolic rates were transformed to natural logarithms for analysis of variance to obtain homogeneity of variance.

In experiments where only one treatment is compared with the control (eg where only one hormone concentration was used), analysis of the differences has been carried out using the Student's t test. Otherwise, the probability tables of Dunnett (1955), for use in an experiment where multiple treatments are compared to a single control, have been applied. The probabilty that the metabolic activity in the presence of a hormone is different from the basal metabolic activity is indicated in each figure. All probabilities greater than 0.05 were considered to be non-significant and are not shown in the figures.

The observed response to a hormone has been expressed as a percentage stimulation above the basal. This has been calculated by subtracting the observed basal rate of metabolism from the observed rate of metabolism achieved by the cells as a result of interaction with the hormone. The difference is then expressed as a percentage of the basal rate, being positive if the hormone has increased the rate and negative if it has decreased it. All maximum observed responses have been calculated using the observed maximum responses and not calculated values derived from the maximum height of a fitted curve.

Since an acid blank (where no metabolism occurred) has been subtracted from each observation to account for background readings, all graphs in which the cellular response has been plotted against time include an observation point at the origin. This is symbolized by a closed circle at the origin of time-course graphs since it applies to all lines plotted. The point 0,0 has been included once in all linear regression analysis of time-course data.

Chapter 3

Incubation of Ovine Adipose Tissue In Vitro

This chapter describes the procedure used to develop and validate the method for preparation and incubation of ovine adipose tissue *in vitro*. Although the techniques for incubation of rat adipocytes and, to a lesser extent, ovine adipose tissue were established in other laboratories, much preliminary work was necessary before incubations could be successfully undertaken in the present study. This involved validation of the *in vitro* model to ensure that the results obtained were a close estimate of the metabolism occurring in the cells and that the metabolism in the cells *in vitro* was a reflection of that occurring in the same cells *in vivo*.

3.1 The choice of animals

Animals used for these experiments were, as far as was possible, all of the same age, weight, sex and nutritional status. They were between the ages of 3 - 11 months and weighed 25 - 40 kg at the time of slaughter. To allow experiments to continue throughout the year, some lambs (used in the late winter and spring) were born out-of-season as a result of artificially-induced ovulation in the mother. Lambs were used only when they were fully weaned and therefore ruminant, thus ensuring that their metabolism was based on the use of acetate as the principle carbon source. All animals were neutered male Coopworth-cross lambs. They were allowed unrestricted access to pasture and water up to the beginning of the experiment. Unless otherwise stated, animals were not given any treatment with hormones or other drugs prior to slaughter.

3.2 Slaughter and tissue collection procedures

Any stress to which the animals were subjected at the time of slaughter would result in the release of catecholamines. Since the presence of catecholamines in the tissue could influence the *in vitro* metabolism, the animals were weighed on the day before the experiment to minimize stress immediately prior to slaughter.

The lambs were killed by an incision in the jugular vein. Once the blood had drained from the body, an area of skin in the lower dorsal region was removed to expose the adipose tissue. Using a sterile scalpel and forceps, small pieces of subcutaneous adipose tissue from the base of the tail and along the dorsal part of the vertebral column were removed and placed immediately in Medium 199 held at 39°C. This task was completed within five minutes of the death of the animal.

The carcass of the animal was skinned and hung for measurement of GR, a method used commercially to assess carcass fatness (Kirton *et al.*, 1985). The GR is defined as the total soft tissue depth between the surface of the carcass and the rib, over the 12th rib and 110 mm from the mid-line. It is expressed in mm. On average, for each 1mm decrease in GR, carcass fatness, measured either by chemical means (Kirton, 1962) or by dissection techniques, decreases about 1% w/w, dissected muscle increases by about 0.5% w/w and carcass bone increases by just under 0.5% w/w.

3.3 *In vitro* methods used to study adipose tissue

The metabolism of adipose tissue *in vitro* can be examined by several different techniques. In the tissue slice or explant technique, the adipose tissue is sliced or minced finely before incubation.Vernon *et al.* (1975, 1976, 1978, 1979b, 1981a, 1982a, 1985a, 1985b), Robertson *et al.* (1981, 1982), Hood and Thorton (1980), Ingle *et al.* (1972), Payne and Masters (1971) and Deeth and Christie (1979) have all used the explant technique to study ovine adipose tissue *in vitro* .

Rodbell (1964) was the first to describe a method to prepare free fat cells from rat epididymal adipose tissue by collagenase digestion. It has been used for experiments on ovine adipose tissue in a number of studies (Vernon *et al.*, 1977a, 1981a, 1981b; Smith and Walsh, 1984; Broad *et al.*, 1983; Yang *et al.*, 1982; Thornton *et al.*, 1979).

In addition, some studies of adipocytes have been carried out using precursor cells to adipocytes, especially 3T3-L1 from Swiss Mouse embryo (Green and Meuth, 1974). These cells have the properties of fibroblasts and are capable of differentiating into adipocytes. (Frigeri *et al.*, 1983; Deutsch *et al.*, 1982;, Morikawa *et al.*, 1982; Reed *et al.*, 1981). Ovine precursor cells to adipocytes which could be propagated are not yet available.

It appeared that either tissue slices or isolated adipocytes from growing lambs would provide the most suitable material for a study of the processes of lipid metabolism in adipose tissue *in vitro* .

3.4 Preparation of isolated adipocytes

This method has been used extensively in the rat where the adipocytes maintain their ability to metabolize glucose and respond to hormones (Rodbell, 1964). Some preliminary experiments were carried out on ovine adipocytes using the following method which has been adapted from the protocol kindly provided by Dr K. Gain, Otago Medical School, Dunedin, New Zealand.

Materials

Adipose tissue from the lower dorsal region of young lambs (as described in sections 3.1 and 3.2) were transported to the laboratory in 37°C modified Krebs-Ringer buffer (see appendix) containing 25 mmol/l Hepes buffer, 1% w/v BSA, 0.25 mmol/l glucose and 1.1 mmol/l sodium acetate at pH 7.4. This buffer containing glucose and acetate was used for all procedures except the collagenase digestion. Collagenase, supplied by Sigma Chemical Co., Poole, Dorset, UK., was prepared at a concentration of 3 mg collagenase dissolved in 4 ml of buffer without glucose and acetate. All treatment of the tissue was carried out in polypropylene vessels.

Method

Approximately 1 g of adipose tissue was rinsed in normal saline at 37°C, transferred to a flask containing collagenase and minced with scissors. The tissue was then digested at 37°C either in a metabolic shaker or held still, with gentle swirling at the end of the incubation period. When ovine adipose tissue was being prepared, it was found that approximately two hours' incubation was required by either method to obtain sufficient isolated adipocytes. On several occasions even this length of time was not sufficient.

After digestion, the suspension was strained through nylon mesh and centrifuged gently for a few seconds. The cells were washed three times in buffer without glucose and acetate and finally suspended in buffer containing glucose and acetate, all at 37°C. The resuspended cells were returned to the metabolic shaker and shaken gently for 15 minutes before use.

3.5 Incubation of isolated adipocytes

Isolated adipocytes were incubated in the same manner as tissue slices (see section 3.11), with the exception that, instead of dispensing the tissue into the incubation vessels, 500 μl of cell suspension was added just prior to commencing the incubation period. The total volume of medium in the incubation vessel was 5 ml. Calculation of substrate incorporation was also the same, except that cell numbers in the suspension were estimated using the dry weight of lipid extracted from the total number of cells used (as described in Section 3.14).

3.6 Media Preparation

Medium 199 is a chemically defined medium which was used for the incubations of ovine adipose tissue slices in preference to Krebs-Ringer buffer in the belief that it more closely resembled the interstitial fluid *in vivo*. It provided the essential amino acids, vitamins and other metabolites which may not necessarily be available in the cell cytoplasm *in vitro*.

In the initial studies, when only lipogenesis was being measured, Gibco Medium 199 (Cat. no. 400-1100) with Earle's salts, L-glutamine and without sodium bicarbonate was used. Later, when lipolysis was being measured, it was found that the phenol red indicator interfered with the spectrophotometric measurement of glycerol in the medium. Since the Gibco Medium 199 was marketed only with phenol red indicator included, the decision was made to change to Difco Medium 199 (Cat. no. 5915-37-D) which was marketed without phenol red. The formulations of both the Gibco and Difco Medium 199 are given in the appendix. The concentration of sodium acetate, which was 0.61 mmol/l in the the Gibco medium and 0.37 mmol/l in the Difco medium, was adjusted to 1.1 mmol/l, making the glucose and acetate concentrations in the two media identical. This concentration of acetate was chosen because circulating acetate levels in sheep were found to be 1.1 ± 0.1 mmol/l (Vernon *et al.* 1981b). Glucose levels in sheep serum are typically 3.2 - 3.6 mmol/l (Vernon and Peaker, 1983; Bergman, 1973). Medium 199 has glucose levels of 5.5 mmol/l and no further additions were made. The use of a Medium with a higher glucose level than that normally seen in sheep was not an optimum situation but was unavoidable when pre-prepared medium initially formulated for use in non-ruminant systems was being used. Hepes buffer was added to give a concentration of 25 mmol/l.

All the medium components were dissolved in de-ionized water (from a Milli-Q ion exchange water purification unit from Millipore, USA) and the pH adjusted to 7.4 with 2 mol/l NaOH. The medium was sterilized using a Sterivex-GS 0.22 μm filter and stored at 4°C in sterile polyethylene flasks until required.

3.7 Preparation of hormone stock solutions

Hormone stock solutions were prepared so that the required amount of hormone could be added to the incubation vessel (containing 5 ml of medium) in the minimum amount of solution possible. The final dilutions of the stock solutions of hormones were made using Medium 199 to minimize the dilution of other components in the medium. These final dilutions and the addition of the hormone to the incubation vessel were all carried out immediately prior to the beginning of the incubation period. All manipulations of hormones were made in plastic containers.

Insulin

The solution of ovine insulin was prepared at a concentration of 1 mg/ml of insulin in 3 mmol/l HCl. The solution was diluted to a concentration of 1163 ng/ml with Medium 199 containing 1% w/v BSA (pH 7.4) and stored at -20°C in polypropylene tubes. It has been shown that the mean immuno-reactive hormone concentration is altered by repeated freeze/thaw cycles (Livesy *et al.*, 1980) and therefore the stock was thawed only once. The insulin stock solution was diluted with Medium 199 and added to the incubation vessel at the required concentration.

Adrenalin and Nor-adrenalin

The initial solutions of adrenalin and nor-adrenalin were prepared freshly for each experiment at a concentration of 1 mg/ml in 0.1 mol/l HCl. Further dilutions were carried out with Medium 199 until the final concentration in the incubation vessel was 50 ng/ml for adrenalin and 11.7 ng/ml for nor-adrenalin.

Insulin-like Growth Factors

The hIGF I from Amgen was dissolved in 10 nmol/l HCl while hIGF I from IMCC was dissolved in 50 mmol/l ammonium acetate at pH 5.5, as recommended by the suppliers. Chemically synthesized IGF II prepared by Professor C.H. Li and biologically derived MSA purified by Professor J.R. Florini were dissolved in 10 mmol/l HCl.

Because of the difficulties associated with the production of these hormones, only extremely small quantities were available for this study and were supplied as 10 or 20 μg of freeze-dried solid. It was therefore not possible to reweigh the amount used and the total quantity was reconstituted and diluted according to the weight given by the supplier. It was also not possible to observe the dissolving process and so, after 20 minutes of gentle agitation at 37°C, it was assumed that the hormone had dissolved. (See Section 7.2 for tests carried out to verify the presence of IGF I in the incubation medium.)

Dilutions of stock solutions were carried out using Medium 199 with 1% w/v BSA to a concentration of 7000 ng/ml. These solutions were stored in polypropylene vials at -20°C until required. Because of the similarities between the structure of the IGFs and insulin and because repeated freeze/thaw cycles are known to alter the mean immuno-reactive hormone concentration of insulin, IGFs also were thawed only once. Further dilutions of the hormone stock with medium were made before addition to the incubation vessel at the stipulated concentration.

Growth hormone

Growth hormone preparations were supplied in sufficient quantities to allow initial weighing in this laboratory. The freeze-dried solid was dissolved just prior to the start of the experiment in 0.1 mol/l $NaHCO_3$ buffer (pH 10) to a concentration of 1 mg/ml. This stock solution was diluted with Medium 199 to the required concentration before addition to the incubation vessel.

3.8 Preparation of radioactively-labelled substrate

^3H_2O

The tritiated water, as purchased, contained 2.5 Ci/ml and was diluted in normal saline to give a stock solution of 25 mCi/ml ^3H_2O. This stock solution, which was frozen until required, was diluted with Medium 199 and added to the incubation vessel to give a final specific activity of 28 μCi/ml ^3H_2O.

The use of ^3H_2O was discontinued early in this study because it became apparent that contamination of the laboratory was extremely difficult to control. Relatively high specific activities in the incubation medium were required to obtain sufficient counts in the fatty acid. Also, it was found that the ^3H_2O was extremely volatile, especially at 39°C and it was suspected that it was passing out of sealed plastic containers as well.

^{14}C-acetate

1 mCi of sodium ^{14}C-acetate was dissolved in 20 ml of distilled water to make up a stock solution which was kept at -20°C until required.

The stock solution was diluted with medium 199 and added to the incubation vessel to give 0.1 μCi/ml in the medium. The total concentration of acetate in the medium was 1.1 mmol/l, giving a specific activity of 90 mCi/mol.

^{14}C-glucose

50 μCi of ^{14}C-glucose was dissolved in 10 ml of distilled water to make up the stock solution which was held at -20°C until required.

The solution was diluted with Medium 199 and added to each incubation vessel to give 0.02 μCi/ml in the medium. The concentration of glucose in the medium was 5.5 mmol/l and the specific activity 3.9 mCi/mol.

3.9 Preparation of incubation vessels.

The incubation vessels were prepared just prior to the slaughter of the animal and tissue collection. Tissue slice incubations were carried out in disposable, sterile, polypropylene vials with screw caps. They were large (65 mm high and 30 mm in diameter) with a total capacity of 40 ml so that 5 ml of medium had a depth of about 20 mm and a large surface area exposed to the air. This was to facilitate gaseous exchange with the air above the medium when the vessels were in the metabolic shaker.

Medium 199 was prepared with BSA or additional acetate where stipulated. Additions were made so that each vessel had the required amounts of hormones, isotope and medium in a total volume of 5 ml. To facilitate the process, each vessel's additions were calculated before beginning the experiment:

vessel No.	hormone ng/ml	hormone stock µl	isotope µl	Medium 199 µl	H_2SO_4 µl
1,2,3	-	-	50	4450	500
4,5,6	-	-	50	4950	-
7,8,9	0.7	40.0	50	4910	-
10,11,12	2.6	150.0	50	4800	-
13,14,15	5.2	300.0	50	4650	-

In vessels 1,2 and 3 only, there was an addition of 500 µl of 1 mol/l H_2SO_4. Tissue slices were added to these vessels as with the rest. but because of the extreme acidity of the medium, there was no metabolic activity. These three samples provided an incubation blank (referred to as "acid blank") for all further determinations. Vessels 4,5 and 6, in which there were no added hormones, were used to assess the basal rate of the metabolic activity under study. The other vessels provided information about the response of the cells to hormone present at various concentrations. Once all the additions had been made, the incubation vessels were capped and placed in a metabolic shaker at 39°C to equilibrate before the addition of the tissue slices.

3.10 Preparation of tissue slices.

The tissue was brought to the laboratory, placed in fresh medium and held at 39°C in a water-bath until required. Slicing was carried out in a specially constructed, temperature-controlled incubation dish. This consisted of a thermostatically controlled heating mantle which heated an aluminium block to a constant temperature. A large, sterile glass petri dish fitted into an indentation in the aluminium block to allow good heat transfer.

The petri dish was filled with sterile medium. Frequent measurements were made to ensure that the temperature of the medium did not vary outside the range of 35 - 39°C since this could cause irreversible damage to the cells. Once the temperature in the petri dish had stabilized, adipose tissue was transferred into it and sliced as thinly as possible using a hand-held razor blade and forceps. The slices did not exceed 10 mm in length or width and were usually 2-3 cells thick.

In order to obtain a good mix of slices from different pieces of adipose tissue in each vessel, all the tissue for the experiment was cut before any slices were dispensed. The preparation of the tissue slices took approximately one hour, timed from the moment of death of the animal. During this time, the medium in the petri dish was changed several times to rinse the cells and ensure that the pH, acetate and glucose levels were all stable. This represented a pre-incubation phase for the tissue, with no isotope or hormones being present.

3.11 Incubation of tissue slices

When all the tissue had been cut, the medium in the petri dish was changed once more. A random selection of slices were quickly dispensed, using sterile forceps, into the prepared incubation vessels containing Medium 199 and any stipulated hormones and isotope. Five to six slices, which contained in total approximately 100 mg of fatty acid, were placed in each vessel which was then capped to prevent water loss. The metabolic shaker was operated at 48 oscillations per minute throughout the incubation period.

Most of the incubation vessels contained isotope to measure the substrate incorporation rate (H_2O, acetate or glucose) into the fatty acids. However, several incubation vessels containing tissue but no isotope were also included. These contained the hormones under test and samples of this medium were taken throughout the incubation period to monitor the degradation of the hormone. Other incubation vessels, containing tissue but no isotope, were used to monitor the pH of the medium. As soon as the pH in these vessels dropped below 7.1, the medium in all vessels was changed. In experiments lasting six hours, the pH of the medium remained stable, owing to the presence of Hepes buffer. However in the longer-term experiment (24-96 hours), the pH did fall below 7.1 and the medium was changed. This was necessary because low pH can inhibit metabolism and also because insulin is known to dissociate from its receptor below pH 7.0.

At timed intervals, usually every two hours, 250 μl of medium was removed from each incubation vessel, placed in a capped glass test-tube and stored immediately at -20°C for later analysis of glycerol. (See section 3.15)

50 μl of isotope stock (the same amount as was dispensed into each incubation vessel) was put in each of three scintillation vials, 950 μl of distilled water and 8 ml of triton-toluol-PPO scintillation fluid was added. The scintillation fluid was prepared using 2 l toluol, 0.5 l triton X100 and 10 g PPO. The CPM was estimated by scintillation counting for one minute and, when corrected for quench (see section 3.14), the average of the three DPM measurements provided an estimate of the total radioactivity in each incubation vessel. In subsequent sections, this is referred to as total count (TC).

At the end of the incubation, the vessels were removed from the metabolic shaker in the order in which they were dispensed and at the same speed. The vessels were placed immediately into ice, the cap removed and, using forceps, the tissue was transferred into 10 ml of ice-cold normal saline to terminate any further metabolism in the cells. To check the efficiency of this termination process, freshly prepared tissue slices were placed in Medium 199 to which ^{14}C-acetate had been added. Instead of placing the incubation vessels in the metabolic shaker at 39°C, they were held on ice for the entire 6 hour incubation time. There was no measurable incorporation of the ^{14}C-acetate into the tissue fatty

acids during this period and it was therefore assumed that lipogenesis had been terminated by the low temperature of the medium.

Medium from the normal incubations carried out at 39°C was stored at -20°C for later measurements of FFA and glycerol. When required, residual ^{14}C-acetate in the medium was also measured to monitor the total amount of substrate used during the incubation period. The tissue, which remained on ice and immersed in normal saline for about half an hour, was then removed, blotted dry on a sheet of filter paper and placed in glass tubes for saponification or Dole's extraction of total lipids (see section 3.14). The tissue was stored at -20°C until required.

Before any experiments were carried out to test the effects of hormones on fatty acid metabolism in ovine adipose tissue, it was necessary to validate the *in vitro* model. One of the most important criteria was that the characteristics of the *in vitro* cellular metabolism observed resembled as closely as possible that occurring *in vivo*. To achieve this, all incubation parameters were set to mimic the cellular environment *in vivo*. Any decisions about alterations of parameters, such as incubation time, media constituents and their concentrations were invariably made by reference to the *in vivo* situation. Only physiologically normal conditions were used. Within these limits, the parameters of the incubation were optimized to give the maximum possible metabolic rate. It was then assumed that any further constraints on the rate of metabolism were due to the cellular components themselves.

3.12 The use of BSA in the medium.

Some batches of BSA exhibit insulin-like activity while others inhibit the normal response to insulin. As seen in Figure 3.1, the BSA used in this study (Sigma Fraction V, catalogue no. A6003, batch no. 104) reduced the basal rate of lipogenesis as well as inhibiting the normal response to insulin. Insulin stimulated the rate of lipogenesis by 48% when BSA was present and by 118% when there was no BSA present.

BSA from several sources was tested prior to beginning this study (see Table 3.1). All the BSA tested appeared to depress the basal rate of lipogenesis and to alter the response of the cells to insulin. BSA supplied by Immuno-chemical Products Ltd (ICP), Auckland, New Zealand inhibited the insulin response while both batches of BSA supplied by Sigma Chemical Co.,Poole, UK enhanced it. However when a further supply of Sigma batch no. 104 was obtained, it depressed both the basal rate of lipogenesis and the response to insulin. This was similar to the effect observed in all subsequent experiments.

Although it was found that BSA did not significantly affect the basal rate of lipolysis (Figure 3.2), its presence enhanced the lipolytic response to IGF I ($p < 0.01$). The lipolytic response to adrenalin was also enhanced ($p < 0.01$) by the presence of BSA (Figure 3.3). Polypep^{R} (a protein digest from Sigma Chemical Co.), restricted the response of the cells to adrenalin when compared with that in the presence of BSA ($p < 0.05$, Figure 3.4).

The batch of BSA which exhibited the least interference and was subsequently used throughout the present study was further investigated for component protein molecules using SDS polyacrylamide gel electrophoresis (Plate 6.2). The BSA sample, which was initially reduced by treatment with 2-mercaptoethanol, was overloaded on the gel so that any subunits present in low concentration were visible. There were a large number of minor components of molecular weight less than 68 kDa and several greater than 100 kDA. These molecules were at such low concentration that they would not normally be seen under more optimal loading volumes and protein concentrations.

Table 3.1 The effect of BSA on acetate incorporation into fatty acid and insulin response

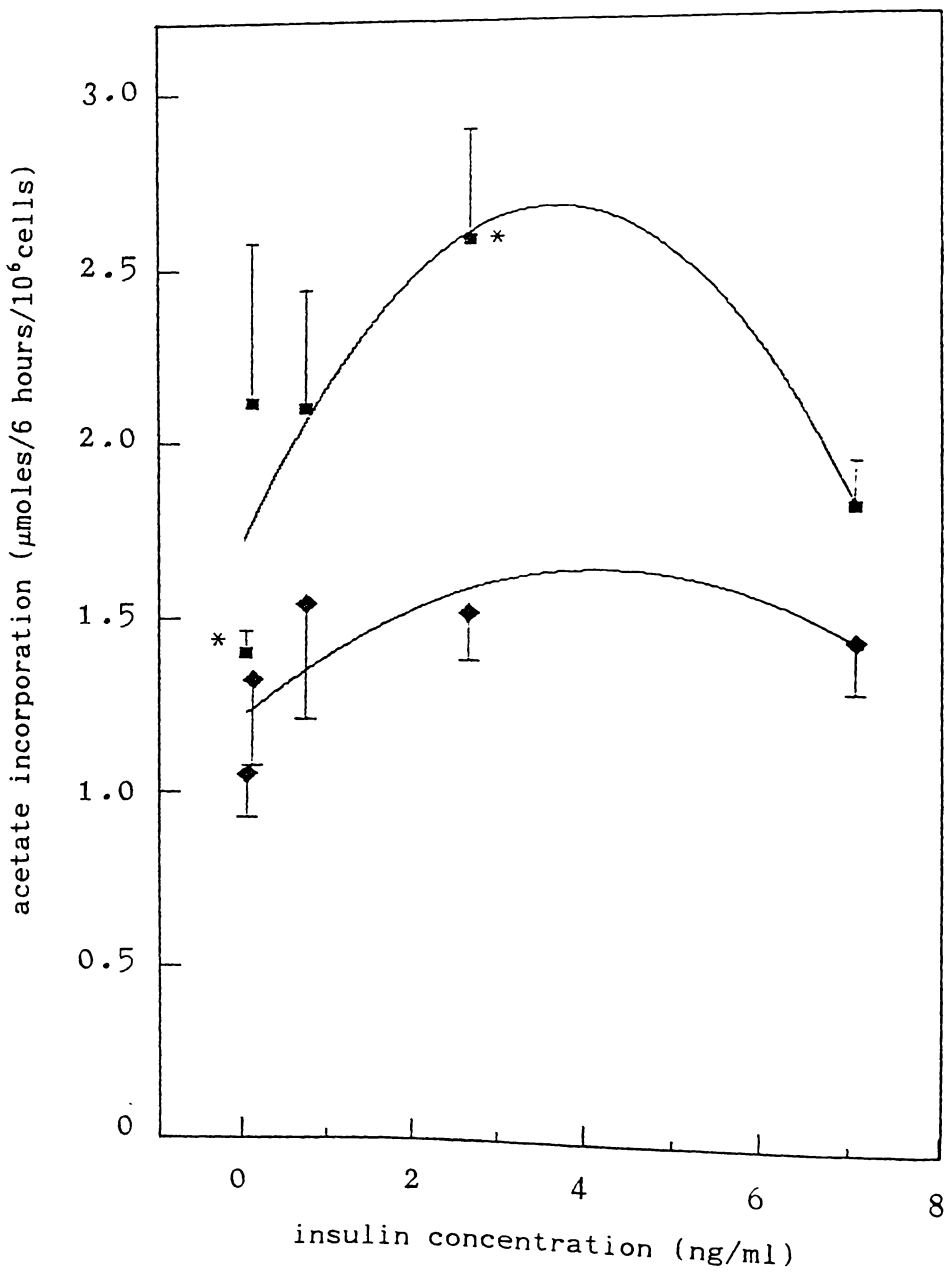
The basal rate of acetate incorporation into fatty acid (μmol/h/10^6 cells) and the rate in the presence of insulin with and without BSA in the medium is shown. Results are expressed as means ± S.E.M. with the number of observations in parentheses. All tissue tested originated from the same animal. Sigma BSA (A6003) was from Sigma Chemical Co. Poole, Dorset, UK and ICP BSA from Immuno-chemical Products Ltd., Auckland, New Zealand.

	Basal rate	Insulin 2.6 ng/ml	Percent stimulation
no BSA	2.0 ± 0.5(3)	5.8 ± 2.8(3)	190%
1% BSA (ICP)	1.75 (1)	2.6 ± 0.6(3)	48%
1% BSA (102*)	0.5 (1)	3.8 ± 0.8(3)	654%
1% BSA (104+)	0.5 (1)	4.8 ± 0.6(3)	860%

* *Sigma BSA batch no. 102*

\+ *Sigma BSA batch no. 104*

Figure 3.1



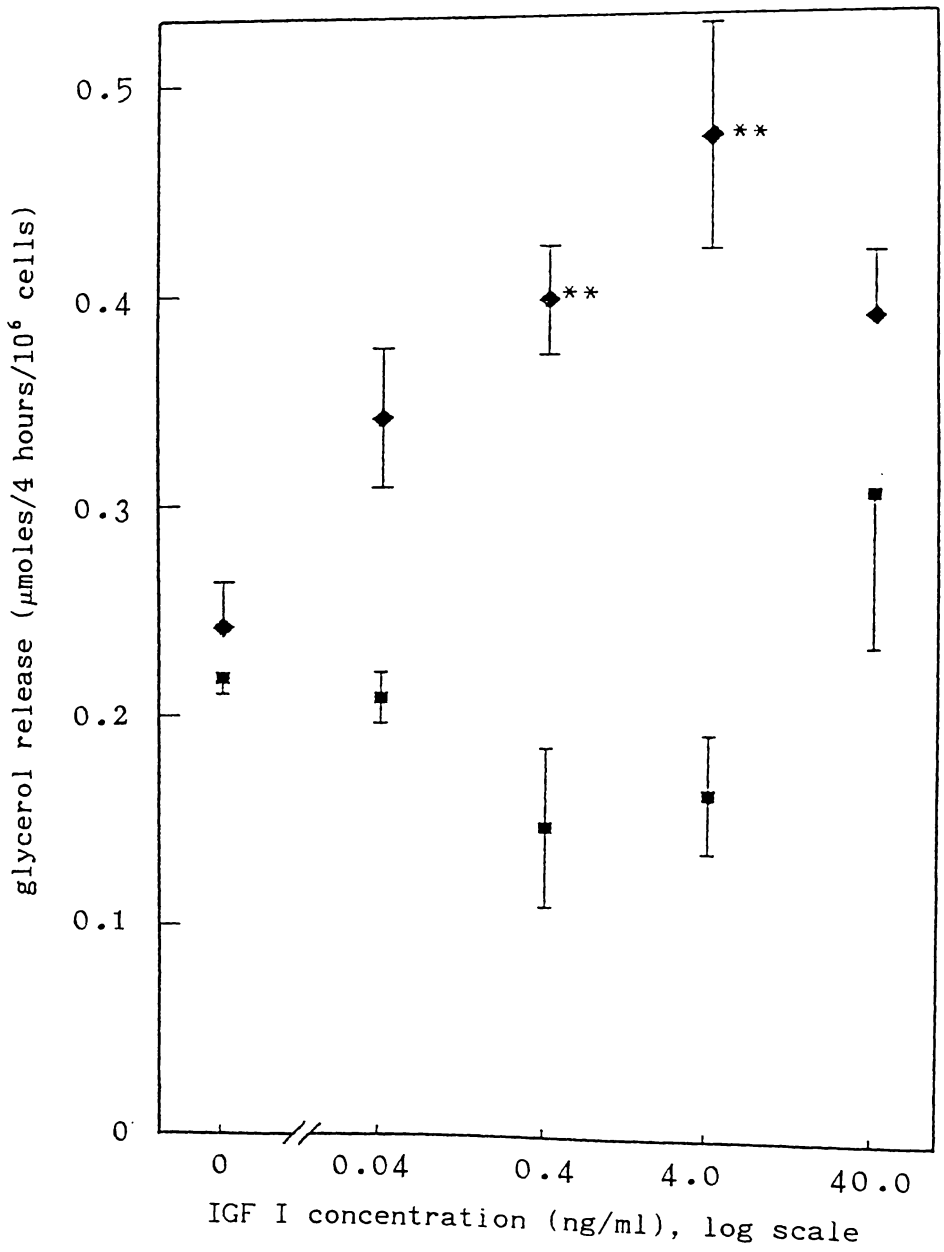
* $p < 0.05$

Figure 3.1 The effect of 1% BSA on insulin stimulation of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 without BSA ■ and with 1% w/v BSA present ◆ . The BSA used was Sigma Fraction V, A6003, batch 104. Acetate incorporation into tissue fatty acids (μmoles/6 hours/10^6cells) is plotted against insulin concentration (ng/ml).

The presence of BSA has depressed the basal rate of lipogenesis ($p < 0.05$) and the rate at all concentrations of insulin. The maximum observed insulin stimulation without BSA is 118% and with BSA 48%, a statistically significant difference ($p < 0.05$).

Figure 3.2



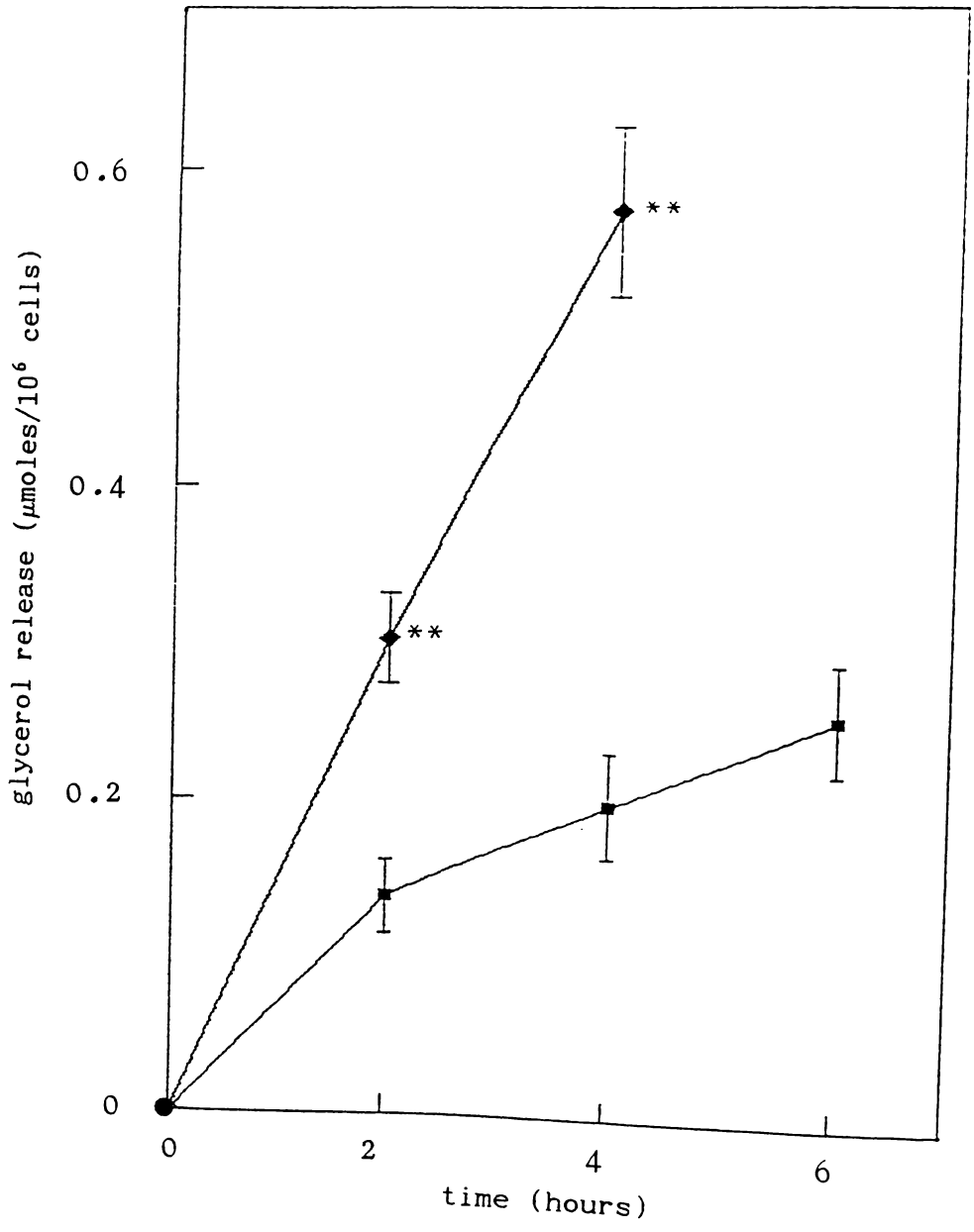
** $p < 0.01$

Figure 3.2 The effect of BSA on IGF I stimulation of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 without BSA ■ and with 1% w/v BSA present ◆ . The BSA used was Sigma Fraction V, A6003, batch 104. Glycerol release into the incubation medium (μmoles/4 hours/10^6cells) is plotted against IGF I concentration (ng/ml), with the x axis drawn on a log scale.

The presence of BSA has significantly enhanced the response to IGF I ($p < 0.01$).

Figure 3.3



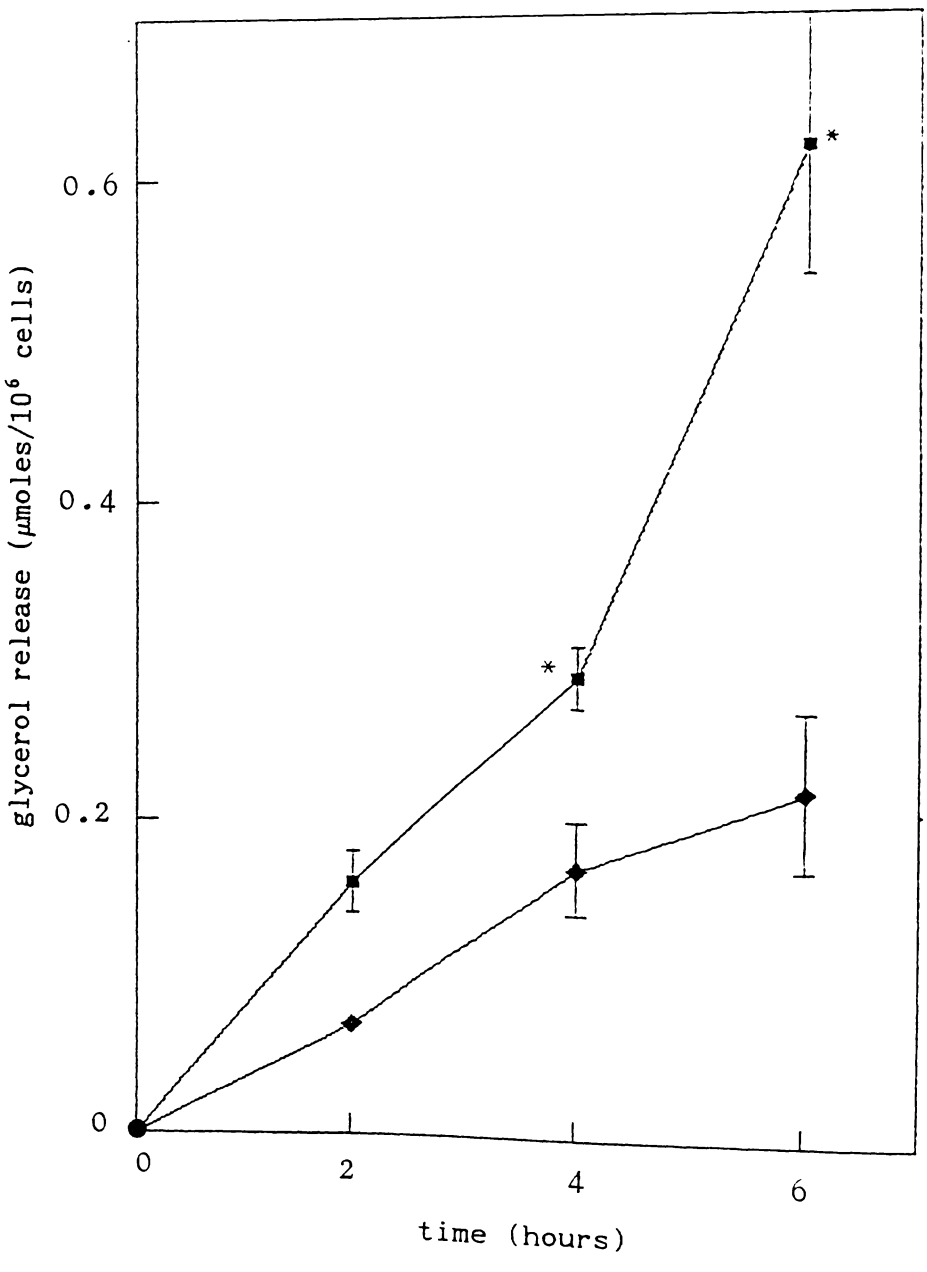
** $p < 0.01$

Figure 3.3 The effect of BSA on adrenalin stimulation of glycerol release from ovine adipose tissue *in vitro*.

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 without BSA ■ and with 1% w/v BSA present ◆ . The BSA used was Sigma Fraction V, A6003, batch 104. Adrenalin was present in all incubations at a concentration of 50 ng/ml. Glycerol release into the incubation medium (μmoles/10^6cells) is plotted against time (hours).

The presence of BSA has significantly increased ($p < 0.01$) the rate of adrenalin stimulated glycerol release.

Figure 3.4



* $p < 0.05$

Figure 3.4 A comparison of the effects of BSA and Polypep^R on adrenalin-stimulated glycerol release from ovine adipose tissue *in vitro*.

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices incubated in Medium 199 with 1% w/v BSA ■ or 1% w/v Polypep^R ◆ present. Polypep^R is a protein digest from Sigma and the BSA used was Sigma Fraction V, A6003, batch 104. Adrenalin, at a concentration of 50 ng/ml, was present in all incubations. Glycerol release into the incubation medium (μmoles/10^6cells) is plotted against time (hours).

The presence of 1% BSA has significantly increased the rate of glycerol release from the cells ($p < 0.05$) when compared with that in the presence of 1% Polypep^R.

3.13 Cellularity and the measurement of cell dimensions

In the preliminary experiments carried out for this study, the rates of lipid metabolism were expressed in terms of wet weight, as described by Vernon (1979b). At the end of the incubation, the tissue was immersed in ice-cold normal saline for ten minutes. It was then removed, blotted dry with filter paper and the wet weight determined.

The ratio of lipid weight (determined by hexane extraction of tissue lipids, see Section 3.14) to wet weight was determined for all cultures in an experiment. The results are given in Table 3.2. In the presence of insulin, the proportion of lipid to wet weight has reduced to 75% of that in the cells incubated without hormone, insulin having a highly significant effect ($p < 0.001$).

If the non-lipid weight is equal to the wet weight minus the lipid weight, then non-lipid weight/wet weight can be expressed as 1 - lipid weight/wet weight. Thus, the non-lipid portion of the basal cells expressed as a fraction of the wet weight is 1 - 0.5175 = 0.4825 and in the presence of insulin changes to 1 - 0.387 = 0.613. This means that insulin increased the cell-associated non-lipid portion by 0.613 - 0.4825 = 0.1305 g non-lipid per g wet weight. Expressed relative to the initial non-lipid portion, this represents a 27% change. The wet weight in this case includes the interstitial fluid and it is possible that at least part of the change is in this component of the tissue.

The second method for expressing metabolic rates is per number of cells. This method involves using an estimation of the number of cells as described by Sjostrom *et al.* (1971). The lipid is extracted from the cell samples, its mass determined and volume calculated by multiplying by the density of lipid. Goldrick (1967) has shown that adipocytes consist almost entirely of lipid, with the cytoplasm in small cells occupying about 8% of cell volume and about 0.8% in large cells. This is also illustrated in Plate 1.1 where the nucleus has been forced into the periphery of the cell, probably by the volume of lipid. Thus a measurement of the volume of lipid provides an estimate of the total volume of each sample of tissue. The total volume can then be divided by the volume of one cell to give the number of cells present in each incubation vessel.

Table 3.2 The ratio of lipid weight to wet weight

The ratio of lipid weight to wet weight for adipose tissue slices incubated in Medium 199 without added insulin (basal) and with 2.6 ng/ml insulin.

	mean ratio $\frac{\text{lipid weight}}{\text{wet weight}}$	S.E.M.	n	variation
Basal	0.5175	0.013	24	2.5%
Insulin	0.387***	0.015	24	4%

*** *The significance of the difference from basal: $p<0.001$.*

The tissue lipid was separated by saponification, acidification and extraction into n-hexane (see Section 3.14). The lipid extract was transferred to a pre-weighed scintillation vial and the hexane evaporated to dryness. The vials containing the lipid were then placed in an oven at 50°C to drive off any moisture and the weight of lipid in each sample was determined. Since the original triacylglycerol molecule contained three fatty acid molecules (with an estimated molecular weight of 285) and one glycerol molecule, the weight of fatty acid was converted to weight of triacylglycerol:

MW fatty acid $285 \times 3 = 855$
MW glycerol 41
MW triacylglycerol 896

Thus weight of TG = (41/855 x weight of FA) + weight of FA
= 4.8% weight of FA + weight of FA

Since density of TG = 0.915 g/ml.

$$\text{Volume of TG} = \frac{\text{weight of TG}}{0.915} \quad \text{ml.}$$

The next part of the estimation of cell numbers involved the assessment of the average volume of the cells in the tissue slice. It is apparent that all adipose tissue has a wide variety of cell sizes, the distribution of which can vary between animals, depots and even within the depot at varying depths (Hood and Thornton, 1979; Thornton *et al.*, 1983). Therefore, for the estimate of the average cell diameter to be meaningful, a large number of cells must be measured.

When Sjostrom *et al.* (1971) prepared sections of adipose tissue for measurement of cell sizes, the tissue was fixed in 35% formaldehyde, frozen in dry ice and sliced with a microtome to a thickness of 200 μm. However, in the present study, it was found that freshly prepared tissue slices, cut thinly for incubation, could be mounted on a microscope slide, bathed in normal saline and viewed directly using an Olympus overhead microscope. A photomicrograph of cells prepared by this method is shown in Plate 3.1. By focussing down through the layers of the section, it was determined that each slice was approximately 2 - 3 cells thick. Since the average diameter of the cells was found to be about 70 μm, this represented an average slice thickness of 210 μm which compares favourably with that obtained with the microtome.

Immediately after all the incubations had begun, several tissue slices were prepared and the mean cell diameter estimated within half an hour. The tissue was held at 39°C until required.

A graticule, mounted in the eye-piece of the microscope, was standardized at the magnification used to view the cells by matching it against a standard 1 mm grid. At the magnification used, one division on the graticule was 5 μm wide. Thus when the cell diameter was estimated against the graticule, the number of divisions were multiplied by 5 to give the diameter of the cell in μm.

It was possible to see when the cells had begun to take in water and increase in size because they developed a honeycomb-like appearance (as illustrated in Plate 3.2). Cells were measured before this occurred. It was found that the cells were not always spherical and so the mean of two measurements of each cell, one at right angles to the other, was used. The two diameters often differed by 5 μm but never more that 15 μm. The average COV (sd/mean) for one hundred measurements (50 pairs) was 21%. The cell diameters varied from 46.2±1.6 to 84.5±1.8 in the animals

studied. The estimations of cell volume using this technique were shown to be proportional ($r = 0.7443$) to fatness of the animal, as measured by GR (Figure 3.5). It is therefore most likely that the variation in cell volumes obtained was due to the variation in fatness of the animal. This may also indicate that it is the cell volume and not the cell numbers in adipose tissue that increase as the lambs fatten (Kirtland and Gurr, 1979; Hood and Thornton, 1979).

Only cell membranes which were sharply in focus were used to determine the diameters, to ensure that the measurements were made at the maximum diameter. Care was taken that the cells were not individually selected for counting because of any given characteristic. To assist this, a group of cells was identified and the diameters of all the cells in the group were measured. The groups were taken randomly from different tissue slices so that no one part of the depot was being used exclusively.

A computer program was used to calculate the mean cell diameter (d), its standard deviation (s), mean cell volume (V) and the number of cells in each tissue sample (c). Cell diameters are normally distributed but the cell volumes, being a function of the cube of the diameter, belong to a skewed distribution. The following equation has been derived by Sjostrom *et al.* (1971) to correct for the skew in calculating the mean cell volume, V. Since the equation includes the standard deviation as one of the parameters, all measurements of cell diameters have been expressed using standard deviation instead of standard error.

$$V = \frac{\pi(3s^2d + d^3)}{6} \qquad \mu m^3$$

(with d measured in μm)

$$\text{volume of 1 million cells} = V \times 10^6 \qquad \mu m^3.$$

$$= V \times 10^{-6} \qquad cm^3$$

$$\text{Thus no. of millions of cells/cc} = \frac{1}{V \times 10^6}$$

To obtain the number of millions of cells per g of lipid (knowing that the density of triacylglycerol is 0.915 g/cc):

$$\frac{\text{No. of millions of cells}}{\text{g lipid}} = \frac{1}{V \times 10^6} \times \frac{1}{0.915}$$

Thus the number of cells in the sample, in millions is

$$c = \frac{\text{g of TG in the tissue slice}}{V \times 10^6 \times 0.915}$$

The number of cells in the sample is referred to in later calculations as "c".

To compare the two methods for expressing the rates of lipogenesis, both wet weight and cell numbers were measured in the same tissue sample after incubation with ^3H_2O. There were six measurements, all of which were replicate samples of tissue from one animal incubated under identical conditions. The results are shown in Table 3.3 where it can be seen that the variation (S.E.M. / mean) in the data expressed as per 10^6 cells is half that of data expressed per wet weight.

Table 3.3 A comparison of methods used to express metabolic data

The two methods used to express the data were either as per wet weight of tissue or per 10^6 cells. There were six replicate 24 hour incubations with identical medium conditions with no hormone present. The tissue was all from the same animal.

wet weight g	lipid weight g	CPM*	DPM* / wet weight	DPM* / 10^6 cells
0.3721	0.2158	1531	9099	2827
0.2882	0.0856	814	6156	2202
0.2494	0.0727	702	6206	2262
0.2207	0.1044	1578	16420	3602
0.3498	0.0858	1303	8396	3619
0.2981	0.1436	1920	14930	3217
		mean ± S.E.M.	10200±1806	2955±258
		variation	18%	9%

*CPM = *counts per minute:*
counts of ^3H from ^3H_2O incorporated into the long-chain fatty acids by the process of lipogenesis.

*DPM = *disintegrations per minute:*
calculated from CPM using efficiencies derived using the channel ratio (see Section 3.14)

Plate 3.1

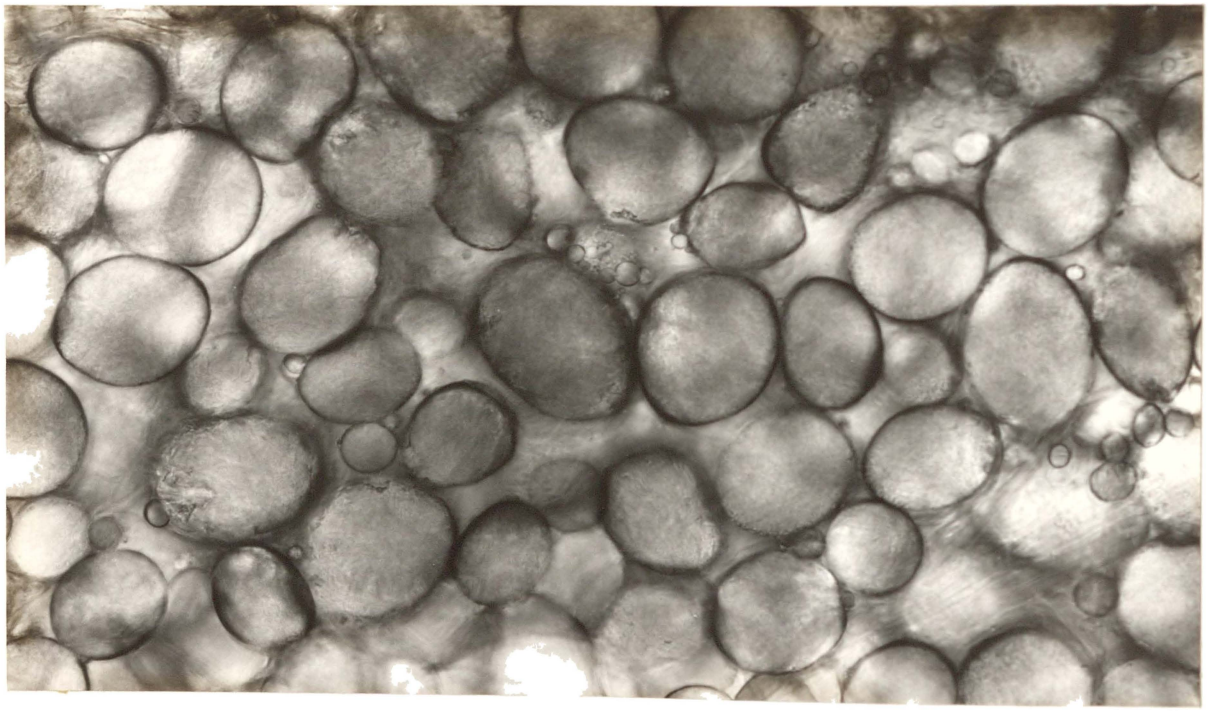
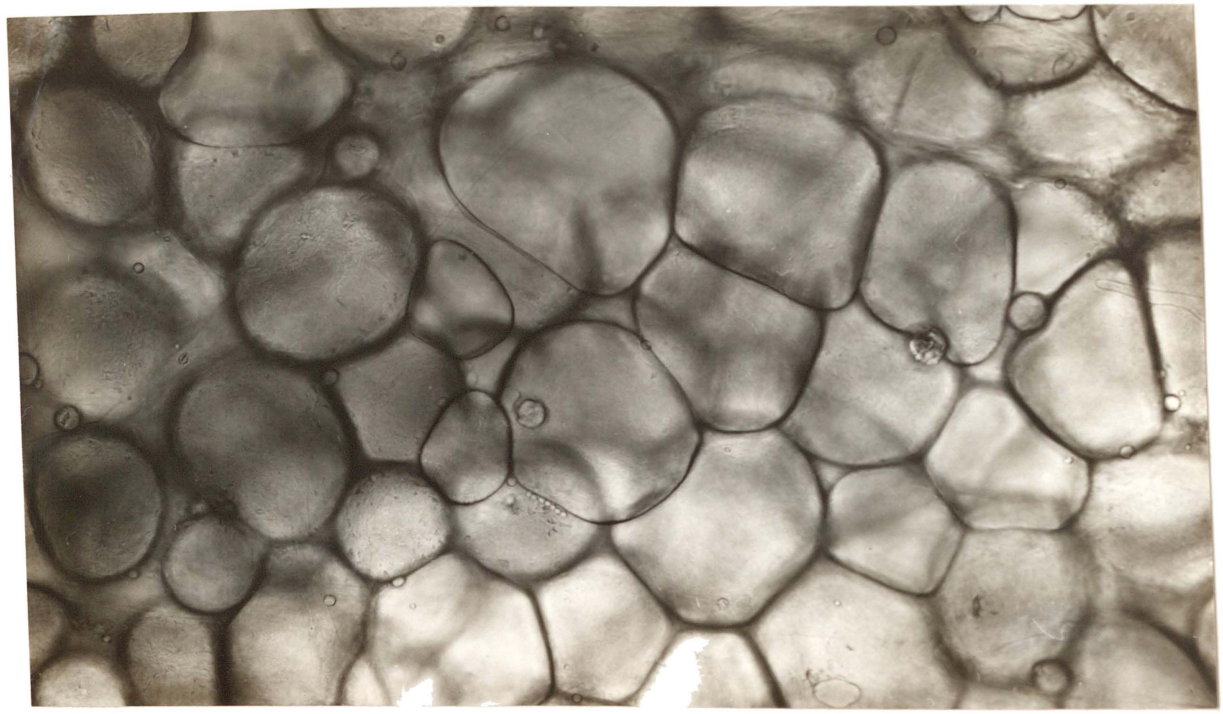


Plate 3.1 A photomicrograph of a normal adipose tissue slice prepared for measurement of cell diameter.

A section of adipose tissue prepared for measurement of mean cell diameter by matching against a standard 1 mm grid. The cells vary in size within each tissue but the mean cell diameter was shown to be proportional to fatness as measured by GR (Figure 3.5).

Plate 3.2



0 50 100 μm

Plate 3.2 A photomicrograph of an adipose tissue slice showing swollen cells.

A section of adipose tissue prepared for measurement of mean cell diameter by matching against a standard 1 mm grid. The cells appear to have taken in water and increased in size shown by the typical honeycomb-like pattern. Cells in this condition were not used for estimation of mean cell diameter.

Figure 3.5

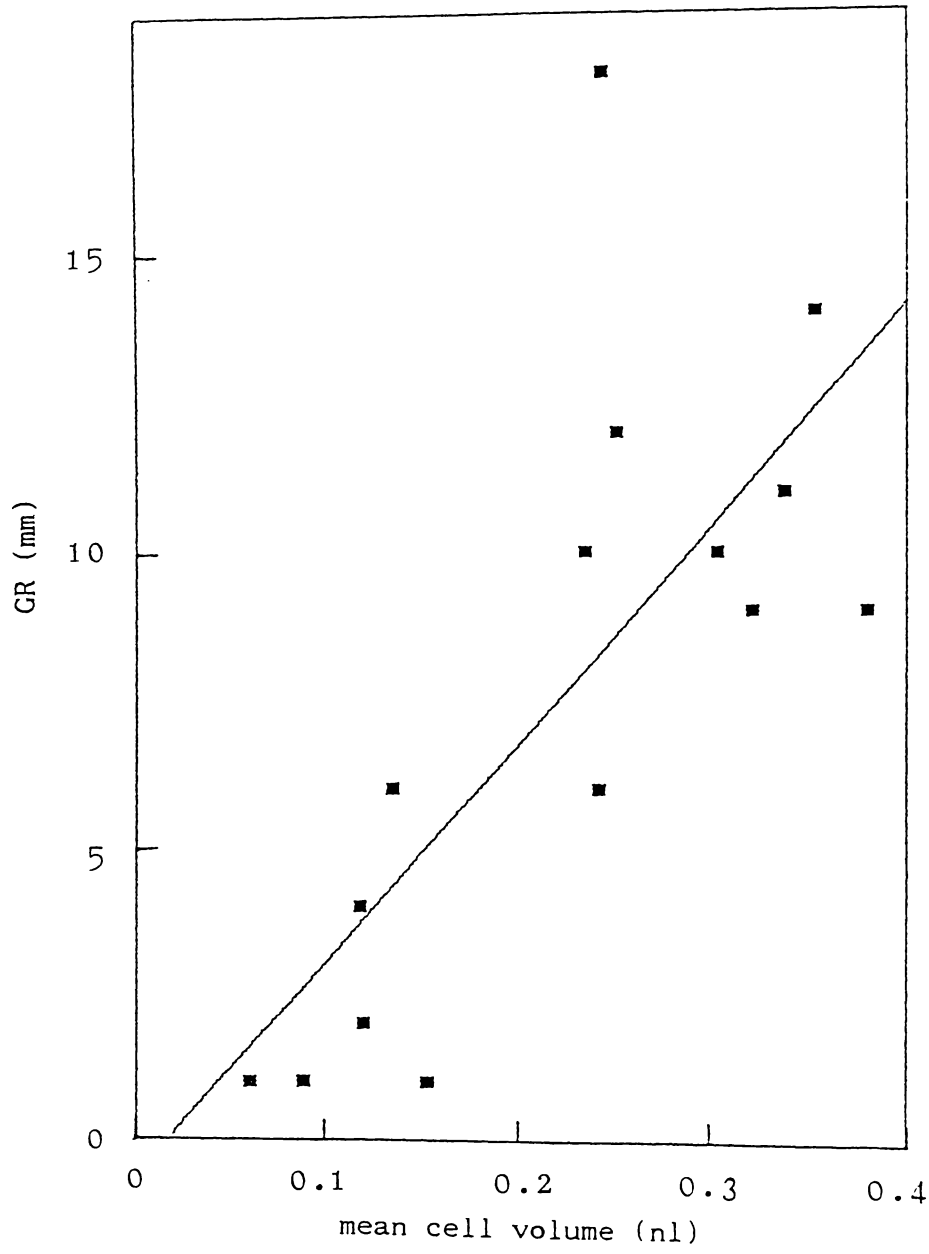


Figure 3.5 The relationship between fatness of the animal, as measured by GR and the mean cell volume.

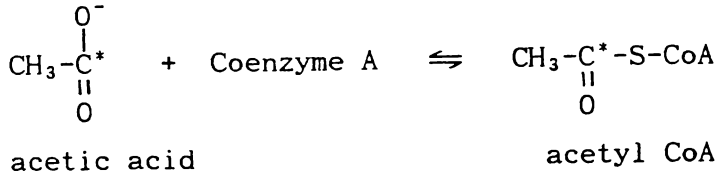
Each point represents the measurement of GR and mean cell volume for a different lamb. The GR (mm) for each animal is plotted against the mean cell volume in the subcutaneous adipose tissue (nl).

The GR has varied from 1 - 18 mm and the mean cell volume from 0.06 - 4.0 nl. There is some evidence for a direct relationship between GR and mean cell volume in the animals used in this study. Using linear regression analysis, the equation for the line is $y = -0.69 + 37.17x$, where y = GR and x = mean cell volume. The error in the estimate of the gradient of the line is 37.17 ± 2.5. The linear regression coefficient (r) is 0.7443. The line could be used to predict mean cell volume from GR (in lambs aged 3 - 11 months) with a confidence level $p < 0.01$.

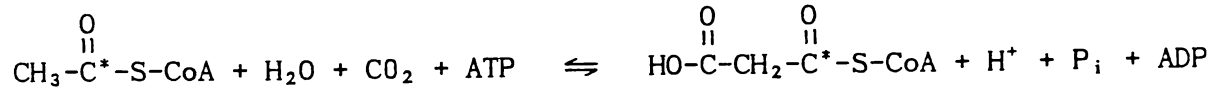
3.14 Measurement of lipogenesis

Using ^{14}C-acetate

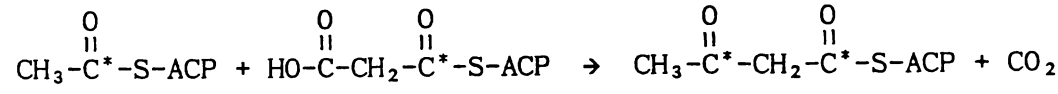
When the adipose tissue slices had been incubated with ^{14}C-acetate present in the medium, the process of lipogenesis resulted in the incorporation of ^{14}C-acetate (denoted below as C*) into the long-chain fatty acids:



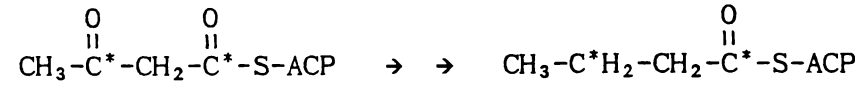
acetic acid — acetyl CoA



acetyl CoA — malonyl CoA

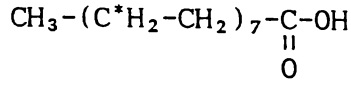


acetyl ACP — malonyl ACP — acetoacetyl ACP



acetoacetyl ACP — fatty acyl ACP

Two carbon atoms, only one of which is labelled, is added during each chain-lengthening cycle and the resulting fatty acid chain has every second carbon atom radioactively labelled. Seven cycles of chain lengthening normally occur, resulting in the 16-carbon chain of palmitic acid with 7 potential labelling sites.



Palmitic acid

Three palmitic acid molecules will combine with a glycerol molecule to form triacylglycerol in which there are potentially 3 x 7 = 21 labelling sites. The actual number of labelled carbon atoms will depend on the specific activity of the ^{14}C-acetate in the medium. The specific activity of ^{14}C-acetate used in these studies was 90 mCi/mol. Frequently, when using this specific activity, the counts in the lipid were higher than was necessary. However, very low rates of lipogenesis were encountered in tissue from some animals. Overall, the range of rates measured varied from a minimum of 0.003 ± 0.0006 to a maximum of 3.0 ± 0.04 μmoles of acetate incorporated per hour per 10^6 cells. Thus it was considered beneficial to continue using this specific activity to ensure that, if the rate of lipogenesis was low, counts in the lipid were not too low to measure.

Purification and extraction of fatty acid

Since ^{14}C-acetate could have been incorporated into either fatty acids or triacylglycerol, saponification was carried out as a first step to release the fatty acids from the triacylglycerol molecules. Saponification was also a convenient method to digest the tissue and obtain a homogeneous solution.

The tissue samples were thawed and 5 ml of 10% potassium hydroxide in ethanol was added. Care was taken to ensure that all the tissue was fully immersed in the ethanolic KOH solution before saponification began. The tissue samples were saponified at 60°C for one hour. It was important that the ethanolic KOH was made up freshly just before the beginning of saponification and that the tubes were not at 60°C for longer than one hour. Both of these were factors which could lead to the development of a yellow colour which subsequently caused colour quenching during scintillation counting. At the end of one hour, the tubes were cooled and 5 ml of 25% concentrated HCl saturated with NaCl was added to each tube. Ten ml of n-hexane was then added to each tube which was shaken thoroughly to ensure mixing of the layers and total extraction of lipid into the hexane layer. As suggested by Folch *et al.* (1957), the presence of mineral salts in the aqueous phase improved the efficiency of subsequent washing procedures by practically eliminating lipids from the aqueous phase. It was also found that, in the absence of NaCl, the interface between the aqueous and hexane layers was not clearly defined.

The pH of the bottom aqueous layer was then checked using universal indicator strips. If the pH was greater than 1, more HCl was added until it was 1. This ensured that ionisation of the fatty acid was completely suppressed since ionised fatty acid is water-soluble and would be lost during the washing procedure.

When the layers had settled, preferably by leaving them to stand for 24 hours, the hexane layer was transferred to another tube using a pasteur pipette. During this process, it was important that none of the aqueous layer was included since it contained the residual ^{14}C-acetate. The original tube was rinsed twice with one ml portions of n-hexane, vortexed and the upper layer pipetted into the second tube. The remaining aqueous layer was discarded.

Five ml of a mixed solution of acetic and sulphuric acids (both at 0.1 mol/l) was added to each of the second tubes containing the hexane-lipid layer. The tubes were vortexed thoroughly and left to settle. The pH of the aqueous layer was checked and, if it was greater than pH 1, more sulphuric acid was added to the washing solution. By washing the hexane layer with acetic acid, any residual ^{14}C-acetic acid molecules still remaining were exchanged with the excess of unlabelled acetic acid.

Once the layers had settled again, the lower aqueous layer was removed and discarded. Washing with the acetic-sulphuric acid solution was repeated twice more. The washed hexane layer was then transferred to a third tube. The last of the hexane was washed in using 2 x 1 ml portions of hexane. Approximately 0.5 g of anhydrous sodium sulphate was added to the hexane to remove any excess water which could contain residual ^{14}C-acetate. The tubes were inverted gently (the bonds between sodium sulphate and water are weak) and left to settle.

Experiments to monitor the efficiency of fatty acid extraction

The accuracy of the fatty acid purification process was investigated by the addition of a quantity of glycerol tri-[9,10-^{3}H]oleate to adipose tissue extracts. The aim of the experiment was to validate the saponification, purification and extraction processes by monitoring any losses of ^{3}H-tri-oleate that occurred as the process was carried out.

Method

Twenty μl quantities of ^{3}H-tri-oleate in n-hexane were added directly to three scintillation vials, evaporated to dryness and the total radioactivity determined by scintillation counting. Further quantities of 20 μl of ^{3}H-tri-oleate were added to each of 36 glass test-tubes, together with approximately 0.1 g of non-radioactive fresh adipose tissue. There were three additional tubes which contained adipose tissue but no isotope to test for background radiation. All tubes were saponified, acidified, washed and extracted as described previously. During each of the processes being monitored, samples of the aqueous and the hexane layers, together with the hexane washings being used to transfer the hexane layer to another tube, were placed in scintillation vials to measure the ^{3}H-tri-oleate content according to the following protocol:

a. *To monitor loss of fatty acid during the removal of the hexane layer immediately after saponification*

(1) all the initial hexane layer
(2) 1st washing with 1 ml hexane
(3) 2nd washing
(4) 3rd washing
(5) 4th washing
(6) aqueous layer

b. *To monitor loss of fatty acid in the acidic washing solutions and test that the anhydrous sodium sulphate is not removing fatty acid from the hexane.*

(7) all the hexane in the tube with the anhydrous Na_2SO_4
(8) 1st washing of anhydrous Na_2SO_4 with 1 ml n-hexane
(9) 2nd washing
(10) 3rd washing
(11) 4th washing
(12) aqueous layer from acetic-sulphuric acid wash.

For the samples of hexane, the total amount in the layers was counted after the hexane was evaporated to dryness and toluol-PPO scintillation fluid added. However, with the aqueous layers, only 1 ml of the solution was counted with toluol-triton-PPO used for scintillation. The CPM was then corrected for quench and multiplied by the total number of milli-liters present in the original layer. Three samples were tested for each procedure and the mean DPM determined.

Results

The mean DPM for the total amount of isotope used in each assay was 1685611. The results of the experiment are given in Tables 3.4a and 3.4b, where corrections for quench and the total volumes present in the aqueous layers have been calculated.

a. total recovered with 4 hexane washes: 92.8%
   total recovered with 2 hexane washes: 92.4%
b. total recovered with 4 washes: 88.9%
   total recovered with 2 washes: 88.7%
   total loss: 11%

Since there were three acetic-sulphuric acid aqueous layers (sample 12 in Table 3.4b) discarded, the total loss by this means would be 1.5%. Together with the loss in the first aqueous layer (sample 6 in Table 3.4b), there was a total loss in the aqueous layers of 2.4% of the fatty acid originally present. Since 3.9% (a - b) was lost during the acid wash and drying with anhydrous sodium sulphate, then 3.9% - 1.5% = 2.4% was lost during exposure to the anhydrous sodium sulphate. Thus approximately 5% was lost by other means, such as adhering to the pipette and test-tube walls.

Conclusions

With the total fatty acid recovered for the entire process being 88.9%, there was an added 0.2% loss by limiting the 1 ml hexane washes to two instead of four. The loss during exposure to anhydrous sodium sulphate was 2.4%.

All of these losses were considered acceptable and it was decided that two 1 ml hexane washes of the layers would suffice.

Table 3.4a Removing the hexane layer immediately after saponification

The transfer of fatty acid molecules between the aqueous and hexane layers and any losses of fatty acid as a consequence of the process of removal of the hexane layer immediately after saponification is monitored using radio-labelled tri-oleate.

		mean DPM*	% ^{3}H-tri-oleate recovered
(1)	hexane layer	1493584	88.6
(2)	1st wash	50827	3.0
(3)	2nd wash	13885	0.8
(4)	3rd wash	5839	0.3
(5)	4th wash	2422	0.1
(6)	1st aqueous layer	15330	0.9

**Sample blanks have been subtracted to account for background radiation.*

Table 3.4b The acetic/sulphuric wash and the anhydrous Na_2SO_4 drying process

The loss of fatty acid molecules by transference into the acidic washing solutions as well as its removal as a consequence of binding onto the anhydrous sodium sulphate molecule is monitored using radio-labelled tri-oleate.

		mean DPM*	% ^{3}H-tri-oleate recovered
(7)	hexane layer	1438737	85.3
(8)	1st wash	47930	2.8
(9)	2nd wash	10887	0.6
(10)	3rd wash	3007	0.2
(11)	4th wash	792	0
(12)	aqueous layer	9030	0.5

*Sample blanks have been subtracted to account for background radiation.

Scintillation counting

The hexane layer was transferred into pre-weighed scintillation vials and the hexane evaporate to dryness using a stream of air. The counting vial containing the purified fatty acid was then placed in an oven at 60°C for at least three hours. The tiny droplets of water were visible in the liquified fatty acid and drying was continued until they were driven off. The water had condensed on the sides of the vial during evaporation of the hexane and the problem was usually only encountered in the humid summer months.

Once the water droplets had been removed, the vials were re-weighed immediately to determine the weight of lipid. The fatty acid was stored in the scintillation vials at 4°C until all the samples from an experiment had been processed. At this stage, 8 ml of toluol-PPO scintillation fluid was added to each vial which was capped and shaken well to ensure that all the fatty acid dissolved. The presence of ^{14}C-fatty acid in each sample was estimated by scintillation counting on two channels for ten minutes using a Tracor Liquid Scintillation counter (Model 6892).

Estimation of quench

The channel ratio (counts in channel A / counts in channel B) was used to estimate the quench in the samples. Quenching is caused by the sample itself absorbing some of the scintillated energy, thus lowering the energy of light picked up by the photomultiplier tube. This shift in energy alters the channel ratio in a manner which is proportional to the amount of quenching. Standard quenched samples of ^{14}-carbon, prepared and sealed by the manufacturer (Amersham International, Amersham, Bucks, UK), were used to establish a quench curve.

The set of 3 quenched standards were counted and the CPM determined. The efficiency for each of the standards, which had increasing amounts of quench, could be calculated by the formula:

$$\%\ \text{efficiency of the count} = \frac{\text{CPM(quenched)}}{\text{DPM(unquenched)}}$$

where DPM (unquenched) was calculated for the appropriate date using an exponential decay equation and the reference DPM supplied by the manufacturer of the quenched standards.

The results are graphed in Figure 3.6. A computer program was used to determine the equation for the quench curve so that channel ratios could be converted to % efficiency of the counting. Once the equation for this quench curve had been entered into the computer, the program then calculated the DPM for every given CPM and channel ratio.

Use of the blank incubation.

Acid blank estimations were obtained by adding sulphuric acid to three incubations which also had normal quantities of tissue, medium and isotope but the presence of the acid ensured that no ^{14}C-acetate was incorporated. The average CPM for the three acid blanks was subtracted for each sample to correct for background radiation. The blank CPM was also used as a quality control check on the efficiency of the fatty acid purification process. Typically, the blank CPM was 50 - 200. If it was any higher, then there was almost certainly contamination of the ^{14}C-fatty acid with residual ^{14}C-acetate and more rigorous washing procedures would have to be adopted.

Thus:

$$\text{DPM} = \frac{\text{CPM} \times 100}{\text{sample efficiency(\%)}} - \frac{\text{Blank CPM} \times 100}{\text{blank efficiency(\%)}}$$

Figure 3.6

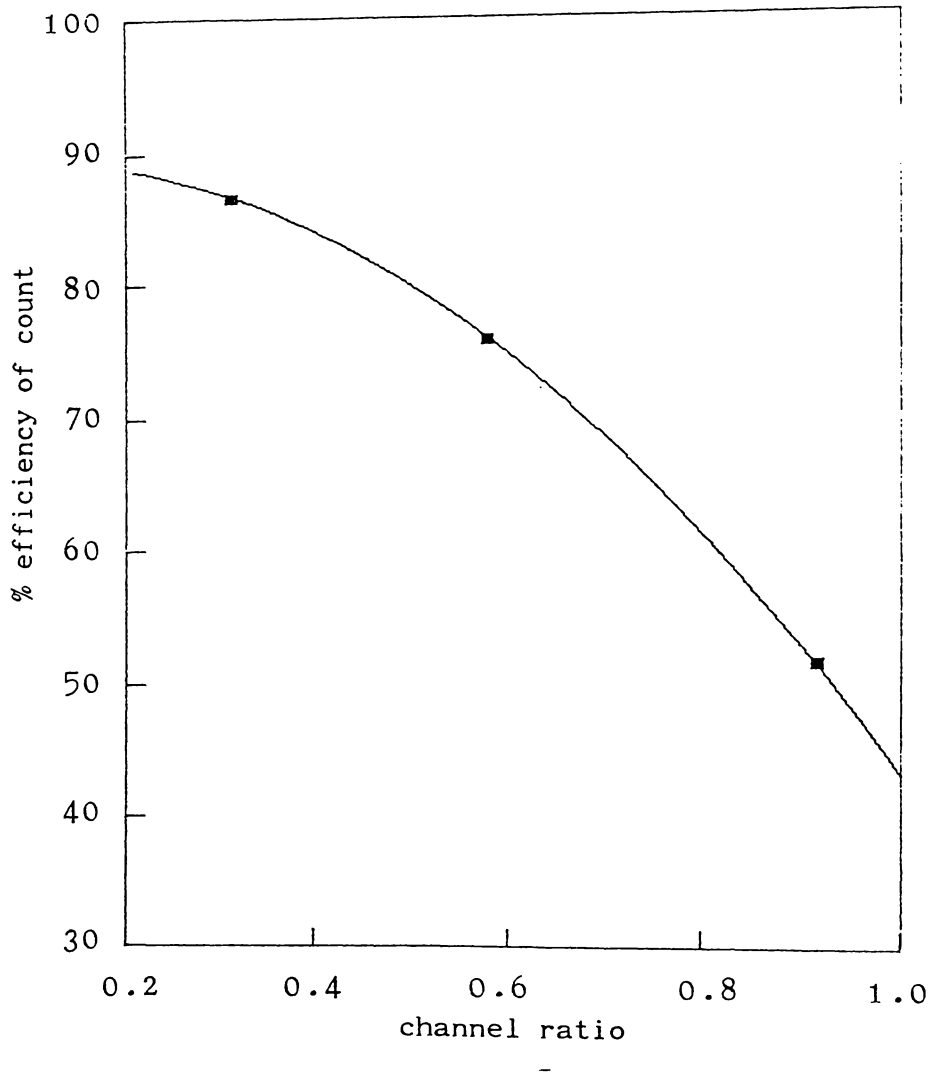

100
90
80
70
60
50
40
30
% efficiency of count
0.2
0.4
0.6
0.8
1.0
channel ratio

Figure 3.6 Quench curve for ^{14}C scintillation counting

The percentage efficiency of scintillation counting is plotted against the channel ratio for 3 prepared quenched standards from Amersham, UK. The method of calculation of percentage efficiency is given in Section 3.14.

The equation for the curve is:

$Y = -54.04X^2 + 7.92X + 89.1$

where Y is percent efficiency of the count

and X is channel ratio

This equation can be used to calculate the percentage efficiency of any count, given the channel ratio.

Calculation of acetate incorporation into lipid

It was assumed that the specific activity of the ^{14}C-acetate in the medium was the same as the specific activity of C_2 units incorporated into the cellular lipids. (See Section 10.1 for a discussion of this assumption.)

Therefore:

specific activity = total counts in medium / moles of acetate in medium.

= total counts in cell lipid/moles acetate incorporated

Thus:

$$\frac{\text{T.C.}}{[\text{acetate}] \times \text{Vol}} = \frac{\text{DPM in lipid}}{\text{moles of acetate incorporated}}$$

Rearranging this equation and dividing by the number of cells in millions gives incorporation per 10^6 cells:

$$\text{moles of acetate incorporated} = \frac{(\text{DPM in lipid}) \times [\text{acetate}] \times \text{Vol}}{\text{T.C.} \times c}$$

units: μmoles acetate incorporated/t hours/10^6 cells.

where: TC = total counts in the medium (DPM)
[acetate] = concentration of acetate in the medium
Vol= total volume of medium (5 ml)
t = time of incubation in hours
c = the number of cells in millions present in the incubation medium.

In summary, the computer program calculated the number of moles of acetate incorporated into lipid using the following equations and input data:

Input: for Quench standards:

original DPM? DPM_o

original date?

date samples counted?

half-life of isotope (^{14}C=5568 years)? hl

Calculate: half-life of isotope in days = h

days since original count of standard = t

use decay curve and half-life to calculate DPM:

DPM (unquenched) = $DPM_o \times 2^{-t/h}$

Input: for each quenched standard:

CPM?

channel ratio?

Calculate: CPM quenched/DPM unquenched = % efficiency of count

Using quadratic regression analysis, fit a curve

efficiency data plotted against channel ratio

and find its equation.

Input: for each blank:

blank CPM?

blank channel ratio?

Calculate: efficiency using formula for quench curve.

$$\text{blank DPM} = \frac{\text{Blank CPM} \times 100}{\%\ \text{efficiency}}$$

Bl = average of the three blank DPMs

Input: for each sample:

sample CPM?

sample channel ratio?

Calculate: efficiency using formula for quench curve.

$$\text{sample DPM} = \frac{\text{sample CPM} \times 100}{\%\ \text{efficiency}} - \text{Bl}$$

= DPM in lipid

Input: for average total counts (TC) in the medium:

TC CPM?

TC channel ratio?

Calculate: efficiency using formula for quench curve.

$$\text{TC DPM} = \frac{\text{TC CPM} \times 100}{\%\ \text{efficiency}}$$

TC = average of the three TC DPMs

Input: for calculation of cell numbers:

(i) to calculate volume of an individual cell:

horizontal diameter?

vertical diameter?

scaling factor? (usually 5 μm)

(ii) to calculate the volume of tissue lipid:

weight of vial?

weight of vial + lipid?

density of TG? 0.915 g/ml

Calculate: mean cell diameter = d

standard deviation = s

volume of one cell (V) = $\frac{\pi(3s^2d + d^3)}{6}$ μm^3/cell

g of FA in tissue=(weight vial + FA) - (weight vial)

g of TG in tissue=(g of FA) x 4.8/100) + (g of FA)

c = number of cells in sample (in millions)

c = (g of TG) / (V x 10^6 x 0.915)

Input: acetate concentration ? [A]

incubation time in hours? t

incubation volume in ml ? Vol

Calculate: μmoles of acetate incorporated/t hours/10^6 cells:

$$= \frac{(\text{DPM in lipid}) \times [A] \times \text{Vol}}{TC \times c}$$

calculate mean and S.E.M. for replicate incubations

The program to make these calculations was written for a Commodore 64 microcomputer by Dr E. Lewis.

A typical calculation:

where: sample CPM = 434436.1

% efficiency = 77.1%

blank DPM (Bl) = 96.9 ± 10.4 (3)

Total Count DPM (TC) = 983207 ± 8652 (3)

weight of vial = 3.6728 g

weight of vial + FA = 3.7809 g

acetate concentration [A] = 1.103 mmol/l

incubation volume (Vol) = 5 ml

incubation time (t) = 6 hours

mean cell diameter (d) = 62.35 μm

standard deviation (s) = 16.69

mean cell volume (V) = 0.1541 nl

millions of cells / cc FA = 6.488

millions of cells/g TG = 7.091

So,

weight of FA = 0.1081 g

weight of TG = 0.1131 g

cells in sample (c) = 0.8020 (in millions)

sample DPM = 563471

therefore:

$$\text{acetate incorporated} = \frac{(\text{sample DPM} - \text{Bl}) \times [\text{A}] \times \text{Vol}}{\text{TC} \times \text{c}}$$

$$= \frac{(\ 563471 - 96.9\ ) \times 1.103 \times 5}{(\ 983207 \times 0.8020\ )}$$

= 3.94 μmoles acetate/6 hours/10^6 cells.

= 0.66 μmoles/hour/10^6 cells

Some basal rates of acetate incorporation into lipids in subcutaneous adipose tissue from sheep have been reported in the literature. They are presented here converted into μmoles/hour/10^6 cells for comparison. To do this it was necessary to calculate the number of cells per g wet weight of tissue, since most of the reported studies with ovine adipose tissue use wet weights. The ratio (DPM / wet weight) : (DPM / 10^6 cells) was calculated for an experiment where both values had been obtained for each incubation. The average ratio for six replicate basal incubations was 5.27.

Thornton *et al.* (1982):
reported incorporation rate: 85 μg/hour/g lipid.
since there are typically 8 x 10^6 cells per g lipid:
incorporation rate = 0.18 μmoles/hour/10^6 cells.

Vernon (1977b):
reported incorporation rate: 0.65 μmoles/hour/g wet weight
= 0.12 μmoles/hour/10^6 cells.

Vernon (1979b):
reported incorporation rate: 588 ± 170 nmole/hour/g wet weight
= 0.11 ± 0.03 μmoles/hour/10^6 cells

Ingle *et al.* (1973):
reported incorporation rate: 0.39 mmoles/3 hours/100 mg tissue
= 0.25 μmoles / hour/10^6 cells

Vernon (1980):
reported incorporation rate: 20 - 74 nmoles/minute/g of tissue
= 0.227 - 0.840 μmoles/hour/10^6 cells
reported incorporation rate: 22 - 87 nmoles/minute/g of tissue
= 0.250 - 0.990 μmoles/hour/10^6 cells

All of these reported values for the rate of lipogenesis in ovine subcutaneous adipose tissue therefore fall within the range of 0.003 - 3.0 μmoles/hour/10^6 cells obtained in the present study. The higher basal rates were all obtained in the latter part of the study. This may be due to a gradual improvement in techniques for tissue preparation and incubation.

Using ^{14}C-glucose

Vernon (1979b) reported that, in ovine adipose tissue *in vitro*, only 5% of the carbon for lipogenesis came from glucose, with the rest coming from acetate. However, glucose is required by the adipose tissue to manufacture NADPH and glycerol. Thus, when ^{14}C-glucose was used in these incubations, the label could be expected to appear principally in the triacylglycerol molecule and there would be very little present in the fatty acid molecule itself. Therefore, when ^{14}C-glucose was used, the saponification step was omitted and the total lipids purified.

Separation of total lipids

The tissue was separated into two equal parts. One half was saponified and the fatty acid molecules were extracted and purified in n-hexane as previously described, except that the hexane layer was washed three times with 1 mol/l sulphuric acid (omitting the acetic acid). The ^{14}C-fatty acid was determined by scintillation counting. The second half was used to assess the total lipid.

The method for extraction of total lipid, as first described by Dole (1956), uses Dole's extraction medium containing propan-2-ol (40 parts), heptane (10 parts) and 1 mol/l sulphuric acid (1 part). Each tissue sample was placed in a glass vial and minced thoroughly with scissors. Five ml of Dole's extraction medium was added to each vial which was left to stand for two days with frequent shaking.

Following extraction, 3 ml of heptane and 2 ml of distilled water were added to each sample. The samples were mixed thoroughly and left to separate the phases. The upper phase, which was the heptane and contained the total lipid, was then washed with three 5 ml portions of 0.01 mol/l sulphuric acid to eliminate any remaining ^{14}C-glucose. The heptane layer was then dried with anhydrous sodium sulphate and transferred to a pre-weighed scintillation vial.

If the intra-cellular free fatty acid was being measured (see Section 3.15), exactly half of the heptane layer was measured by pipetting and left in the tube. In this case, the amount of intra-cellular free fatty acid, the weight of lipid for estimation of cell numbers and the DPM in the total lipids obtained had to be multiplied by two.

The heptane was evaporated to dryness and the lipid dried in an oven at 60°C. The amount of ^{14}C-triacylglycerol present was determined by scintillation counting.

Calculation of glucose incorporation into lipid

A slightly modified computer program was used to calculate the incorporation of ^{14}C-glucose into triacylglycerol and fatty acids. The alterations were:

(i) for triacylglycerol, there was no need to add the correction for glycerol loss as in the fatty acids (*i.e.* 4.8% of the weight of fatty acid) since this time the amount of triacylglycerol present was measured directly rather than being calculated from the amount of fatty acid present.

(ii) glucose concentration (usually 5.5 mmol/l) was used to determine the specific activity of the medium.

Otherwise, the calculations were identical to those shown for the incorporation of ^{14}C-acetate.

Using $^{3}H_2O$

Although the rate of lipogenesis from a particular precursor such as ^{14}C-acetate or ^{14}C-glucose can be measured, this does not give an estimate of the total lipogenesis from all precursors. Jungas (1968) described the use of $^{3}H_2O$ to measure the total fatty acid synthesis in adipose tissue from all precursors. He showed that 14 tritium atoms were incorporated into each palmitic acid molecule, or one for every methyl group present.

After incubation with ^3H_2O, the ^3H-labelled lipid was saponified, acidified and extracted with hexane, as described for ^{14}C-acetate. However, the hexane layer was washed three times with 0.01 mol/l sulphuric acid to remove the last traces of ^3H_2O. Once the hexane layer had been washed, it was dried with anhydrous sodium sulphate and transferred to a pre-weighed scintillation vial. When the hexane had been evaporated to dryness in a fume hood, the fatty acid extracts were not placed at 60°C for further drying owing to the hazard caused by the volatility of any residual ^3H_2O but instead were left overnight under a stream of air in a fume hood. Blank incubations were used to check for residual ^3H_2O and washing procedures could be continued until they were consistently low. The samples were weighed, dissolved in toluol-PPO and the amount of ^3H-fatty acids was measured using scintillation counting. Standard quench samples of ^3H were used to estimate quench and the sample DPM was calculated.

Calculation of H atom incorporation into lipid

Incorporation of H atom into tissue lipids was calculated from the DPM of ^3H-fatty acids in the lipids and the specific activity of ^3H_2O in the incubation medium. The specific activity in the medium was assumed to be the same as the specific activity in the tissue lipid:

Therefore:

$$\text{moles H atoms incorporated}/10^6 \text{ cells} = \frac{\text{DPM}}{c} \times \frac{2 \times \text{moles } H_2O}{TC}$$

where c = no of cells in millions

TC = total count in medium

Jungas (1968) showed that, when ^3H_2O was incorporated into fatty acids during lipogenesis, the ratio of H:C atoms incorporated was 0.87.

Using this factor:

$$\text{moles C atoms incorporated} = \frac{\text{moles H atom incorporated}}{0.87}$$

3.15 Measurement of lipolysis

As a result of the process of lipolysis in adipose tissue, glycerol and fatty acids are released. The fatty acids can then either be recycled back to triacylglycerol by re-esterification, be oxidised for cell maintenance or pass out of the cell (Figure 3.8). Once outside the cell, they attach to albumin molecules for transportation in the blood. Glycerol, on the other hand, is not re-used within the cell after lipolysis has occurred because the adipose cells lack the enzyme necessary (glycerol kinase) to convert it to a molecular form suitable for re-esterification. Therefore, the measurement of glycerol released from the cells provides the best means of estimating the total lipolysis occurring within the cell.

Measurement of glycerol in the medium.

Glycerol concentrations were measured in 250 μl quantities of medium using the spectrophotometric method described by Galletti (1967). The assay was carried out in glass tubes since some plastics have been found to interfere with the reaction (Dr S. Hannon, personal communication). Standard glycerol solutions were made from a stock solution of 1 mg glycerol dissolved in 1 ml distilled water. The stock was diluted to concentrations of 3.0, 7.5, 14.5, 25.0, and 50.0 μg/ml glycerol to produce a standard curve (Figure 3.7). Medium 199 was used as the diluent when preparing the standards so that background readings caused by the medium constituents would be included. A blank sample of Medium 199 was also included in assays. The materials required and method for the assay are detailed in Appendix D. The intra-assay variation was 8% and the inter-assay variation 12%.

Interference of varying glucose concentrations with the glycerol assay

When Medium 199 is the diluent for the standards, there is a moderately high background reading caused by the medium constituents (Figure 3.7). It has been suggested (Lambert and Neish, 1950) that under some conditions, periodate oxidises glucose to formaldehyde. Since glucose was present in the incubation medium at varying concentrations due to its utilization by the cells during incubation, an experiment was

designed to determine what effect changing concentrations of glucose had on the glycerol assay.

Method

A series of samples were prepared, each containing 14.5 μg/ml glycerol and different concentrations of glucose. Glycerol assays were then performed on each sample in triplicate.

Table 3.5 Glycerol detected in medium samples containing differing concentrations of glucose.

The Galletti method was used to detect glycerol in samples of medium which contained different concentrations of glucose. Since glucose present in the Medium 199 was initially at 5.5 mmol/l, a series of concentrations less than this were chosen to represent the decreasing glucose concentration as it was utilized by the cells. All samples contained 14.5 μg/ml glycerol and were assayed at each glucose concentration in triplicate. Results are expressed as mean ± S.E.M.

Concentration of glucose mmol/l	glycerol detected μg/ml
0	13.25 ± 0.85
0.625	14.7 ± 0.31
1.25	14.6 ± 0.03
2.50	14.8 ± 1.26
5.00	16.4 ± 0.50
overall variation -	*11.4%*

Conclusions

Since the variation for the detection of glycerol in the presence of all the concentrations of glucose tested was very similar to the normal inter-assay variation obtained, it was concluded that, if glucose was having a slight effect on the assay, it was within acceptable limits.

Figure 3.7

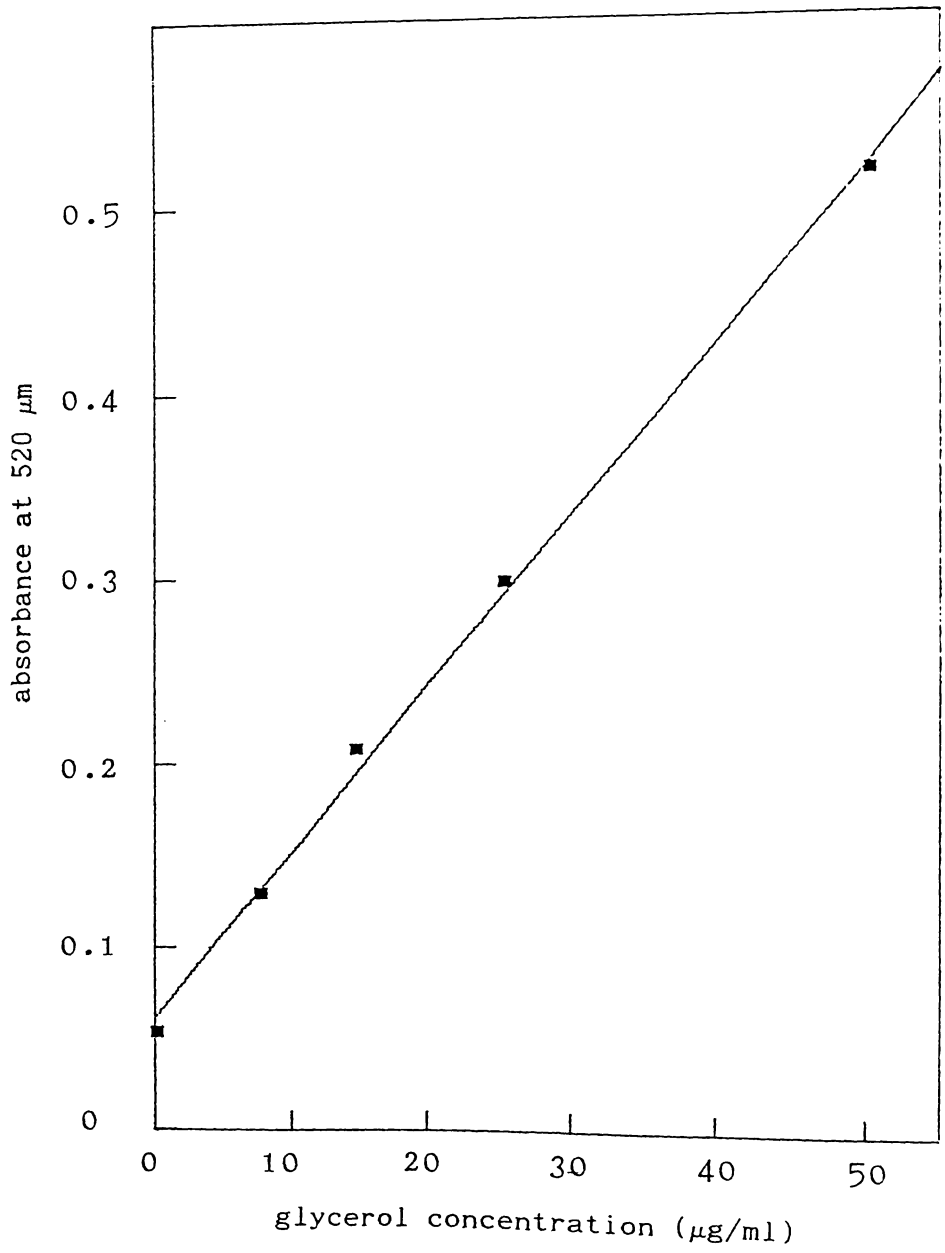

absorbance at 520 µm
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
glycerol concentration (µg/ml)

Figure 3.7 Glycerol standard curve

The standard glycerol solutions were analysed using the Galletti method of glycerol determination(See Appendix D). Absorbance at 520 μm is plotted against the original glycerol concentration in the standard solutions which were prepared using Medium 199 as the diluent.

Using linear regression analysis, the equation for the line was found to be:

$$Y = 9.256 \times 10^{-3}X + 0.061$$

where Y is absorbance at 520 μm

and X is glycerol concentration

The linear regression coefficient (r) is 0.9989

The equation can be used to predict glycerol concentration, given the absorbance reading for a sample. From the equation for the line, it can be calculated that, when Y = 0, X = 0.061. Thus 0.061 represents the absorbance of the blank reading and results from interference by constituents of the Medium 199 with the assay.

Interference of other components of used medium with the glycerol assay.

There was also the possibility that, as a result of metabolic activity in the adipose tissue, there was a component released into the medium which would interfere with the glycerol assay. An experiment was carried out to investigate this.

Method

Known quantities of glycerol were added to medium in which adipose tissue had been incubated for six hours. Thus, the glycerol present in the blank was that released from the cells. Any interference by metabolic products would mean that not all of the "spiked" glycerol would be measured in the assay. Medium from two separate incubations were used and glycerol was added to a final concentration of 7.5 and 14.5 μg/ml. The results are given in Table 3.6.

Table 3.6 **Glycerol detection in used medium**

Adipose tissue slices were incubated in two samples of Medium 199 for six hours. Known amounts of glycerol were then added to the "used" medium which was subsequently assayed for glycerol using the Galletti method. All samples were spiked in triplicate and the results are expressed as mean ± S.E.M.

Sample	Glycerol added (μg/ml)	Total Glycerol measured (μg/ml)	Total glycerol expected (μg/ml)	% recovered
1	0	4.0 ± 0.4		
	7.5	11.5 ± 0.07	11.5	100%
	14.5	17.8 ± 0.21	18.5	96%
2	0	3.5 ± 0.17		
	7.5	11.5 ± 0.10	11.0	104%
	14.5	17.2 ± 0.7	18.0	95%

Conclusions

All of the glycerol present in the medium, except at the most 5%, was detected by the Galletti method. There is therefore no evidence that the components of "used" medium are interfering with the ability of the assay to detect all the glycerol present in it.

Calculation of glycerol release from the cells

The concentration of glycerol in the medium was expressed in μg/ml. This was converted to total glycerol released/hour/10^6 cells using the formula:

$$\text{Total Glycerol released} = \frac{(\mu g/ml \text{ glycerol}) \times (\text{total incubation vol. ml})}{\text{number of cells in the sample in millions}}$$

where glycerol concentration was read off the glycerol standard curve. The number of cells in the sample was obtained from the measurements of total lipid and cell dimensions described in Section 3.13. The formula gave the result in μg glycerol/10^6 cells which was converted to μmoles/10^6 cells by dividing by 92.1

In some incubations, 250 μl of medium was removed from the incubation vessel every two hours throughout the incubation period. As a consequence, the total volume of medium present reduced by 250 μl for each sample: at 2 hours, the total volume was 5000 μl, at 4 hours was 4750 μl and at 6 hours was 4500 μl

The amount of glycerol removed with the 250 μl sample at two and four hours was added to the total glycerol using the formulae:

at 2 hours:

$$\frac{((\mu g/ml \text{ at 2 hours}) \times 5.00)}{\text{no of cells in sample in millions}}$$

at 4 hours:

$$\frac{((\mu g/ml \text{ at 4 hours}) \times 4.75) + (\mu g/ml \text{ at 2 hours}/4)}{\text{no of cells in sample in millions}}$$

at 6 hours:

$$\frac{((\mu g/ml \text{ at 6 hours}) \times 4.50 + (\mu g/ml \text{ at 2 hours}/4) + (\mu g/ml \text{ at 4 hours}/4)}{\text{no of cells in sample in millions}}$$

A computer program similar to the one described in Section 3.14 was used to calculate these results.

Measurements of free fatty acid in medium and tissue extracts.

FFA was measured in 3.0 ml aliquots of either incubation medium or the heptane layer following Dole's extraction of total lipids using the semi-automated method described by Crane and Lane (1977). The method is detailed in Appendix E.

Calculation of FFA release from cells

Since 1 ml of the standard was used and 3 ml of medium sample, the value obtained from the standard curve for each sample was divided by 3. (This step was necessary to bring the samples into the range of the auto-analyser.)

The FFA present in the original extract or incubation medium was calculated using the formula:

$$\frac{\mu\text{mole FFA}}{\text{no of cells in millions}} = \frac{((\mu\text{mol/l FFA}) \times \text{TV}/1000) - (\mu\text{moles FFA in blank})}{\text{no of cells in the sample in millions}}$$

where:

μmole/l FFA is the concentration of FFA in the original sample.

μmol/l FFA in blank is the measure of FFA in fresh medium (=0 for Dole's extraction of tissue.)

TV is the total volume of sample used (in ml)

The number of cells in the samples were calculated using the total lipid and cell dimensions of the tissue slices as described in Section 3.14.

A computer program similar to the one described in Section 3.14 was used to calculated these results.

3.16 Re-esterification and mass balance

The rate of triacylglycerol breakdown and fatty acid re-esterification can be calculated if the net changes of glycerol and FFA are known. This method makes the assumption that most of the glycerol released when lipolysis occurs is released from the cell into the medium. The FFA thus formed will not necessarily be released however. It could be either re-esterified into triacylglycerol, remain in the cell as FFA, be oxidized within the cell or, in the presence of albumin, pass out of the cell into the interstitial space. These processes are summarised in Figure 3.8. From this it can be seen that any measurement of the mass balance of fatty acid in the system would have to take into account the changing concentrations of fatty acid in all depots. The term "mass balance" is used to describe the process of balancing the number of moles of fatty acid in the system assuming an overall conservation of mass.

If all the glycerol formed during lipolysis is assumed to pass out of the cell, then the total number of moles of fatty acid released at the same time will be three times the number of moles of glycerol found in the medium. If the amount of FFA passing out of the cell is subtracted from the amount of fatty acid released by lipolysis (3 x glycerol), the remainder represents the amount of fatty acid remaining in the cell after lipolysis has occurred. For the purposes of simplification, it has been assumed that most of the fatty acid remaining is re-esterified and will be referred to as "fatty acid re-esterified". However, as already mentioned, it may also be oxidized or remain as FFA inside the cell at this stage. It is likely that the proportions of fatty acid being re-esterified, oxidized or remaining as tissue-associated FFA will vary with changing physiological conditions, both before the animal is slaughtered or later during incubation. The hormonal influences on the total fatty acid in this pool will be investigated but not the proportions going to each of the three subsequent intracellular processes.

The total amount of fatty acid accumulated in the cell during the incubation time can be calculated by subtracting the amount of fatty acid passing out into the medium from the amount of new fatty acid that was manufactured in the cell during the incubation period. The percentage of fatty acid recycling which occurs in the cell is defined as the percentage of the fatty acid released within the cell by lipolysis which was subsequently re-esterified. It is calculated by dividing the amount of fatty acid re-esterified by the amount fatty acid released during the process of lipolysis (3 x glycerol).

The following is a summary of the calculations:

FA released = 3 x glycerol release

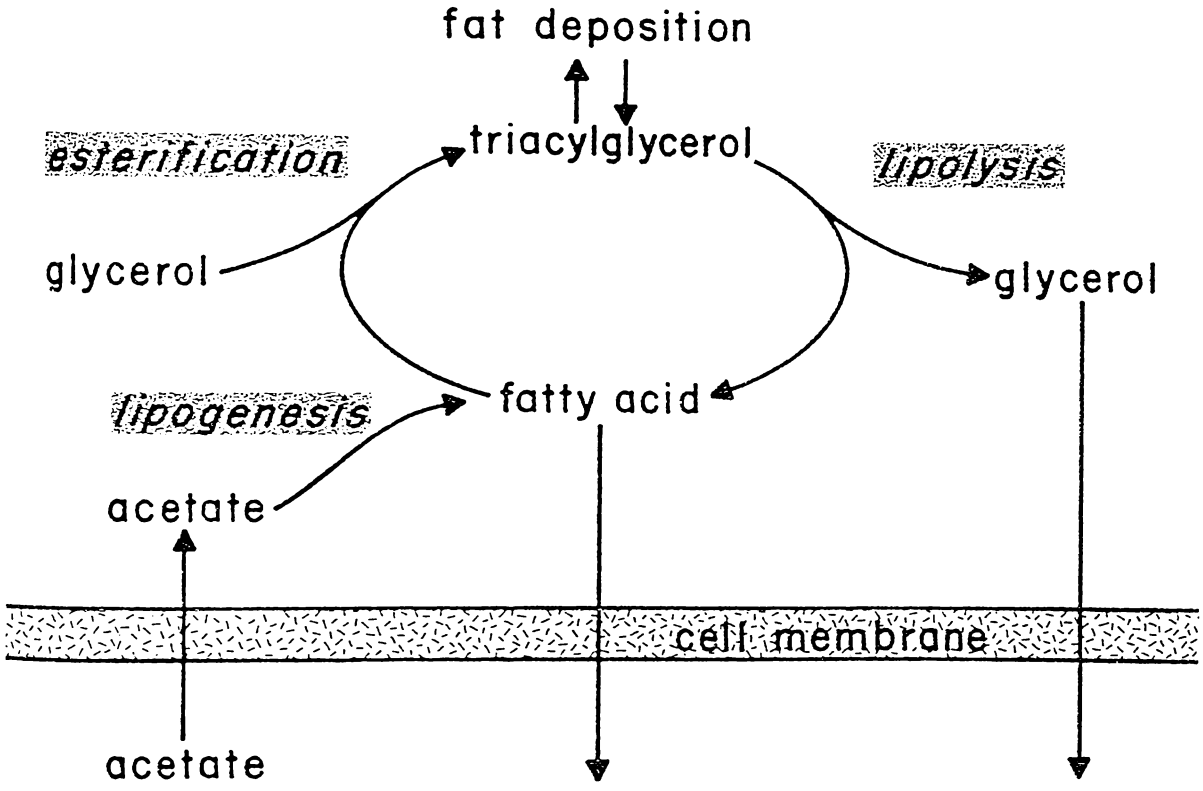
FA re-esterified = FA released - FFA leaving the cell

FA accumulation = FA manufactured - FFA leaving the cell

% recycling of FA = (FA re-esterified / FA released) 100

The assumptions made in making these calculations are discussed in more detail in Section 10.1.

Figure 3.8 Fatty acid turnover in an adipocyte



Chapter 4

Insulin

4.1 Introduction

There have been conflicting reports published in recent years regarding insulin's role in regulating the rate of acetate incorporation into fatty acids in ruminant adipose tissue *in vitro* (see Section 1.6). Some studies indicated that insulin will stimulate the rate by 10 - 50% (reviewed by Vernon, 1980), while several more recent reports suggest that insulin's role in altering the rate may be negligible (Thornton *et al.*, 1982; Vasilatos *et al.*, 1983). Further study of the action of insulin in this tissue was therefore required. The following experiments have been carried out with the aim of defining the role of insulin in ruminant adipose tissue.

4.2 The stability of insulin preparations.

Gliemann and Sonne (1978) have demonstrated that as much as 50% of insulin can be degraded by rat adipocytes *in vitro*. It is thought that this loss is due to proteolytic activity leaked into the medium by either by ruptured cells spilling their contents or by cells which are able to release proteolytic enzymes without cell rupture.

It has been reported that increasing albumin concentrations will reduce insulin degradation *in vitro* (Gliemann and Sonne, 1978; Etherton *et al.*, 1984). Etherton *et al.* (1984) also found that incubation and washing of the cells before the addition of insulin markedly reduced the amount of insulin degradation by swine adipocytes. In addition, Livesey and Donald (1982) showed that non-specific binding of insulin to the walls of polypropylene vessels, can be a significant problem.

Experiments were therefore conducted to measure the stability of insulin during incubation with ovine adipose tissue slices.

Table 4.1 The stability of insulin in Medium 199 at 39°C in the absence of tissue.

Samples of insulin were made up at the stipulated concentration in Medium 199 with 1% w/v BSA added where specified and placed at 39°C for 4 hours. There was no tissue present in the incubation vessels. Samples of medium were analysed for insulin by radioimmunoassay (RIA) at the end of the incubation period. The results given are the means of duplicate observations.

Insulin concentration by weight (ng/ml)	Insulin concentration by RIA (ng/ml)	
	1% BSA	no BSA present
0	<0.1	<0.1
0.9	1.0	1.0
2.6	2.2	1.8
3.5	3.2	2.9
4.4	4.5	3.2
5.3	5.1	3.9

Method

Samples of hormone and Medium 199, containing 1% w/v BSA where indicated, were made up at the required concentration and placed in the polypropylene incubation vessels. Since isotope could possibly interfere with the radioimmunoassays, it was excluded from the medium incubated for hormone analysis. Samples of medium were taken from each vessel, before tissue was introduced, to measure the quantity of immuno-reactive hormone present at the start of the experiment. The samples were held at 4°C until analysed.

Table 4.2 The effects of BSA on insulin stability in the presence of ovine adipose tissue slices *in vitro*.

Samples of insulin were made up at the specified concentration in Medium 199 with BSA added where specified and placed at 39°C for 4 hours. Ovine adipose tissue slices were present in all the incubation vessels. Samples of medium were analysed for insulin by radioimmunoassay (RIA) at the end of the incubation period and the values given represent the means of duplicate observations. The percentage loss of insulin is calculated using the concentration of immuno-reactive insulin following the specified incubation period compared to that at the start of the experiment.

Insulin concentration by weight (ng/ml)	BSA	Insulin by RIA (ng/ml) t = 0	% loss of insulin		
			2 hours	4 hours	6 hours
2.6	1%	2.3	43%	43%	48%
4.4	1%	3.6	16%	28%	41%
2.6	no	2.8	*	53%	64%
4.4	no	4.8	*	57%	71%

* *not measured.*

The incubation vessels were placed in a metabolic shaker at 39°C and equal quantities of tissue slices were placed in those vessels specified (half of the vessels contained no tissue). Samples of medium were removed for analysis at 2, 4 and 6 hours and placed at 4°C. Some samples of tissue were pre-incubated in excess medium containing 1% w/v BSA before the above experiment was repeated. When all the samples had been collected, the amount of immuno-reactive hormone present in each sample was measured using radioimmunoassay.

Table 4.3 The effect of 24 hours pre-incubation of the tissue slices on insulin stability in the subsequent incubation.

Ovine adipose tissue slices were pre-incubated in excess Medium 199 for 24 hours. Samples of insulin were made up at the specified concentration in Medium 199 with BSA added where indicated. Pre-incubated ovine adipose tissue slices were placed in the incubation vessels which were incubated at 39°C for 4 hours. Samples of medium were analysed for insulin by radioimmunoassay (RIA) at the end of the incubation period and the values given represent the means of duplicate observations. The results are expressed as the percentage loss of insulin when compared to the immuno-reactive concentration at the start of the experiment.

Insulin concentration by weight (ng/ml)	BSA	Insulin by RIA (ng/ml) t = 0	% loss of insulin		
			2 hours	4 hours	6 hours
2.6	1%	2.3	0	17%	100%
4.4	1%	4.3	32%	23%	100%
2.6	no	1.5	67%	47%	80%
4.4	no	3.0	30%	50%	67%

Results and conclusions

The results of the radioimmunoassays, carried out by Mr T. Braggins, are given in Tables 4.1, 4.2 and 4.3. It can be seen in Table 4.1 that, in the absence of adipose tissue slices, there is relatively little insulin lost from the medium. It is possible that BSA gives some protection although the differences are small. However, in Tables 4.2 and 4.3, there has been considerable degradation (averaging 50% of the initial immuno-reactive concentration of the hormone) in almost all incubations containing adipose tissue slices. BSA does appear to protect the insulin from this degradation, at least in the first few hours of incubation. Pre-incubation of the tissue for 24 hours did not reduce the amount of insulin degradation occurring in the subsequent incubation.

4.3 The effects of insulin on the rate of lipogenesis

Acetate and H atom incorporation into tissue fatty acid

The rate of lipogenesis in ovine adipose tissue slices *in vitro* were estimated using the rate of incorporation of either acetate or H atoms into fatty acid. Incubations were terminated at different times for measurement of the rate of incorporation of the radioactively labelled substrate into the tissue fatty acids. Substrate incorporation is plotted against time in the time-course graph. As seen in Figure 4.1, insulin stimulated the overall rate of acetate incorporation into fatty acid by 460% ($p < 0.001$). However, in another experiment, there was no significant stimulation by insulin throughout the 6 hour incubation (Figure 4.2). Insulin stimulated the rate by 52% ($p < 0.05$) at 4 hours (Figure 4.3) and, in another experiment, by 47% after 6 hours ($p < 0.05$), with the stimulation at 2 and 4 hours not significant (Figure 4.4).

In Figure 4.5, insulin stimulated the rate of H atom incorporation by 67% after 24 hours ($p < 0.001$), by 171% ($p < 0.001$) at 72 hours and by 96% ($p < 0.001$) at 96 hours. The stimulation at 48 hours was not statistically significant. Insulin was seen to stimulate lipogenesis by up to 6-fold, with the average being about 70% in all the animals tested ($n = 30$). Insulin failed to significantly stimulate lipogenesis in tissue from approximately one quarter of these animals.

The basal rate of H atom incorporation into fatty acid was 1.68 μmoles H/hours/10^6cells (Figure 4.5). Using the formula for conversion to C incorporation given in Section 3.14, this represents a basal rate of C incorporation into fatty acid of 1.94 μmoles C/hour/10^6cells or 0.97 μmoles C_2/hour/10^6cells. This is similar to the rate of acetate incorporation for Sheep G in Table 4.4 (1.00 μmoles acetate/hour/10^6cells).

Table 4.4 A comparison of the action of insulin on the rate of acetate incorporation into fatty acid in adipose tissue from 4 sheep

The four sheep were slaughtered on the same day and tissue from each was incubated separately under identical incubation conditions. The rate of lipogenesis was estimated using the rate of acetate incorporation into fatty acids in adipose tissue slices incubated in Medium 199 with no BSA present.

Sheep identity	Age (days)	GR (mm)	Basal rate of lipogenesis (μmol/h/10^6cells)	Stimulation by insulin %	p
D	234	18	0.22 ± 0.02	+90	<0.05
E	233	12	0.15 ± 0.01	+90	<0.001
F	231	10	0.45 ± 0.02	+30	<0.05
G	228	9	1.00 ± 0.05	+40	<0.001

In the experiment referred to in Table 4.4, tissue was taken from 4 sheep slaughtered at the same time. The basal rate of lipogenesis and the response of the cells to insulin was tested in tissue from each animal separately but under identical incubation conditions. The basal rate was seen to vary from 0.15 - 1.0 μmoles acetate incorporated/hour/10^6 cells and the insulin stimulation from 30 - 90% of the basal rate.

In Table 4.5, the basal rate of lipogenesis and insulin response are shown for two lambs which were twins. Their ages, nutritional status and fatness at slaughter were identical but, although their genetic backgrounds were very similar, it is not known whether they were identical. The basal rate of lipogenesis and the response to insulin in tissue from these two animals was similar.

The rate of acetate incorporation into fatty acid is plotted against concentration of insulin in the dose-response graphs. The rate is seen to increase with insulin concentration up to 4.5 ng/ml (Figure 4.6). Insulin stimulated the rate of lipogenesis by a maximum of 350% ($p < 0.01$).

Table 4.5 A comparison of the action of insulin on the rate of acetate incorporation into fatty acid in adipose tissue from twin lambs

Each experiment was carried out using tissue from a single lamb. The two lambs used were twins although it is not known if they were identical. The lambs were slaughtered several days apart and incubation conditions for each experiment were the same. The rate of lipogenesis was estimated using the rate of acetate incorporation into fatty acids in adipose tissue slices incubated in Medium 199 with no BSA present.

Lamb identity	Age (days)	GR (mm)	Basal rate of lipogenesis (μmol/h/10^6cells)	Stimulation by insulin %	p
H	129	1	0.85 ± 0.05	+30	<0.05
J	133	1	0.55 ± 0.02	+50	<0.05

In another experiment, the rate also initially increased in the presence of increasing concentrations of insulin but decreased back to a rate similar to the basal rate at concentrations higher than 3.6 ng/ml (Figure 4.7). Insulin stimulated the rate of lipogenesis by a maximum of 50% in this experiment.

A study was made of the effects of increasing acetate concentration on the rate of lipogenesis. As seen in Figure 4.8b, where there was no insulin present, there is an initial increase in the rate of acetate incorporation with increasing concentration of acetate, followed by a flattening of the Michaelis-Menton curve when little further increase in the rate occurred. The V_{max} for this curve was 1.58 ± 0.16 μmoles acetate incorporated/4 hour/10^6 cells and the K_m 0.49 ± 0.08 mmol/l acetate, as calculated from the Lineweaver-Burk Plot (Figure 4.8a). In Figures 4.9a (Lineweaver-Burk Plot) and 4.9b (Michaelis-Menton curve), where the incubations were all carried out in the presence of 2.6 ng/ml insulin, the V_{max} was 2.024 ± 0.20 μmoles/4 hours/10^6cells and the K_m 0.607 ± 0.09 mmol/l. Although insulin has caused an increase of 28% in the V_{max} and 24% in the K_m, the difference was not statistically significant at the 95% level of confidence.

Table 4.6 A comparison of the effects of insulin on glucose and acetate incorporation into tissue lipid *in vitro*

Sheep 1

All incubations have been carried out in triplicate with tissue from one animal. The rates of incorporation into lipid have been taken from the gradient and standard error of the gradient for time-course plots using linear regression analysis (e.g. for glucose incorporation into triacylglycerol and fatty acid for this experiment, see Figures 4.10 and 4.11). The rate of glucose incorporation is multiplied by 3 to give the rate of incorporation of C_2 units. The rate of glucose incorporation into glycerol has been calculated by subtracting the rate of glucose incorporation into fatty acids from the rate of glucose incorporation into triacylglycerol.

	rate of C_2 incorporation (nmol/h/10^6 cells)	% change with 2.6 ng/ml insulin	p
acetate → FA	198.2 ± 8.0	0	NS
glucose → TG	86.7 ± 6.0	+44%	<0.05
glucose → FA	47.1 ± 5.0	+69%	<0.05
glucose → glycerol	39.5 ± 8.0	+15%	NS

Glucose incorporation into tissue lipids

Ovine adipose tissue slices were incubated in Medium 199 containing ^{14}C-glucose or ^{14}C-acetate where specified and no BSA. Glucose into tissue triacylglycerol (Figure 4.10) and tissue fatty acid (Figure 4.11) is plotted against time. Linear regression analysis of these plots gives an estimate of the contribution made by glucose to the rate of lipogenesis from all precursors in ovine adipose tissue. The rate of glucose incorporation into either triacylglycerol or fatty acid varied linearly with time and insulin stimulated the rate of glucose incorporation into triacylglycerol by 44% ($p < 0.05$) and into fatty acid by 69% ($p < 0.05$).

Table 4.7 A comparison of the effects of insulin on glucose and acetate incorporation into tissue lipids *in vitro*

Sheep 2

Experimental conditions and calculation of components were as given in Table 4.6 but the time-course plots are not shown.

	rate of C_2 incorporation (nmol/h/10^6 cells)	% change with 2.6 ng/ml insulin	p
acetate → FA	45.2 ± 4.0	+60%	<0.01
glucose → TG	105.9 ± 5.1	-13%	<0.05
glucose → FA	28.2 ± 5.7	-50%	<0.01
glucose → glycerol	73.4 ± 8.0	<0.1%	NS

These results are summarised in Table 4.6 and are compared with the rates of glucose incorporation into glycerol and acetate incorporation into fatty acid in tissue from the same animal (Sheep 1). Insulin did not significantly affect the rate of acetate incorporation into fatty acid or glucose incorporation into glycerol in tissue from this animal. Since all rates of incorporation have been converted to the contribution of C_2 units in the tissue lipids, they can be directly compared. The ratio of acetate incorporation to glucose incorporation into fatty acid is:

for the basal incubations, 4:1 (20% from glucose)
for the incubations with insulin, 2.5:1 (28% from glucose)

The number of molecules of fatty acid produced by both acetate and glucose incorporation was compared with the number of molecules of glycerol simultaneously produced. In making these calculations, it was assumed that all fatty acid produced had 16 carbon atoms. In the basal incubation, there were 27.2 nmoles of fatty acid produced for every 26.3 nmoles of glycerol. In the insulin-stimulated cells, there were 31.0 nmoles of fatty acid produced for 30.2 nmoles of glycerol. Therefore, the ratio of fatty acid produced to glycerol produced for both the basal and insulin stimulated cells was 1:1.

The experiment was repeated on tissue from a second animal (Sheep 2) and the results are given in Table 4.7. In this case, insulin did significantly stimulate the uptake of acetate into fatty acid ($p < 0.01$) while depressing the rate of glucose uptake into triacylglycerol ($p < 0.05$) and fatty acid ($p < 0.01$). Insulin had no effect on the uptake of glucose into glycerol. Using the contributions of C_2 units made to the lipid, the ratio of acetate incorporation to glucose incorporation into fatty acid is:

for the basal incubations,	2:1	(33% from glucose)
for the incubations with insulin,	6:1	(14% from glucose)

In the basal incubations, there were 8.1 nmoles of fatty acid produced for every 48.9 nmoles of glycerol. At the same time, in the incubations with insulin present, there were 9.59 nmoles of fatty acid produced for every 48.93 nmoles of glycerol. The ratio of the number of molecules of fatty acid produced by both acetate and glucose incorporation to the number of molecules of glycerol simultaneously produced is:

for the basal incubations,	1:6
for the incubations with insulin,	1:5

The effect of increasing insulin concentrations on glucose incorporation into triacylglycerol in isolated ovine adipocytes was examined and the results are plotted in Figure 4.12. Isolated adipocytes prepared from sheep subcutaneous adipose tissue were incubated in modified Krebs-Ringer buffer containing ^{14}C-glucose and 1% BSA. There was increasing incorporation of glucose into triacylglycerol with increasing insulin concentration. A similar experiment was carried out using isolated rat adipocytes (taken from the epididymal fat pad of a young rat weighing less than 200g) using the same incubation conditions as with the sheep isolated adipocytes. Glucose incorporation into triacylglycerol is plotted against increasing insulin concentration in Figure 4.13. The basal rate of glucose incorporation in the ovine adipocytes was 0.13 μmoles/hour/g lipid and the rat adipocytes was 0.18 μmoles/hour/g lipid. Insulin stimulated the basal rate of glucose uptake in ovine adipocytes by 4-fold and rat adipocytes by 12-fold.

4.4 The effects of insulin on the rates of lipolysis and mass balance of fatty acid

Ovine adipose tissue slices were incubated in Medium 199 containing 1% BSA to investigate the effects of insulin on the rate of release of glycerol into the incubation medium. The results are presented in Figure 4.14 with glycerol release from the tissue slices plotted against incubation time. The rate was decreased overall by 29% ($p < 0.001$) in the presence of 2.6 ng/ml insulin during the 6 hour incubation. Linear regression analysis of the line gave a correlation coefficient of 0.9947.

The change in the rate of release of glycerol with increasing insulin concentration is seen in Figure 4.15. The maximum observed decrease in the rate of glycerol release was 36% ($p < 0.01$) at insulin concentrations of 0.7 and 70 ng/ml.

The effect of insulin on the mass balance of fatty acid in ovine adipose tissue slices is seen in Table 4.8 for Sheep 3. Insulin increased fatty acid manufacture by 92% ($p < 0.01$) while in the same time increasing the fatty acid accumulation in the cell by 132% ($p < 0.001$). The increases in the amount of fatty acid released by lipolysis and in the rate of fatty acid re-esterification within the cell were not statistically significant. The amount of FFA leaving the cell decreased by 61% ($p < 0.001$). The percentage of fatty acid recycled within the cell was 91% in the basal incubations, increasing to 97% in the presence of 2.6 ng/ml insulin.

The experiment was repeated with tissue from a different animal (Sheep 4) and the results are presented in Table 4.9. In this experiment, 2.6 ng/ml insulin increased fatty acid manufacture by 34% ($p < 0.05$) while increasing fatty acid accumulation within the cell by 50% ($p < 0.05$). Fatty acid released within the cell decreased by 12% ($p < 0.05$) while the decrease in the amount of fatty acid re-esterified was not statistically significant. The amount of FFA leaving the cell decreased by 52% and the percentage recycling of fatty acid within the cell increased from 89% to 94% in the presence of insulin.

The results of an experiment with tissue from a third animal, Sheep 5, is presented in Table 4.10. Here, the effects of insulin on the rate of fatty acid manufacture, fatty acid release by lipolysis or on the amount of this fatty acid being re-esterified were not statistically significant. However, insulin decreased the amount of fatty acid leaving the cell by 64% ($p < 0.05$) and increased the amount of fatty acid accumulated within the cell by 61% ($p < 0.05$). The percentage of fatty acid released by lipolysis that was recycled within the cell increased from 74% to 89% in the presence of insulin.

For a discussion of the results presented in this Chapter, refer to Section 10.2.

Table 4.8

	μmoles/4 hours/10^6cells			
	basal rate	rate with insulin	change in rate (% change)	p
FA manufactured	0.62 ± 0.06	1.19 ± 0.12	+0.57 (+ 92%)	**
FA released	1.50 ± 0.04	1.55 ± 0.05	+0.05 (+ 3%)	NS
FFA leaving cell	0.13 ± 0.01	0.05 ± 0.01	-0.08 (- 61%)	***
FA re-esterified	1.37 ± 0.04	1.51 ± 0.05	+0.14 (+ 10%)	NS
FA accumulation	0.49 ± 0.06	1.14 ± 0.06	+0.65 (+132%)	***
% FA recycling	91%	97%		

** p < 0.01 *** p < 0.001

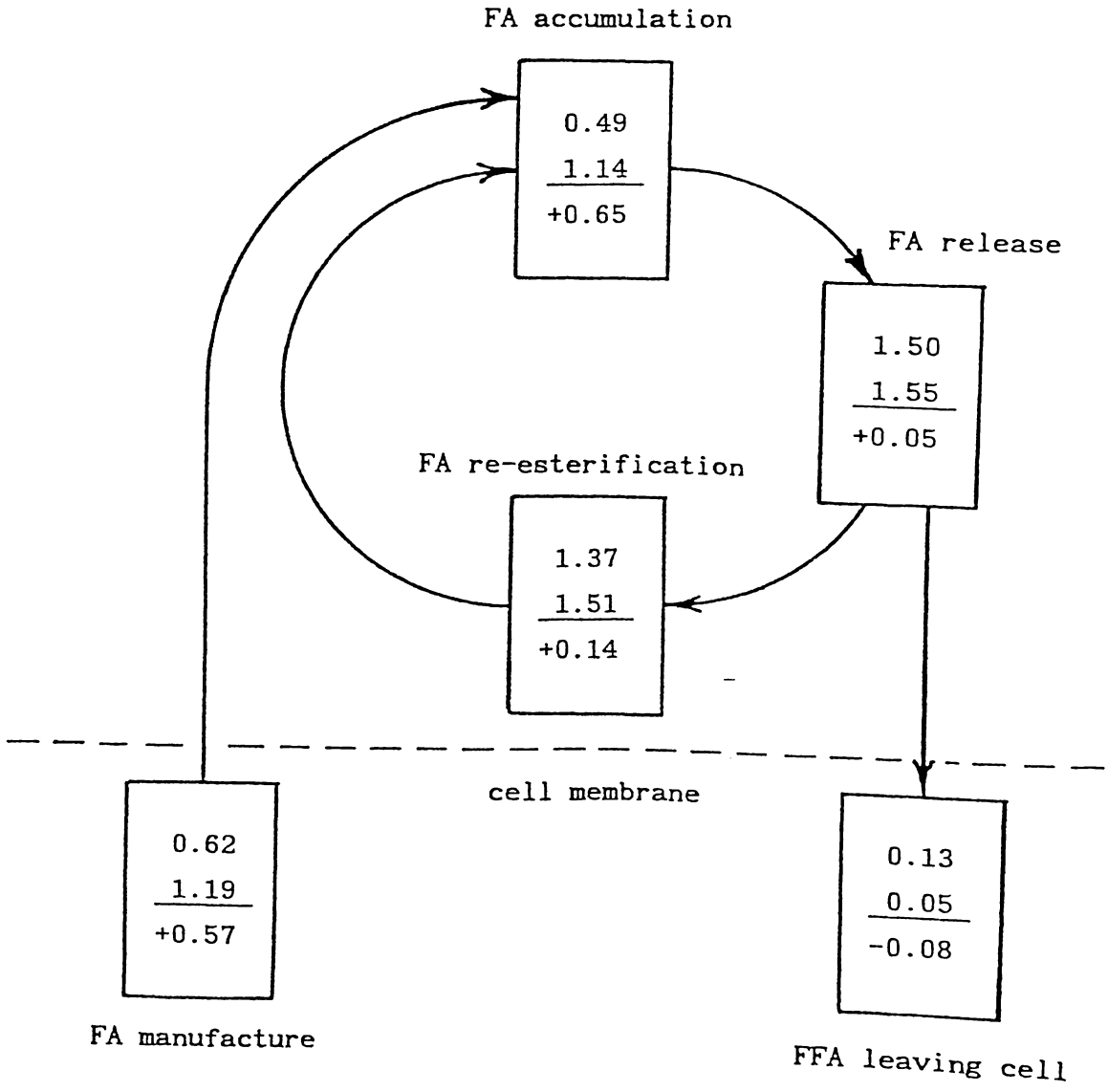


Table 4.8 The effect of insulin on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Sheep 3

Each rate represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with insulin) of ovine adipose tissue slices in Medium 199 with 1% w/v BSA. Insulin was present where specified at a concentration of 2.6 ng/ml.

"FA manufactured" was measured by acetate incorporation into tissue fatty acid.

"FA released" is the fatty acid released by lipolysis within the tissue and was measured by glycerol release into the incubation medium (x3).

"FFA leaving cell" is the amount of fatty acid released into the incubation medium.

"FA re-esterified" is the amount of fatty acid that remained in the tissue after lipolysis and was presumably re-esterified.

"FA accumulation" is the amount of fatty acid that accumulated in the tissue during the time of incubation (4 hours).

"% FA recycling" is the percentage of the fatty acid released which was subsequently re-esterified.

For a description of the method of calculation of each variable, refer to Section 3.16.

The diagram, which can be compared with Figure 3.8, is a summary of the data presented in the Table and represents the mass balance of the fatty acid in the cell and incubation medium. The boxes at each stage in the cycle represent the pools of fatty acid present. The first number in each box is the basal rate (in μmoles fatty acid/4 hours/10^6 cells), the next the rate with insulin and the third is the change in the rate which occurred in the presence of insulin.

Table 4.9

	μmoles/4 hours/10^6cells			
	basal rate	rate with insulin	change in rate (% change)	p
FA manufactured	1.82 ± 0.14	2.44 ± 0.25	+0.62 (+34%)	*
FA released	2.63 ± 0.08	2.32 ± 0.14	-0.31 (-12%)	*
FFA leaving cell	0.29 ± 0.04	0.14 ± 0.05	-0.15 (-52%)	*
FA re-esterified	2.34 ± 0.05	2.18 ± 0.10	-0.16 (-7%)	NS
FA accumulation	1.53 ± 0.15	2.29 ± 0.28	+0.76 (+50%)	*
% FA recycling	89%	94%		

* $p < 0.05$

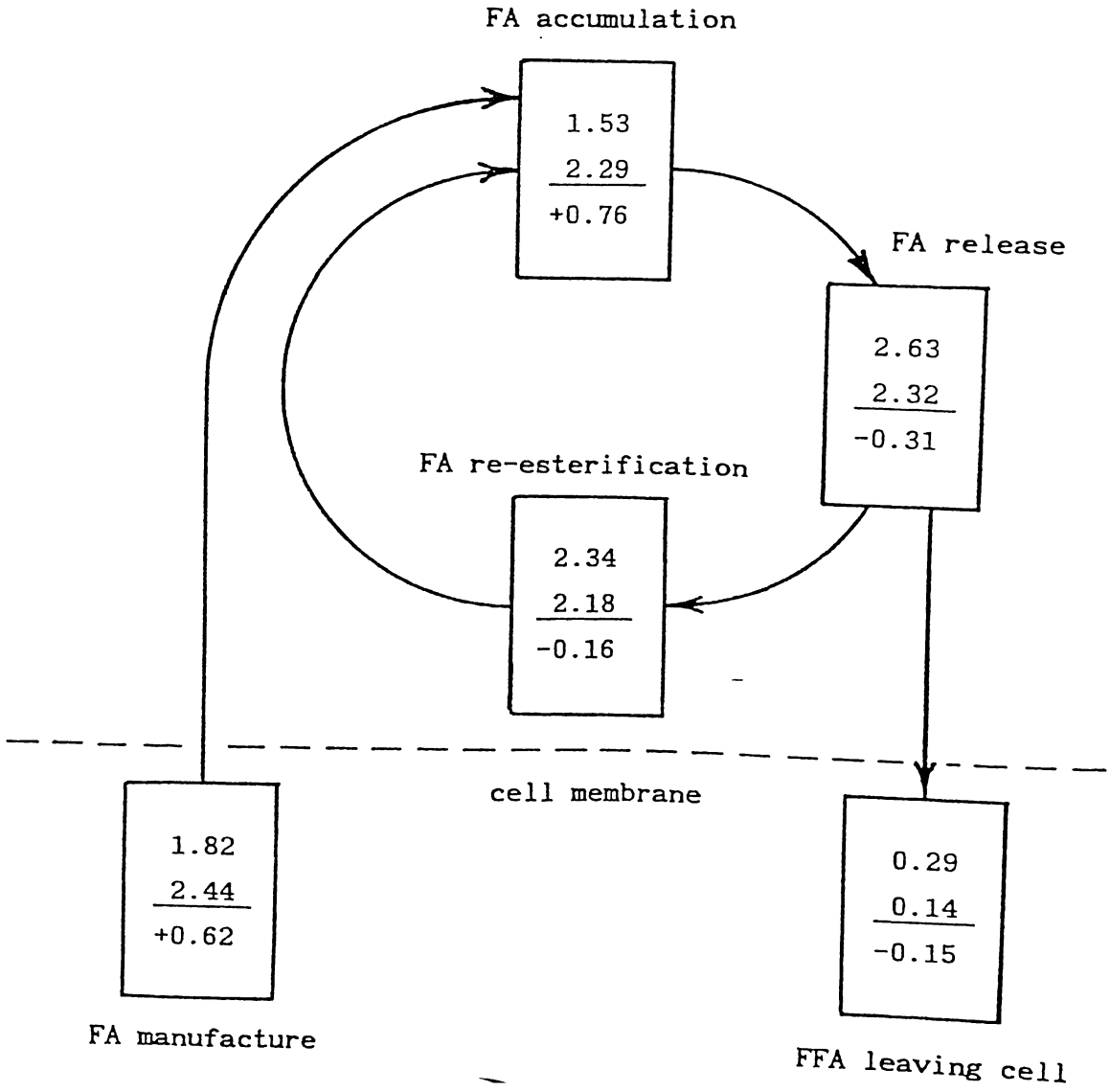


Table 4.9 The effect of insulin on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Sheep 4

Experimental conditions and the definition of terms are the same as those given in Table 4.8.

Table 4.10

	μmoles/6 hours/10^6cells			
	basal rate	rate with insulin	change in rate (% change)	p
FA manufactured	2.36 ± 0.07	2.57 ± 0.06	+0.21 (+9%)	NS
FA released	3.80 ± 0.33	2.99 ± 0.80	-0.81 (-21%)	NS
FFA leaving cell	0.98 ± 0.22	0.34 ± 0.03	-0.64 (-64%)	*
FA re-esterified	2.82 ± 0.19	2.65 ± 0.77	-0.17 (-6%)	NS
FA accumulation	1.38 ± 0.20	2.23 ± 0.05	+0.85 (+61%)	*
% FA recycling	74%	89%		

* $p < 0.05$

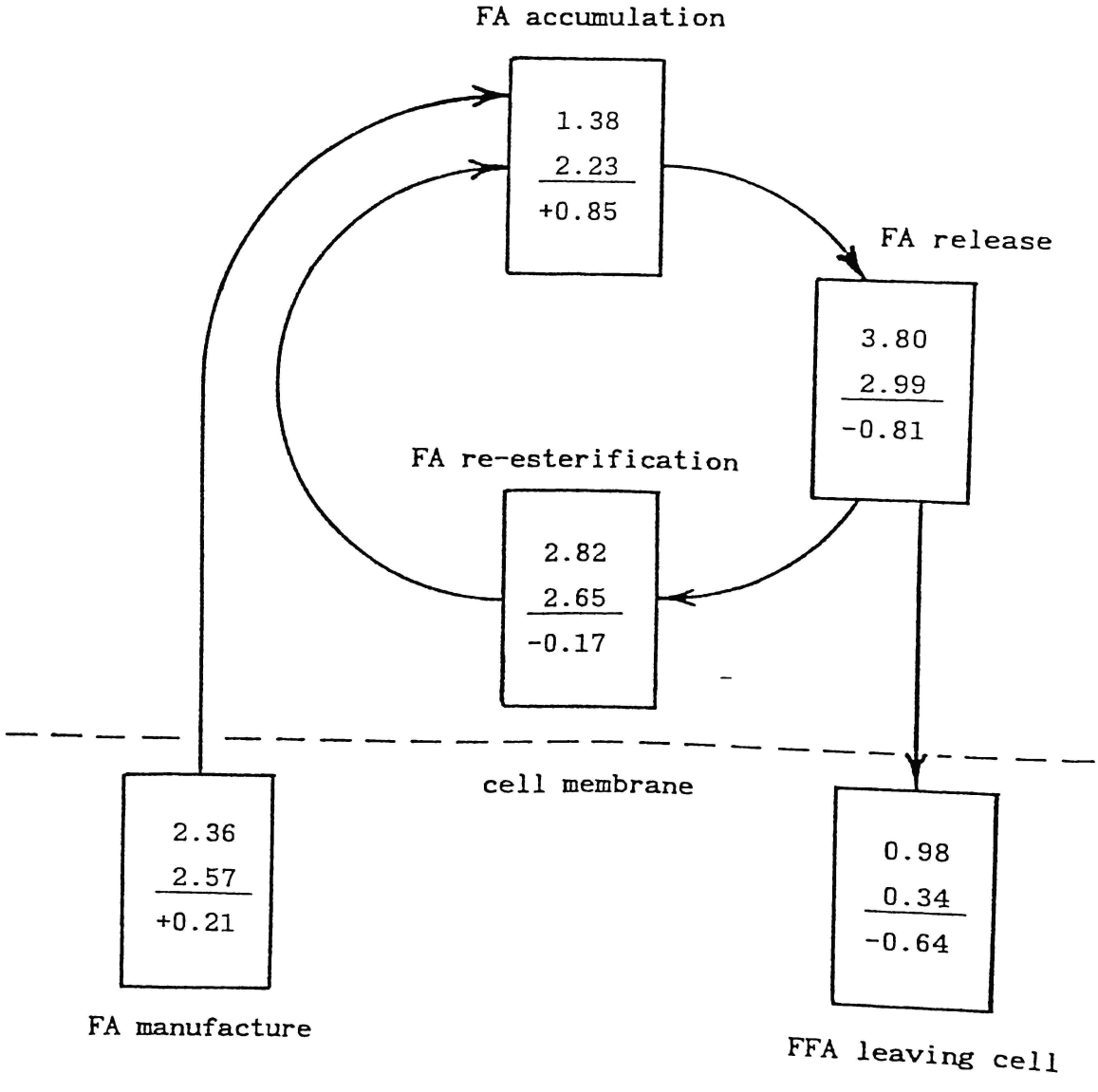


Table 4.10 The effect of insulin on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Sheep 5

Experimental conditions and the definition of terms are the same as those given in Table 4.8 except that the incubation time was 6 hours.

Figure 4.1

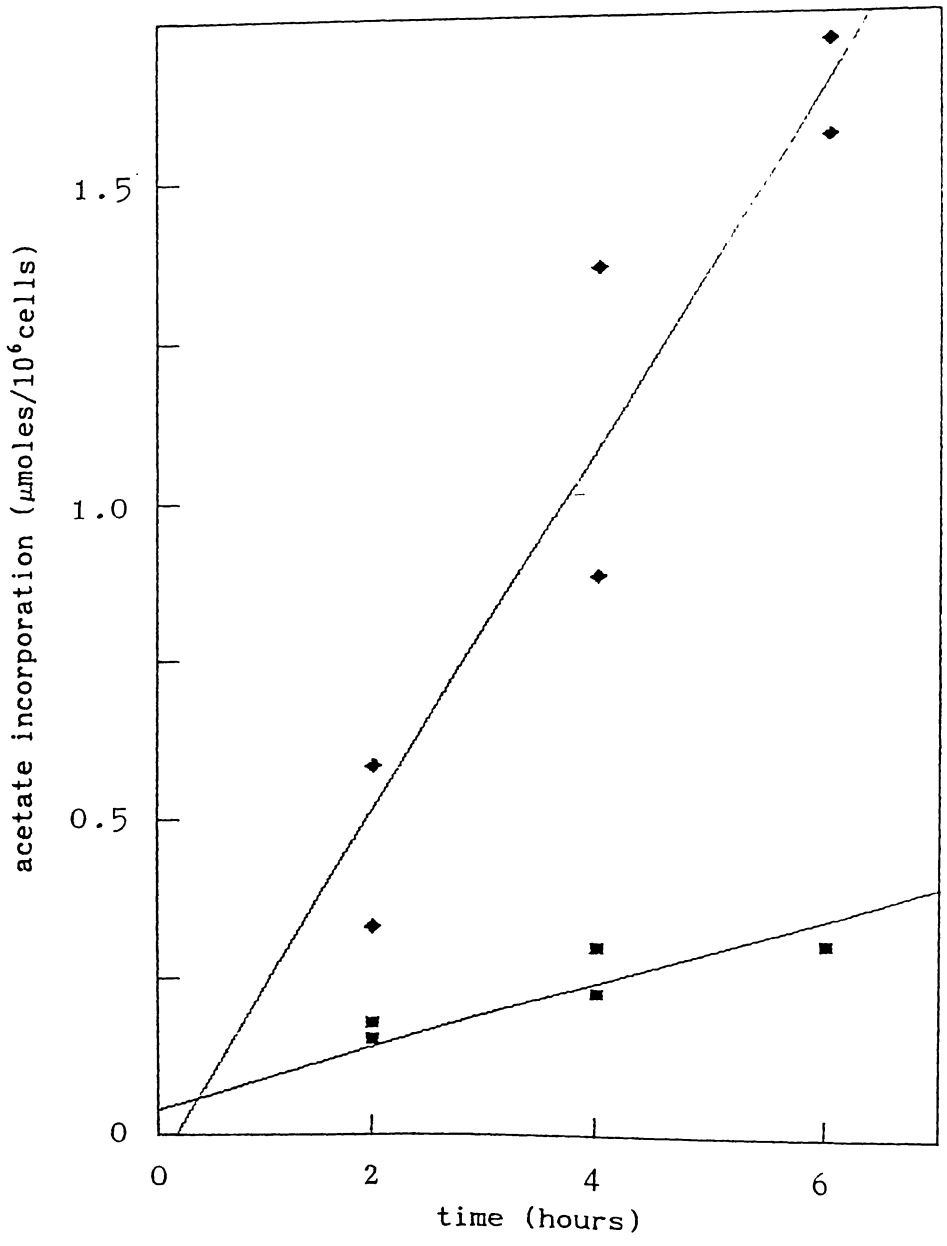

acetate incorporation (μmoles/10^6cells)
1.5
1.0
0.5
0
0
2
4
6
time (hours)

Figure 4.1 The effect of insulin on the rate of acetate incorporation into fatty acid *in vitro*

Each point represents a single incubation of ovine adipose tissue slices in Medium 199 with no BSA present. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ is defined by the equation Y = 0.04 + 0.05X, where Y = acetate incorporation into fatty acid and X = time (r = 0.9336). Acetate incorporation into fatty acid in the presence of 2.6 ng/ml insulin ◆ is defined by the equation Y = -0.05 + 0.28X (r = 0.9655).

Linear regression analysis was used to estimate the overall rates of incorporation. Insulin stimulated the rate of acetate incorporation from 0.050 ± 0.004 to 0.283 ± 0.014, a 460% increase ($p < 0.001$).

Figure 4.2

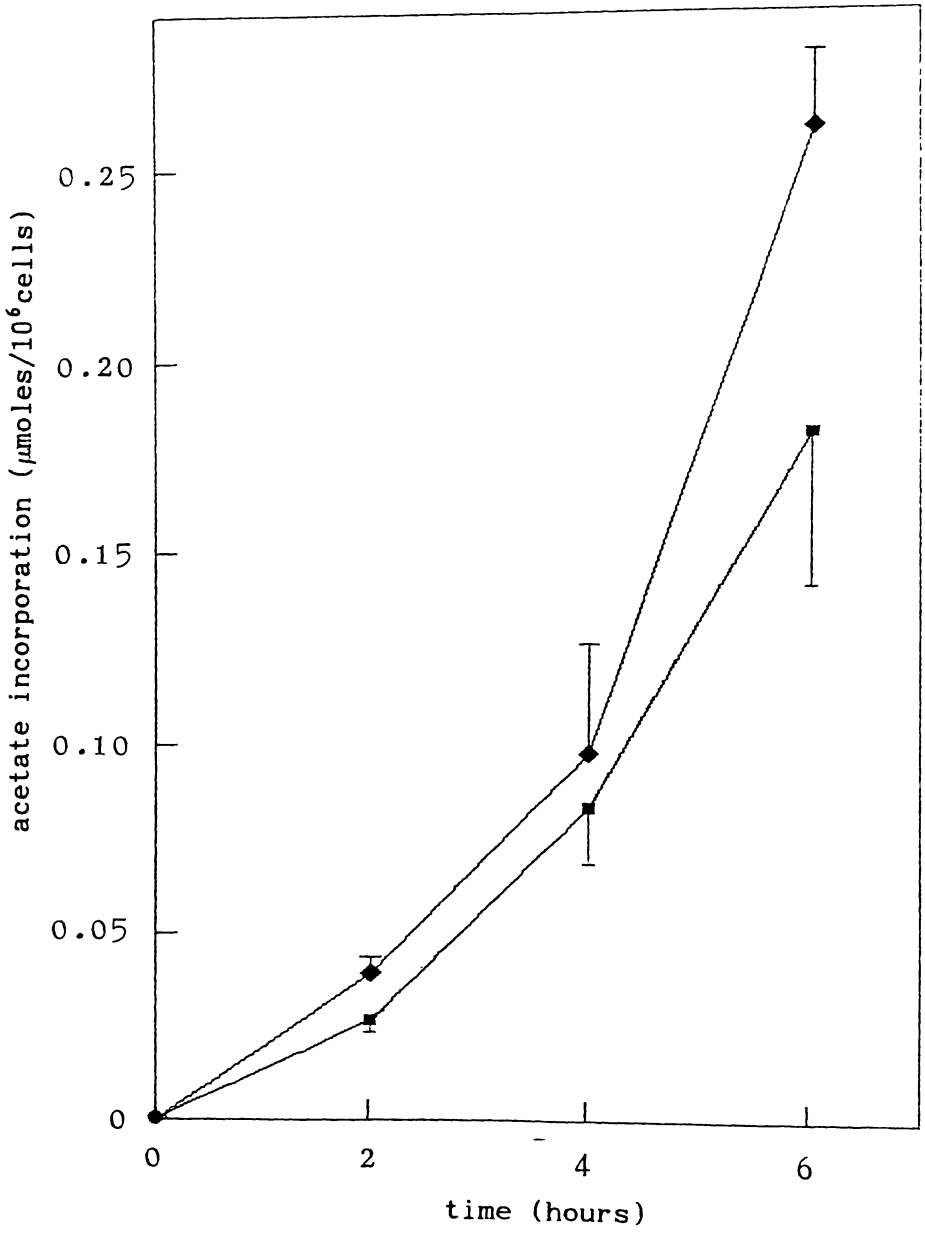
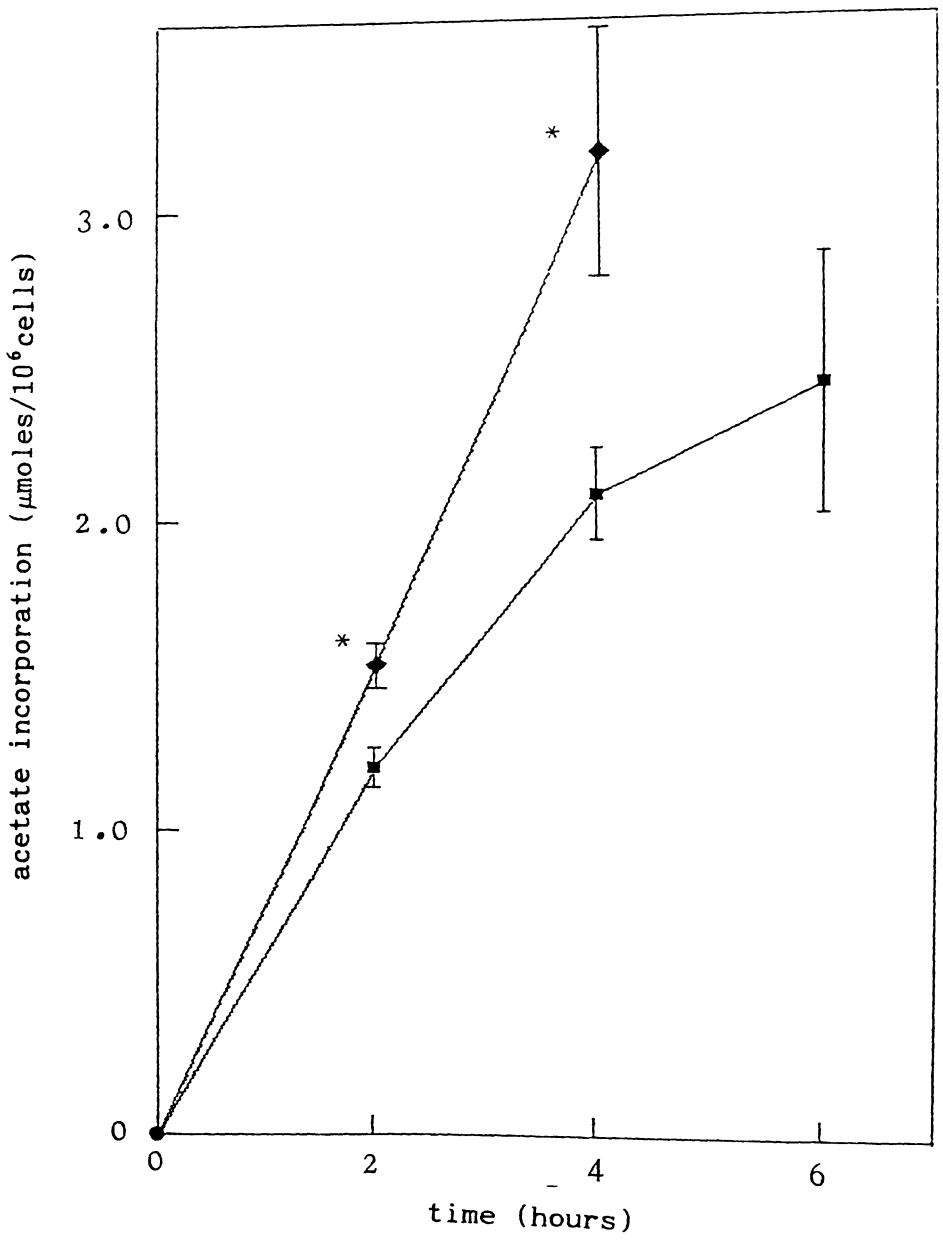

0.25
0.20
0.15
0.10
0.05
0
acetate incorporation (μmoles/10^6cells)
0
2
4
6
time (hours)

Figure 4.2 The effect of insulin on the rate of acetate incorporation into fatty acid *in vitro*.

Experimental conditions were as given in Figure 4.1. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ and in the presence of 2.6 ng/ml of insulin ◆ are shown.

Figure 4.3



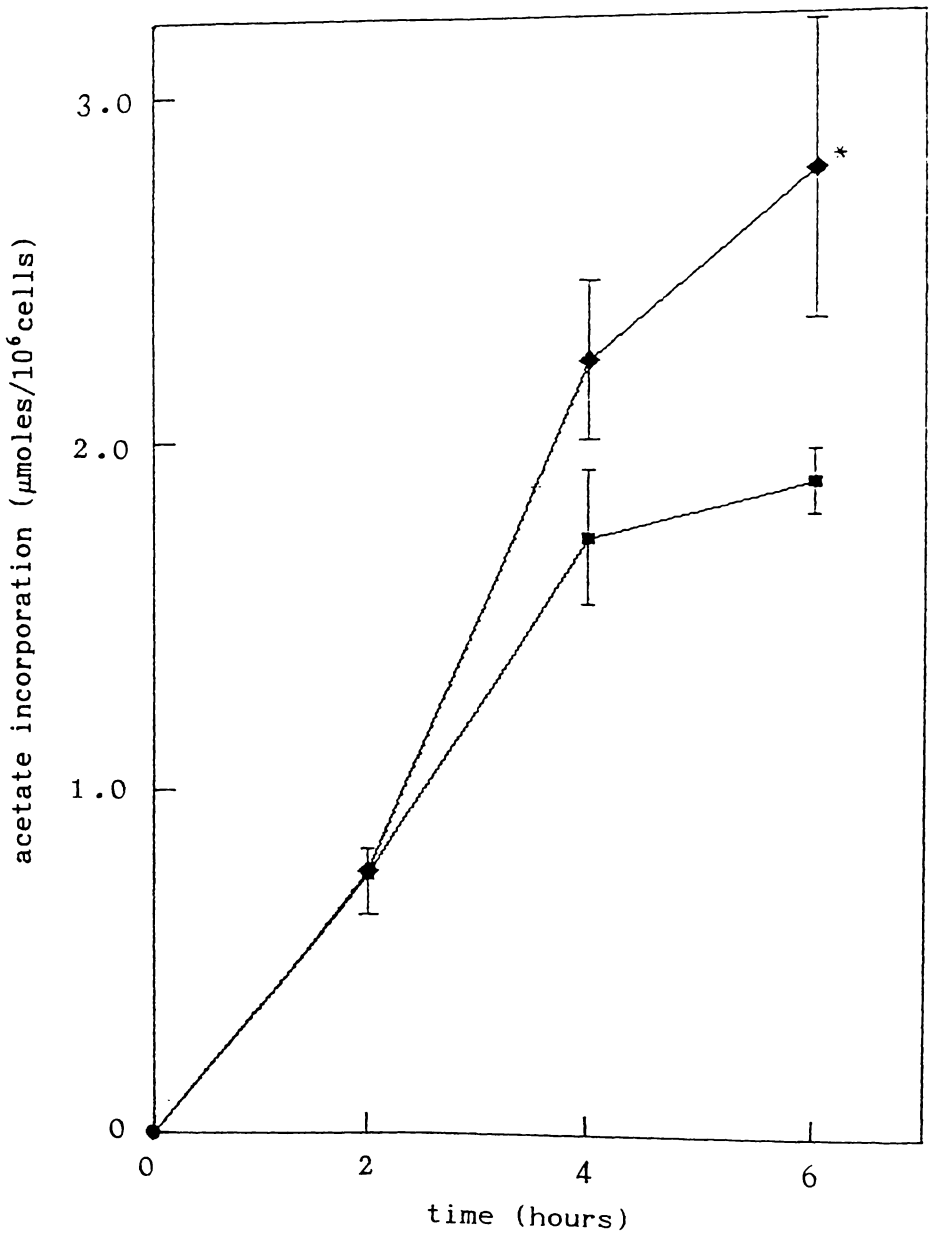
* $p < 0.05$

Figure 4.3 The effect of insulin on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 4.1. Acetate incorporation into tissue fatty acids (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ and in the presence of 2.6 ng/ml of insulin ◆ are shown.

Figure 4.4



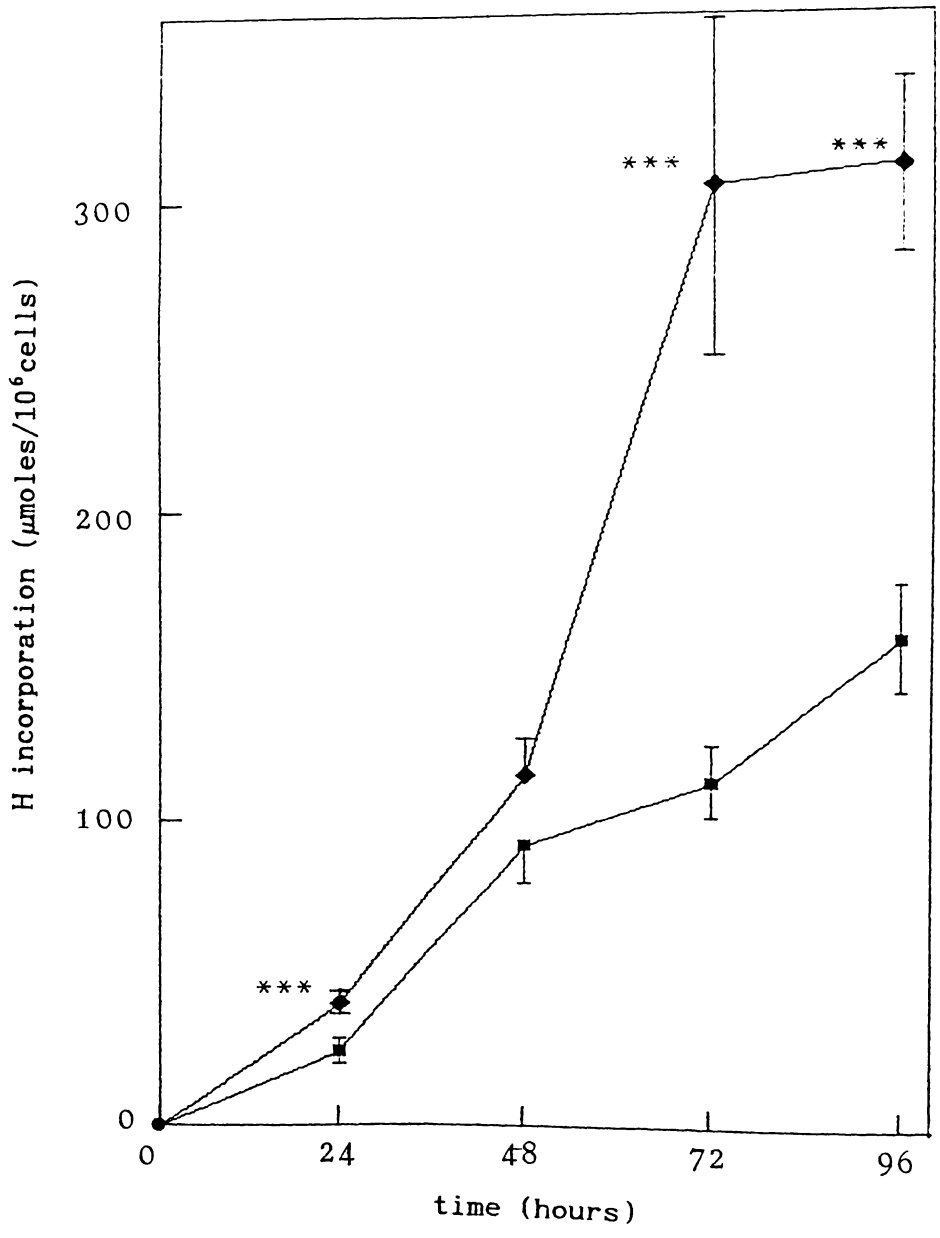
* $p < 0.05$

Figure 4.4 The effect of insulin on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 4.1. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ and in the presence of 2.6 ng/ml insulin ◆ are shown.

Figure 4.5



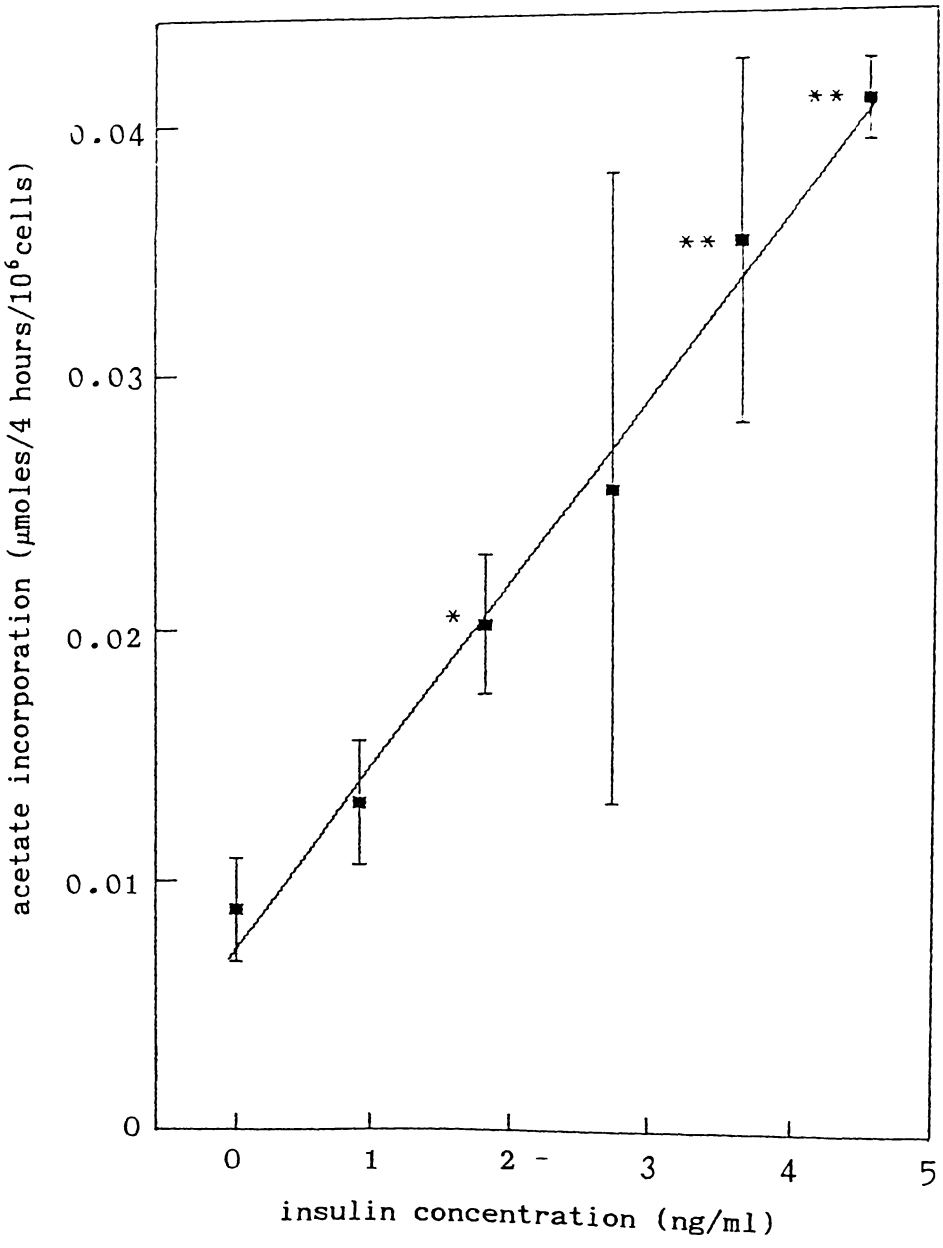
*** p < 0.001

Figure 4.5 The effect of insulin on the rate of H atom incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of six replicate incubations of ovine adipose tissue slices in Medium 199 with no BSA present. H atom incorporation from ^3H_2O into fatty acid (μmoles/10^6cells) is plotted against time (hours).

H incorporation into fatty acid with no hormone in the medium ■ and in the presence of 2.6 ng/ml insulin ◆ are shown.

Figure 4.6



* $p < 0.05$

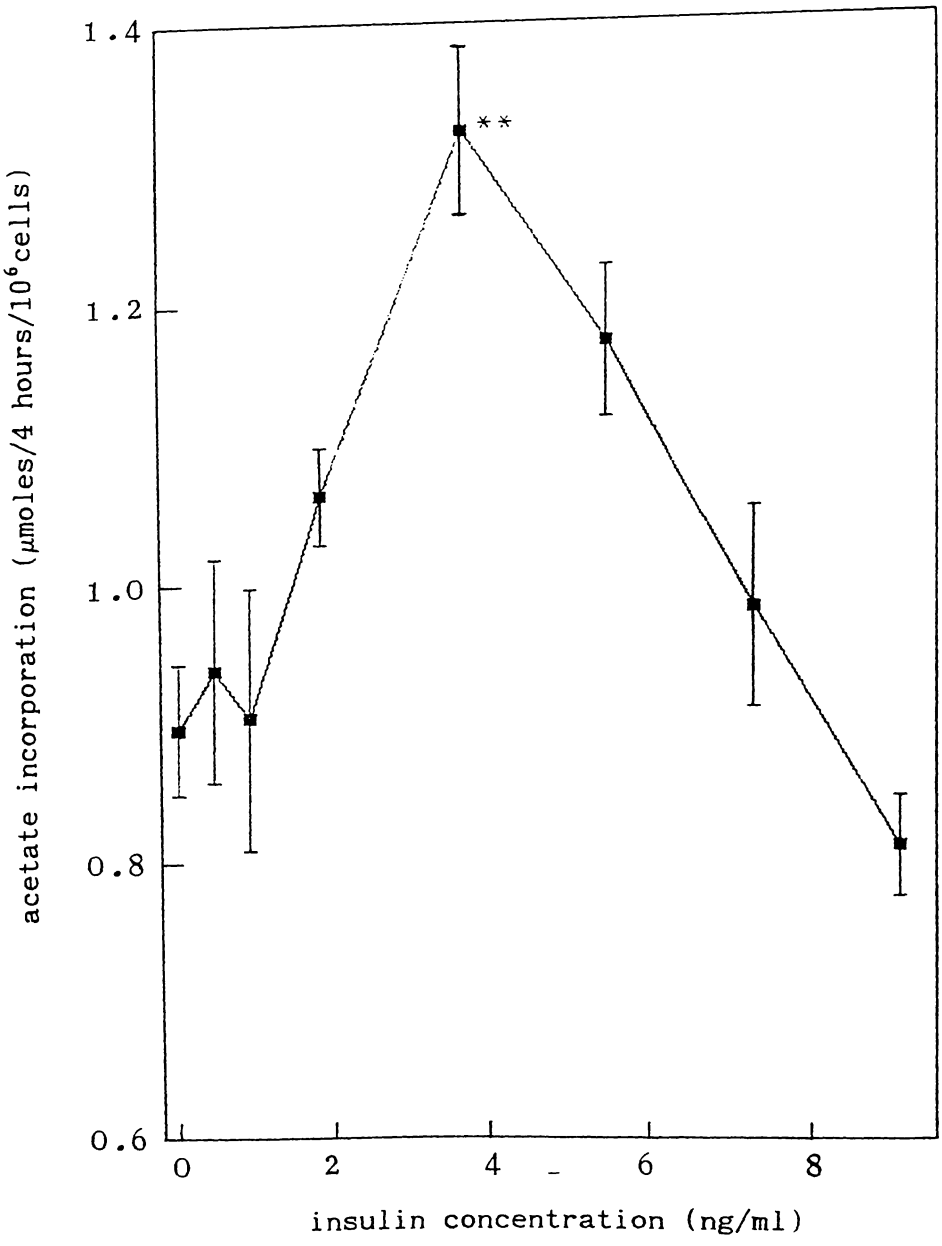
** $p < 0.01$

Figure 4.6 The effect of insulin concentration on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 4.1. The rate of acetate incorporation into tissue fatty acids (μmoles/4 hours/10^6cells) is plotted against insulin concentration (ng/ml).

The changing rate of acetate incorporation into fatty acid with insulin concentration is approximately linear (r = 0.9868).

Figure 4.7



** p < 0.01

Figure 4.7 The effect of insulin concentration on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 4.1. The rate of acetate incorporation into tissue fatty acids (μmoles/4 hours/10^6cells) is plotted against insulin concentration (ng/ml).

Figure 4.8a

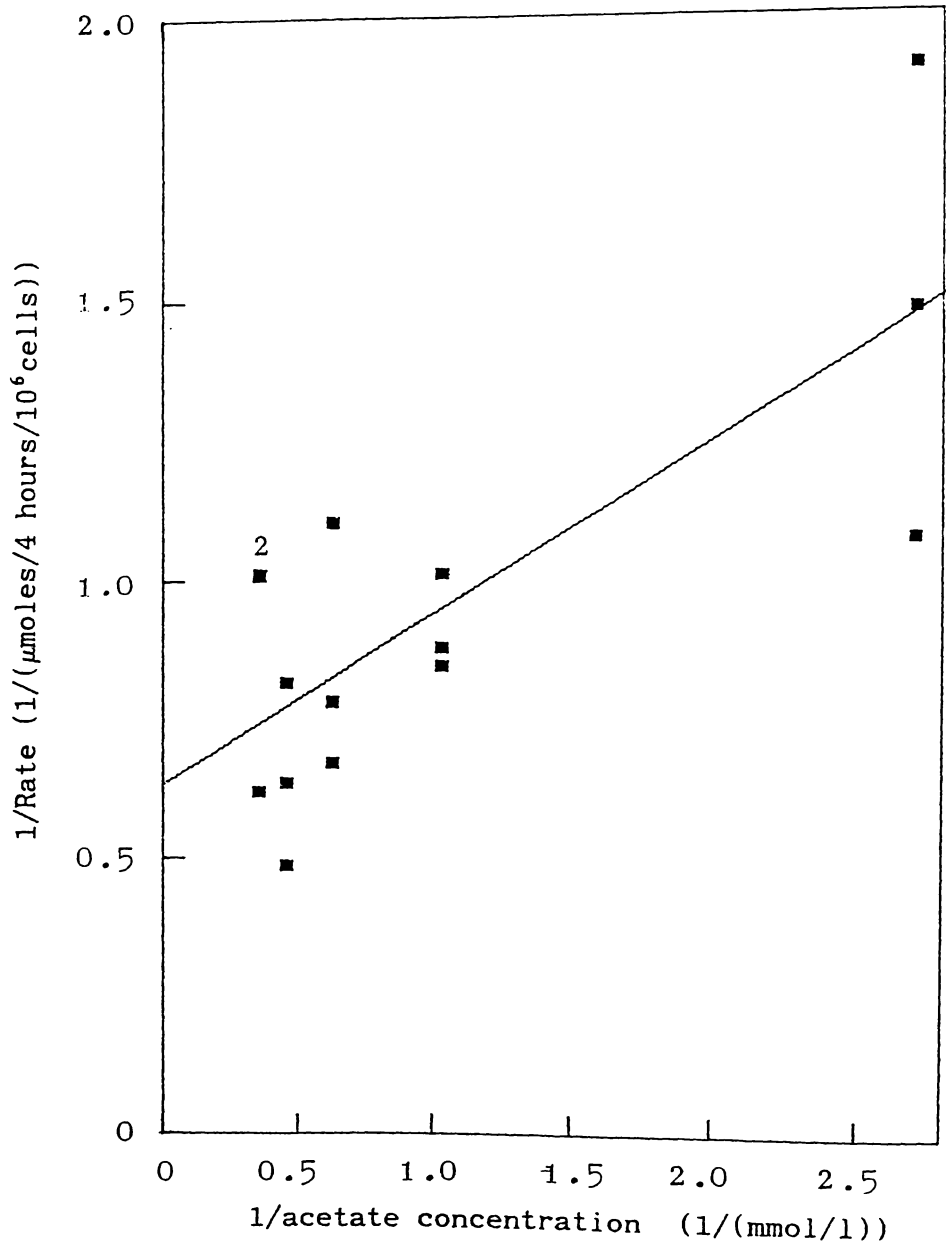

2.0
1.5
1.0
0.5
0
1/Rate (1/(μmoles/4 hours/10^6cells))
2
0
0.5
1.0
1.5
2.0
2.5
1/acetate concentration (1/(mmol/l))

Figure 4.8 The effect of acetate concentration on the basal rate of acetate incorporation into fatty acid *in vitro*

Figure 4.8a is a Lineweaver-Burk plot used to calculate V_{max} and K_m for the data plotted in Figure 4.8b.

Figure 4.8a Lineweaver-Burk double reciprocal plot of the basal rate of acetate incorporation into fatty acid against acetate concentration

Each point represents a separate incubation of ovine adipose tissue slices in Medium 199 with no BSA or hormone added. The reciprocal of acetate incorporation into tissue fatty acids (1/(μmoles/4 hours/10^6cells)) is plotted against the reciprocal of acetate concentration (1/(mmol/l)).

There is some indication of a linear trend in the data (r = 0.7736) and the equation for the line is Y = 0.63 + 0.312X where Y = 1/(rate of acetate incorporation into fatty acid) and X = 1/(acetate concentration). There is 10% error in the estimation of the X intercept (c) and 6% error in the estimate of the gradient (m).

V_{max} = 1/c = 1.58 ± 0.16 μmoles/4 hours/10^6cells.
K_m = m/c = 0.49 ± 0.08 mmol/l

Figure 4.8b

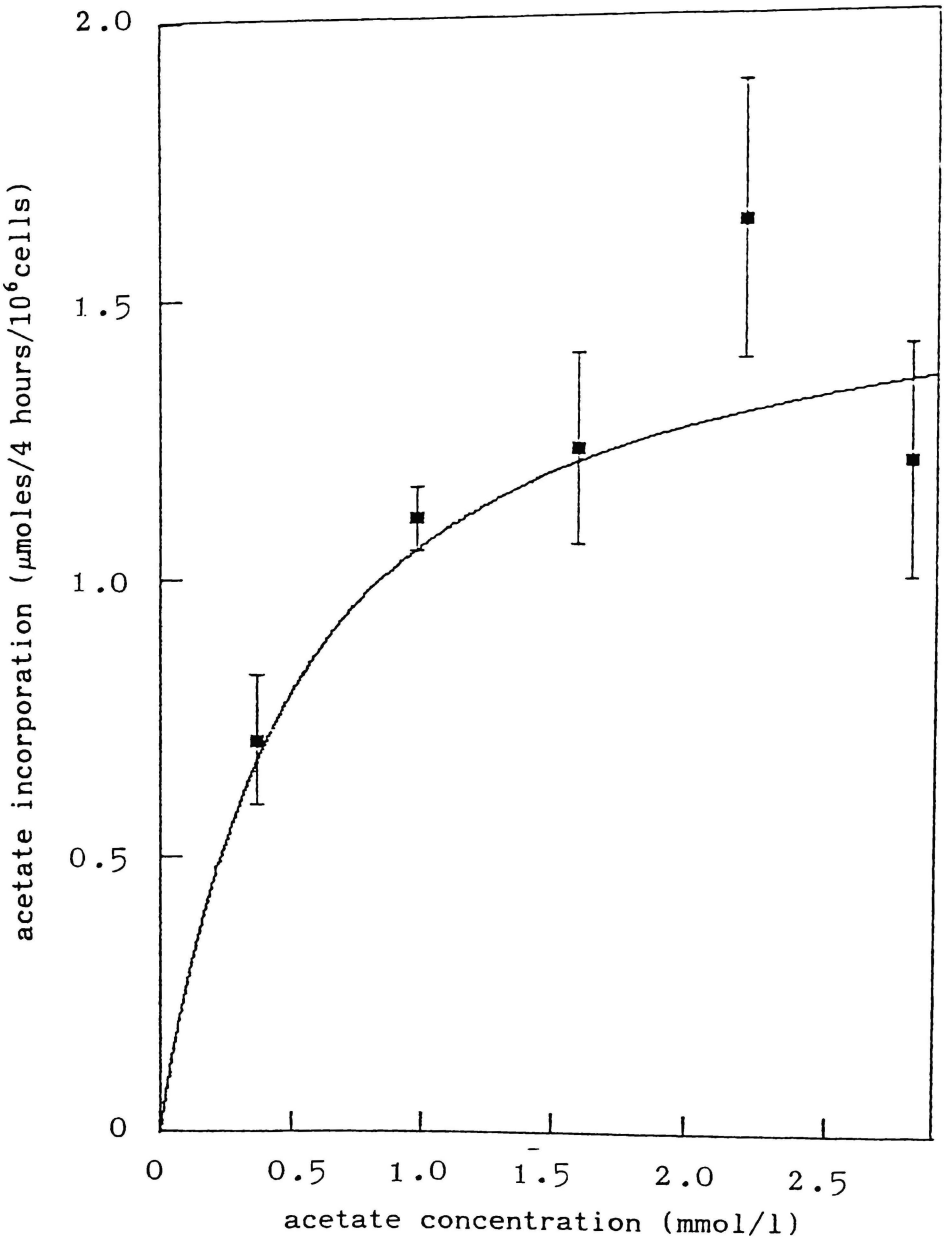

2.0
1.5
1.0
0.5
0
acetate incorporation (μmoles/4 hours/10^6cells)
0
0.5
1.0
1.5
2.0
2.5
acetate concentration (mmol/l)

Figure 4.8b The effect of acetate concentration on the basal rate of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 with no hormone or BSA present. The rate of acetate incorporation into tissue fatty acids (μmoles/4 hours/10^6cells) is plotted against acetate concentration (mmol/l).

The curve plotted on the graph has been calculated for each acetate concentration using the Michaelis-Menton equation:

$$V = \frac{V_{max}\,[S]}{[S] + K_m}$$

where V_{max} is the maximum rate of acetate incorporation into fatty acid approached as the acetate concentration tends towards infinity and the enzymes become saturated with substrate (= 1.58 ± 0.16 μmoles/4 hours/10^6cells as calculated in Figure 4.8a)

K_m is the concentration of acetate at which the rate of acetate incorporation into fatty acid is half-maximal (= 0.49 ± 0.08 mmol/l as calculated in Figure 4.8a)

[S] is acetate concentration (mmol/l)

V is rate of acetate incorporation into fatty acid (μmoles/4 hours/10^6cells)

Figure 4.9a

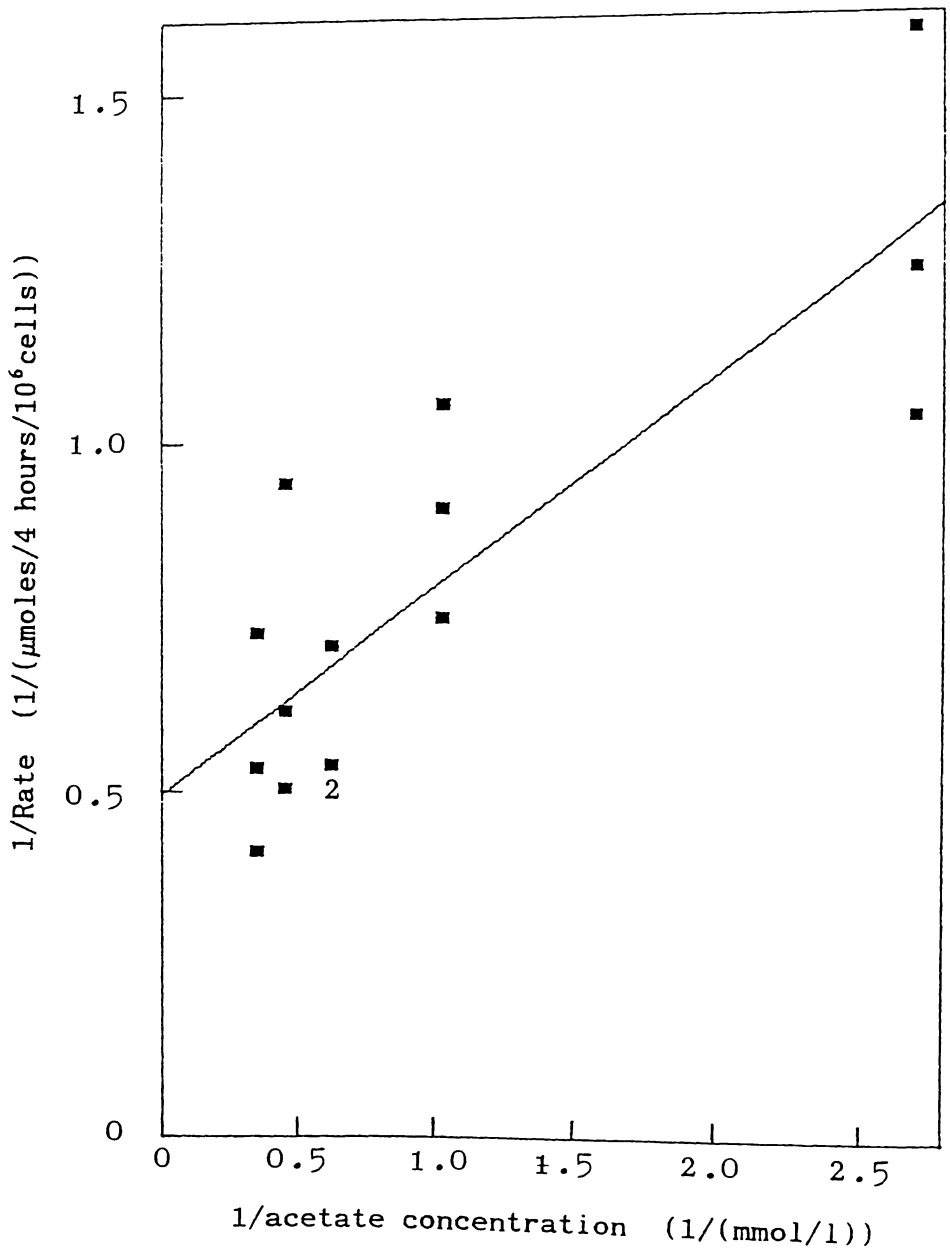

1.5
1.0
0.5
0
1/Rate (1/(μmoles/4 hours/10^6cells))
2
0
0.5
1.0
1.5
2.0
2.5
1/acetate concentration (1/(mmol/l))

Figure 4.9 The effect of acetate concentration on the rate of acetate incorporation into fatty acid in the presence of insulin *in vitro*

Figure 4.9a is a Lineweaver-Burk plot used to calculate V_{max} and K_m for the data plotted in Figure 4.9b. The tissue was from the same animal as that used for Figure 4.8. The results presented in Figure 4.8 represent the basal rate of acetate incorporation into fatty acid for the incubations carried out for Figure 4.9 where insulin was present in all incubation vessels. The incubations for both Figures 4.8 and 4.9 were carried out at the same time.

Figure 4.9a Lineweaver-Burk double reciprocal plot of the insulin-stimulated rate of acetate incorporation into fatty acid against acetate concentration

Each point represents a separate incubation of ovine adipose tissue slices in Medium 199 with no BSA present. Insulin, at a concentration of 2.6 ng/ml, was present in all incubations. The reciprocal of acetate incorporation into fatty acid after it has been stimulated by insulin (1/(μmoles/4 hours/10^6cells)) is plotted against the reciprocal of the acetate concentration (1/(mmol/l)).

There is some indication of a linear trend in the data ($r = 0.835$) and the equation for the line is $Y = 0.494 + 0.300X$ where Y = 1/(rate of acetate incorporation into fatty acid) and X = 1/acetate concentration. There is 10% error in the estimation of the X intercept (c) and 5% error in the estimation of the gradient (m).

$V_{max} = 1/c = 2.024 \pm 0.20$ μmoles/4 hours/10^6cells.

$K_m = m/c = 0.607 \pm 0.09$ mmol/l

Figure 4.9b

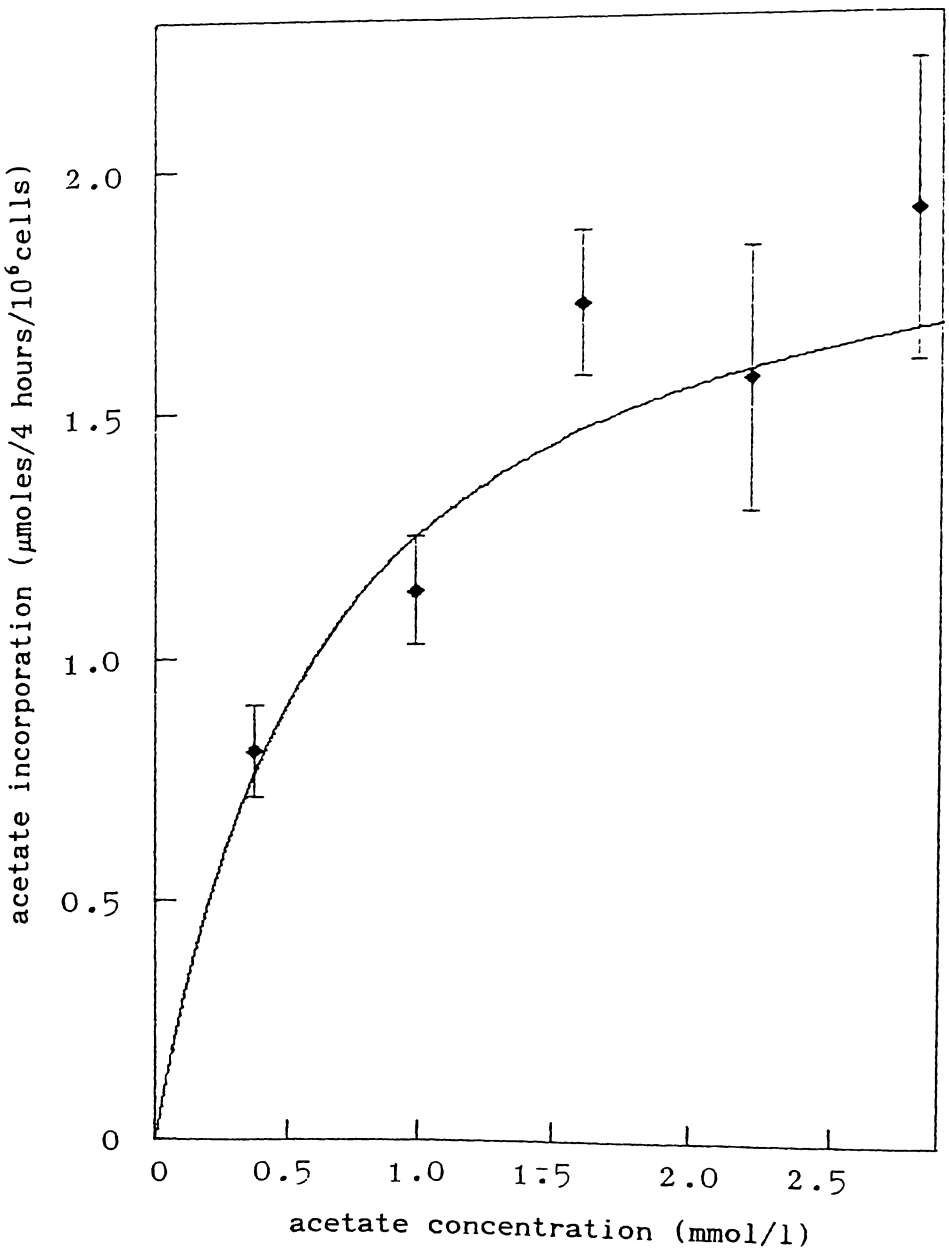

acetate incorporation (μmoles/4 hours/10^6cells)
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
acetate concentration (mmol/l)

Figure 4.9b The effect of acetate concentration on the insulin-stimulated rate of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 with no BSA present. Insulin, at a concentration of 2.6 ng/ml was present in all incubations. Acetate incorporation into fatty acid (μmoles/4 hours/10^6cells) is plotted against acetate concentration (mmol/l).

The curve plotted on the graph has been calculated for each acetate concentration using the Michaelis-Menton equation (given in Figure 4.8b).

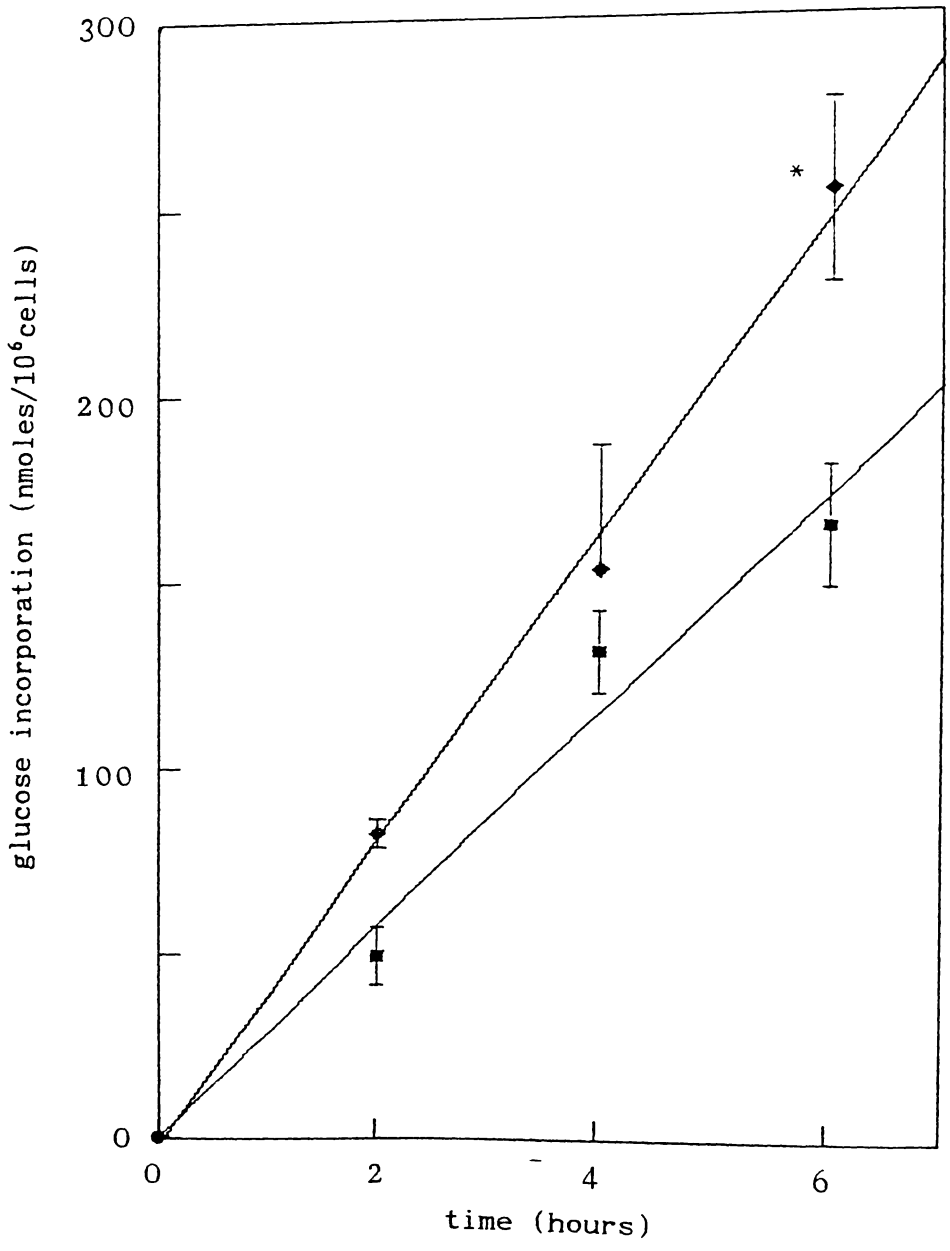
where V_{max} is 2.024 ± 0.20 μmoles/4 hours/10^6cells

and K_m is 0.607 ± 0.09 mmol/l

(as calculated in Figure 4.9a)

Since the tissue used in the experiments described in Figures 4.8 and 4.9 was all from the same animal and the experiments were carried out simultaneously, the rates of acetate incorporation can be compared. Thus the action of insulin has increased the V_{max} from 1.58 ± 0.20 to 2.024 ± 0.20 μmoles/4 hours/10^6cells (NS) and increased the K_m from 0.49 ± 0.08 to 0.607 ± 0.09 (NS).

Figure 4.10



* $p < 0.05$

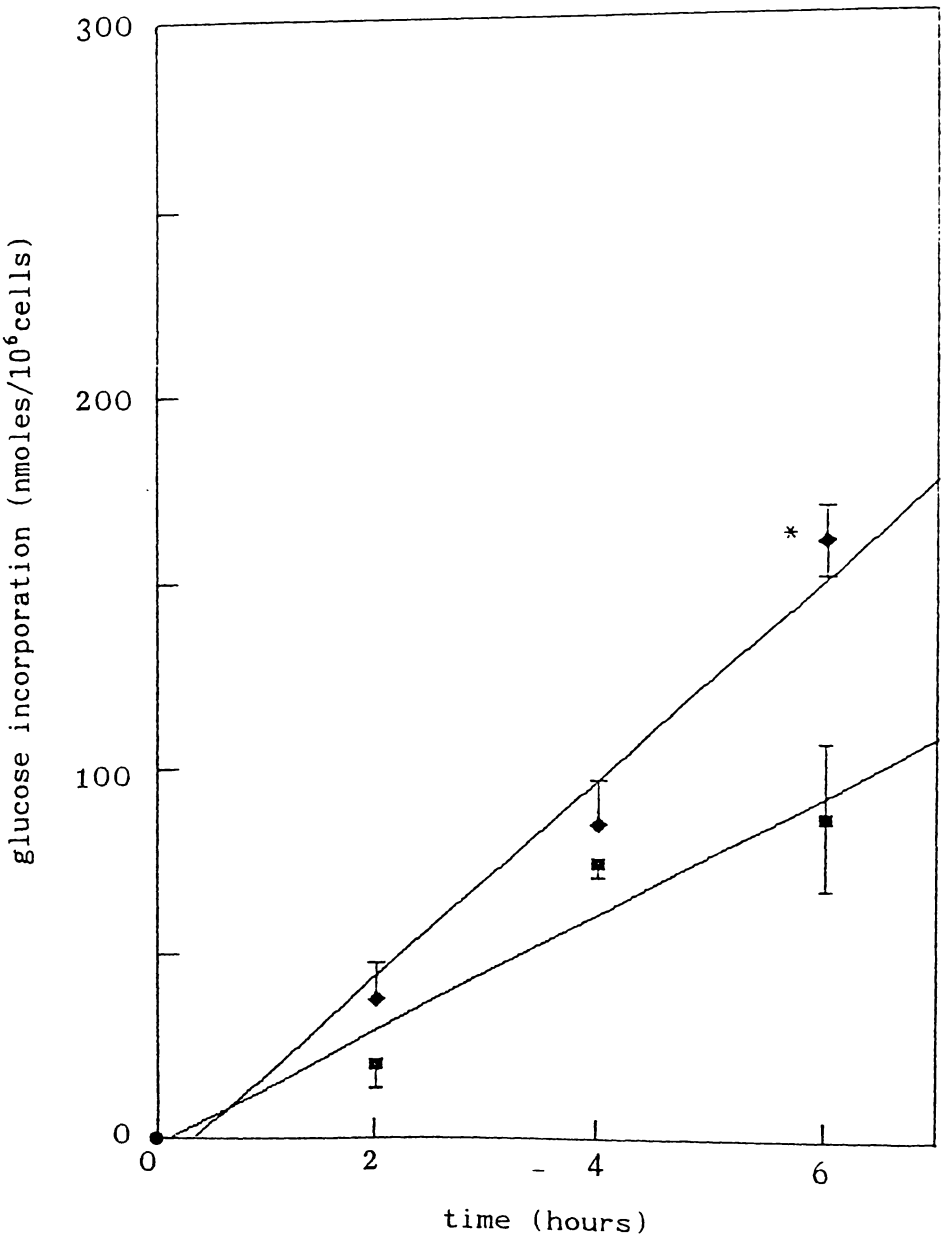
Figure 4.10 The effect of insulin on glucose incorporation into triacylglycerol *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 with no BSA present. Glucose incorporation into triacylglycerol (nmoles/10^6cells) is plotted against time (hours).

Glucose incorporation into triacylglycerol with no hormone present in the medium ■ is defined by the equation $Y = -23.9 + 28.9X$ where Y = glucose incorporation and X = time ($r = 0.9880$). Glucose incorporation into triacylglycerol in the presence of 2.6 ng/ml insulin ◆ is defined by the line $Y = -2.6 + 41.7X$ ($r = 0.9977$).

Linear regression analysis was used to estimate the overall rates of incorporation. Insulin stimulated the rate of incorporation from 28.9 ± 1.8 to 41.7 ± 1.2 nmoles/h/10^6cells, a 44% stimulation ($p < 0.05$).

Figure 4.11



* $p < 0.05$

Figure 4.11 The effect of insulin on glucose incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 4.10. Glucose incorporation into fatty acid (nmoles/10^6 cells) is plotted against time (hours).

Glucose incorporation into fatty acid when no hormone is present ■ is defined by the equation Y = -1.7 + 15.7X where Y = glucose incorporation and X = time (r = 0.9705). Glucose incorporation into fatty acid in the presence of 2.6 ng/ml insulin ◆ is defined by the line Y = -8.6 + 26.6X (r = 0.9868).

Linear regression analysis was used to estimate the overall rates of incorporation. Insulin increased the rate of glucose incorporation into fatty acid from 15.7 ± 1.6 to 26.6 ± 1.8, a 69% stimulation ($p < 0.05$).

Figure 4.12

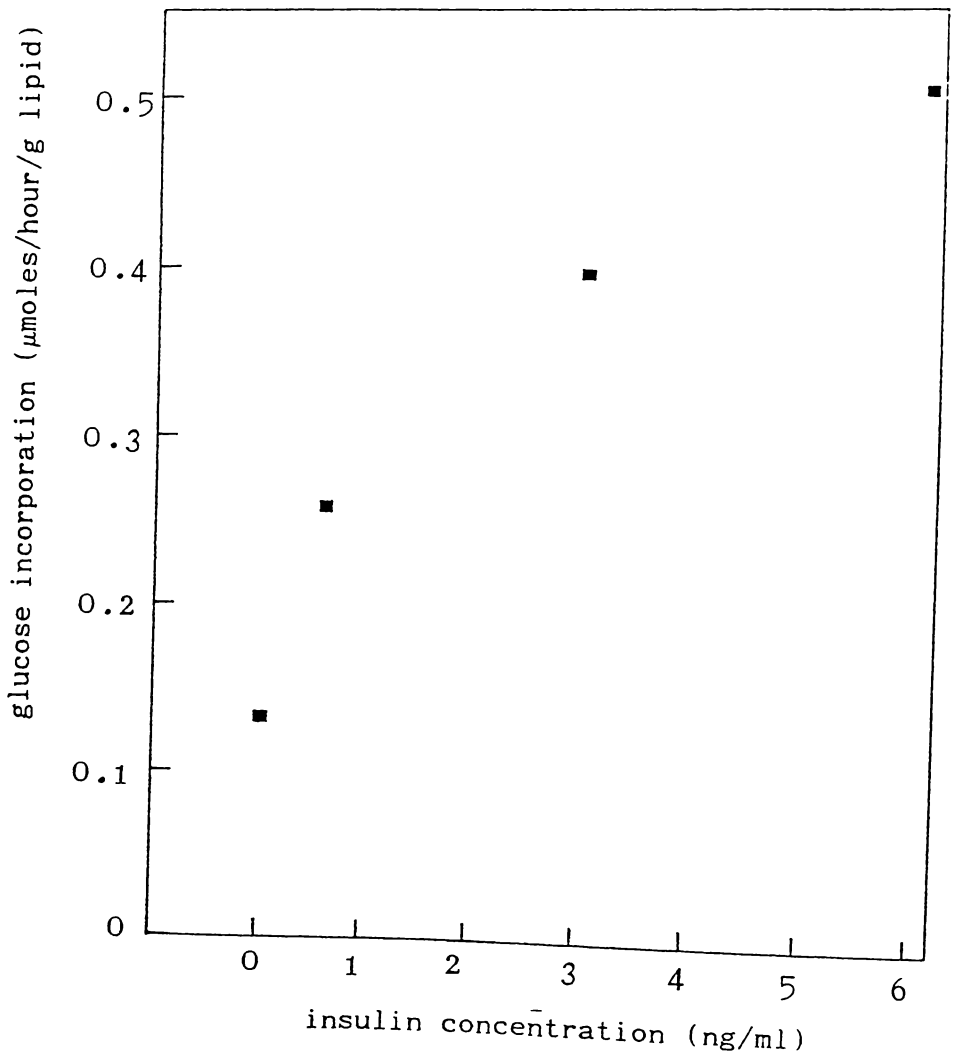

glucose incorporation (μmoles/hour/g lipid)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
insulin concentration (ng/ml)

Figure 4.12 The effect of insulin concentration on glucose incorporation into triacylglycerol in isolated ovine adipocytes *in vitro*

Each point represents the mean of duplicate incubations of ovine isolated adipocytes in modified Krebs-Ringer buffer containing 1% w/v BSA, 0.25 mmol/l glucose and no acetate. The rate of glucose incorporation into triacylglycerol (μmoles/hour/g lipid) is plotted against insulin concentration (ng/ml).

Figure 4.13

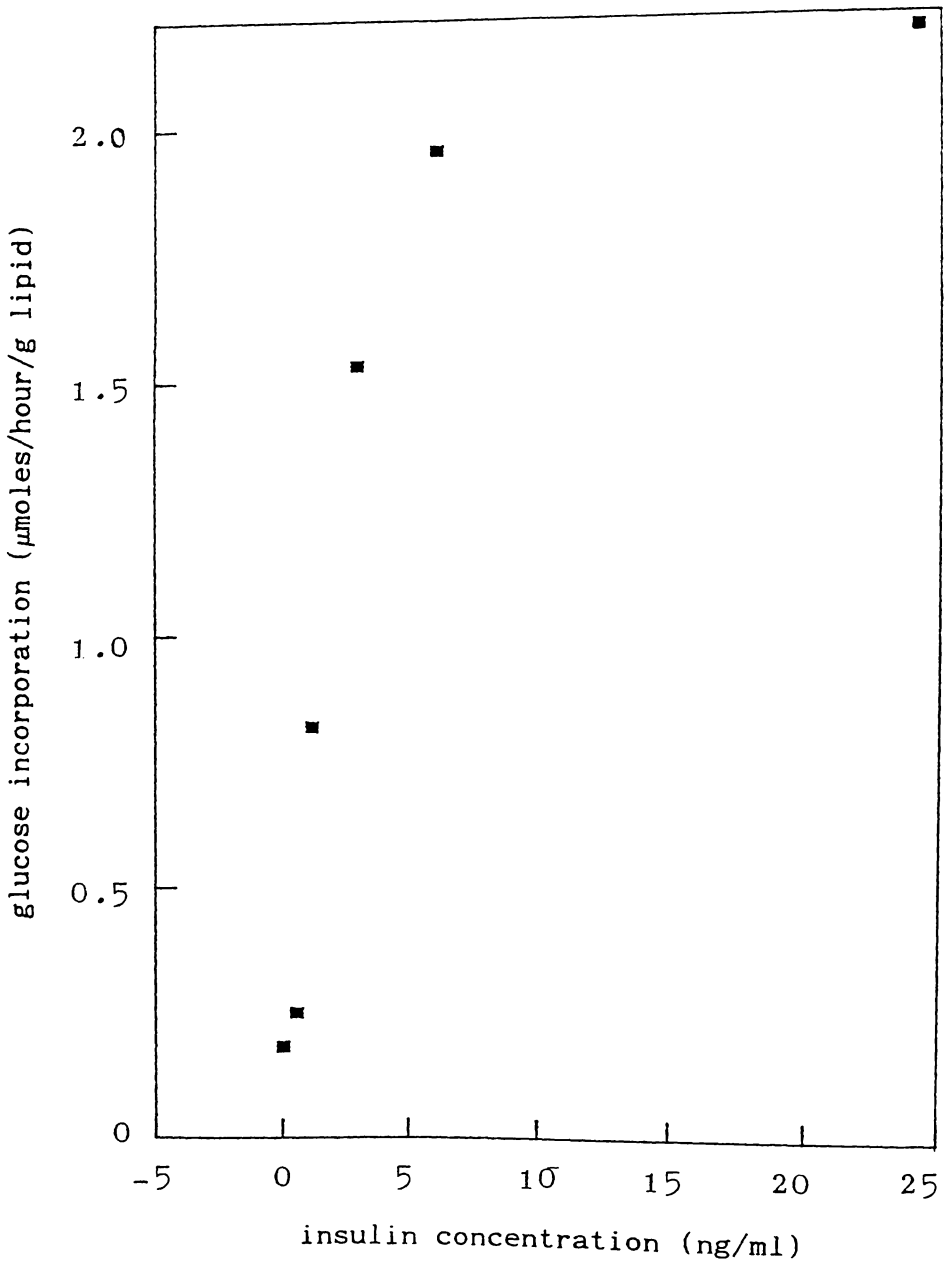
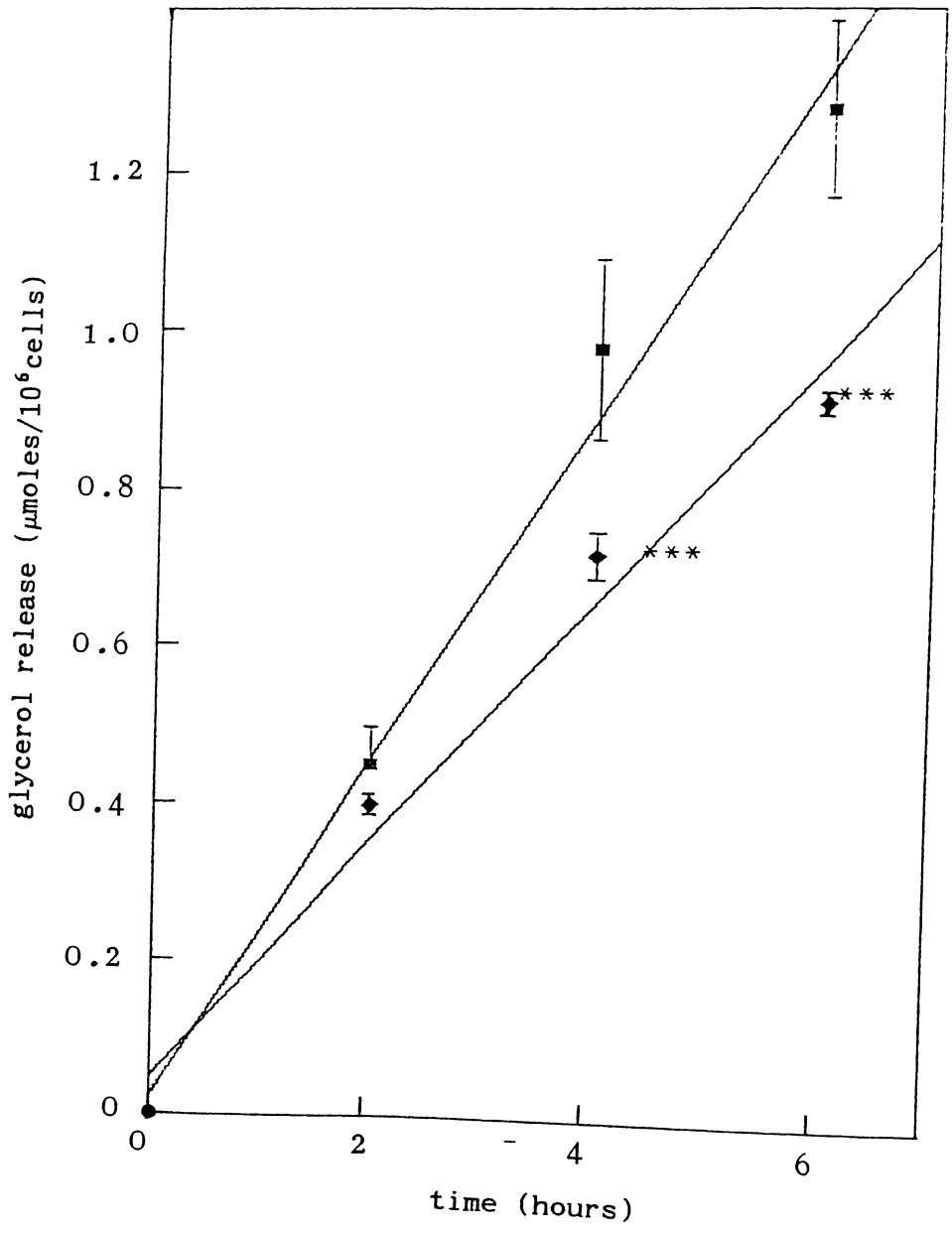

glucose incorporation (μmoles/hour/g lipid)
2.0
1.5
1.0
0.5
0
-5
0
5
10
15
20
25
insulin concentration (ng/ml)

Figure 4.13 The effect of insulin concentration on glucose incorporation into triacylglycerol in isolated rat adipocytes *in vitro*

Experimental conditions were as given in Figure 4.12. The rate of glucose incorporation into triacylglycerol (μmoles/hour/g lipid) is plotted against insulin concentration (ng/ml).

Figure 4.14



*** p < 0.001

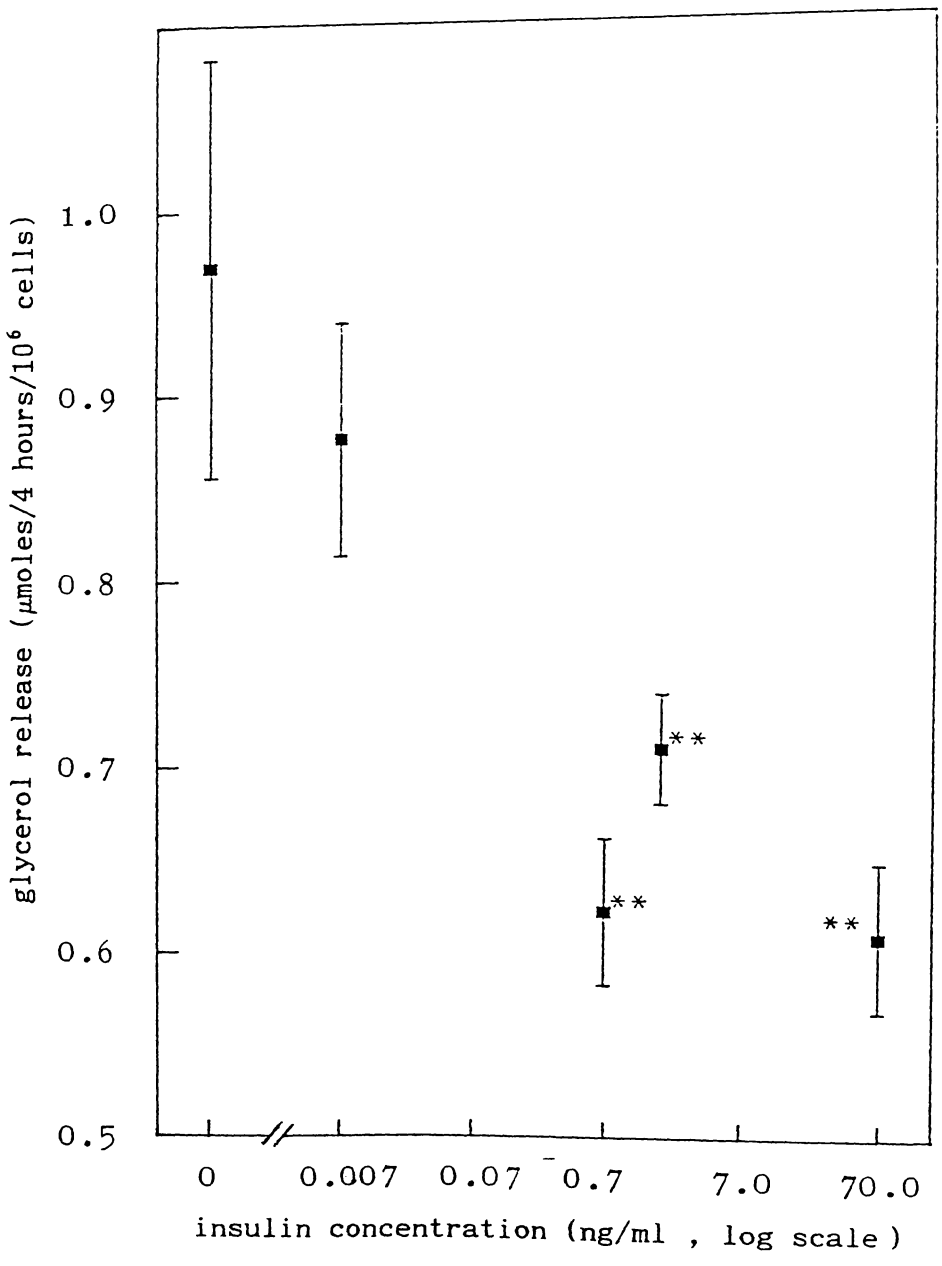
Figure 4.14 The effect of insulin on the rate of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 with 1% w/v BSA present. Glycerol release into the medium (μmoles/10^6cells) is plotted against time (hours).

Glycerol release when no hormone was present in the medium ■ and in the presence of 2.6 ng/ml insulin ◆ are both linear (r = 0.9947 and 0.9897 respectively).

Linear regression analysis was used to estimate the overall rates of incorporation. Insulin decreased the rate of glycerol release from 0.216 ± 0.009 to 0.152 ± 0.009 μmoles/hour/10^6cells, a 29% decrease ($p < 0.001$).

Figure 4.15



** p < 0.01

Figure 4.15 The effect of insulin concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 4.14. The rate of glycerol release into the incubation medium (μmoles/4 hours/10^6cells) is plotted against insulin concentration (ng/ml).

Chapter 5

Adrenalin and Nor-adrenalin

5.1 Introduction

The function of adrenalin and nor-adrenalin in both ruminants and non-ruminants is to mobilize body resources during periods of emergency and stress. The hormones must therefore be capable of acting immediately they are released and will probably only be required for a short length of time. For the adipose tissue, in the presence of these hormones, the priority becomes to release as much energy in the form of fatty acid as possible. It is also essential that the adipose tissue is prevented from removing nutrient molecules, such as glucose, from the blood to store as lipid. These nutrient molecules will be required more urgently by other tissues, for example the brain or muscle, to cope with the impending emergency.

Since the functions of these hormones are well established in the literature, at least for non-ruminants, adrenalin has been used in the present study as a standard against which the lipolytic effects of other hormones could be compared. Initially, however, experiments were carried out to confirm that adrenalin and nor-adrenalin were acting in their classical role in ovine adipose tissue slices.

5.2 The purity and stability of adrenalin preparations

Adrenalin was used at the one concentration (50 ng/ml) throughout this study. Since this was the concentration found in the blood of sheep under extreme stress (Pearson, 1979), it was assumed that this concentration would elicit a maximal response. Nor-adrenalin was used only at a concentration of 11.7 ng/ml as was found in sheep under extreme stress (Pearson, 1979).

The stock solutions of the hormones were prepared freshly from freeze-dried solid for each experiment. When the biological activity was found to be normal under the specified experimental conditions, no further checks were made on the stability or purity of the hormones.

5.3 The effects of adrenalin on the rate of lipolysis

The rate of release of glycerol into the incubation medium was used as a method to estimate the rate of lipolysis in ovine adipose tissue *in vitro*. Adrenalin stimulated the rate of glycerol release by 108% ($p < 0.05$) at 2 hours, by 192% ($p < 0.01$) at 4 hours and by 182% ($p < 0.01$) at 6 hours (Figure 5.1). In another experiment, adrenalin stimulated the rate by 248% ($p < 0.01$) at 2 hours and by 137% ($p < 0.01$) at 4 hours (Figure 5.2). Adrenalin stimulated the rate of lipolysis in all animals tested in this study ($n = 15$). The percentage stimulation varied from 23 to 300%.

The actions of adrenalin and insulin on the rate of lipolysis in this tissue were compared and the results are seen in Figure 5.3. Adrenalin increased the rate of glycerol release by 120% ($p < 0.01$) and insulin decreased the rate by 38% ($p < 0.05$).

5.4 The effects of adrenalin and nor-adrenalin on the rate of lipogenesis

As seen in Figure 5.4, adrenalin decreased the rate of acetate incorporation into fatty acid by 27% at 2 hours, by 40% at 4 hours and by 35% at 6 hours. The inhibition was significant only at 4 hours ($p < 0.05$). In another experiment, there was no change at 2 hours but adrenalin decreased the rate of lipogenesis by 17% ($p < 0.05$) at 4 hours and by 13% (NS) at 6 hours (Figure 5.5). The effects of 11.7 ng/ml nor-adrenalin on the rate of lipogenesis are shown in Figure 5.6. Nor-adrenalin had no effect on the rate of acetate incorporation into fatty acid at 2 hours but depressed the rate by 17% ($p < 0.05$) at 4 hours and by 19% ($p < 0.05$) at 6 hours. Since the data presented in Figures 5.5 and 5.6 were from a single experiment with tissue from one animal, they can be directly compared. Thus adrenalin and nor-adrenalin both depressed the rate of lipogenesis by 17 - 19% in tissue from this animal.

A comparison of the effects of adrenalin and insulin on the rate of lipogenesis was made (Figure 5.7). After 2 hours of incubation, insulin increased the rate of acetate incorporation into fatty acid by 220% ($p < 0.01$) and adrenalin by 112% ($p < 0.01$). However after 4 hours, although there was no longer a significant response to insulin, adrenalin decreased the rate by 38% ($p < 0.05$).

5.5 The effects of adrenalin on the mass balance of fatty acid

The effects of adrenalin on the mass balance of fatty acid in ovine adipose tissue from Sheep A after 6 hours incubation *in vitro* are shown in Table 5.1. Although the effect of adrenalin on the rate of fatty acid manufacture was not statistically significant, adrenalin increased the rate of fatty acid release within the cell (lipolysis) by 183% ($p < 0.01$). The rate of release of free fatty acid into the incubation medium increased by 573% ($p < 0.01$) and the rate of fatty acid re-esterification increased by 142% ($p < 0.01$). However, the rate of fatty acid accumulation within the tissue decreased by 38% ($p < 0.01$).

The effects of adrenalin on the mass balance of fatty acid in adipose tissue from Sheep B after 4 hours incubation are shown in Table 5.2. Adrenalin's effect on the rate of fatty acid manufacture was also not statistically significant but adrenalin increased the rate of fatty acid release within the cell by 23% ($p < 0.001$), the rate of release of free fatty acid into the medium by 38% ($p < 0.05$) and the rate of fatty acid re-esterification by 21% ($p < 0.01$). On the other hand, adrenalin had no significant effect on the rate of fatty acid accumulation within the tissue.

In tissue from sheep A, the percentage of fatty acid released by lipolysis which was subsequently recycled decreased from 91% in the tissue with no hormone in the medium to 77% in the adrenalin-stimulated tissue. In tissue from sheep B, the percentage of fatty acid recycling without hormone in the medium and in the presence of adrenalin remained largely unchanged at 90 - 91%.

5.6 The effects of adrenalin on insulin stimulation of lipogenesis

As seen in Figure 5.8, insulin at a concentration of 2.6 ng/ml increased the rate of H atom incorporation into fatty acid by a maximum of 171% ($p < 0.001$). Adrenalin, present at a concentration of 50 ng/ml, decreased the rate by a maximum of 35% ($p < 0.05$). However, when insulin (at 2.6 ng/ml) and adrenalin (at 50 ng/ml) were present in the medium together, the rate of H atom incorporation was stimulated by 129% ($p < 0.01$). The difference between the response to insulin alone and that when adrenalin was present as well was not significant.

For a discussion of the results presented in this chapter, refer to Section 10.3

Table 5.1

	μmoles/6 hours/10^6cells			
	basal rate	rate with adrenalin	change in rate (% change)	p
FA manufactured	2.25 ± 0.09	2.01 ± 0.26	-0.24 (-11%)	NS
FA released	1.14 ± 0.14	3.23 ± 0.78	+2.09 (+183%)	**
FFA leaving cell	0.11 ± 0.03	0.74 ± 0.30	+0.63 (+573%)	**
FA re-esterified	1.03 ± 0.11	2.49 ± 0.49	+1.46 (+142%)	**
FA accumulation	2.14 ± 0.11	1.27 ± 0.33	-0.87 (-41%)	**
% FA recycling	90%	77%		

** $p < 0.01$

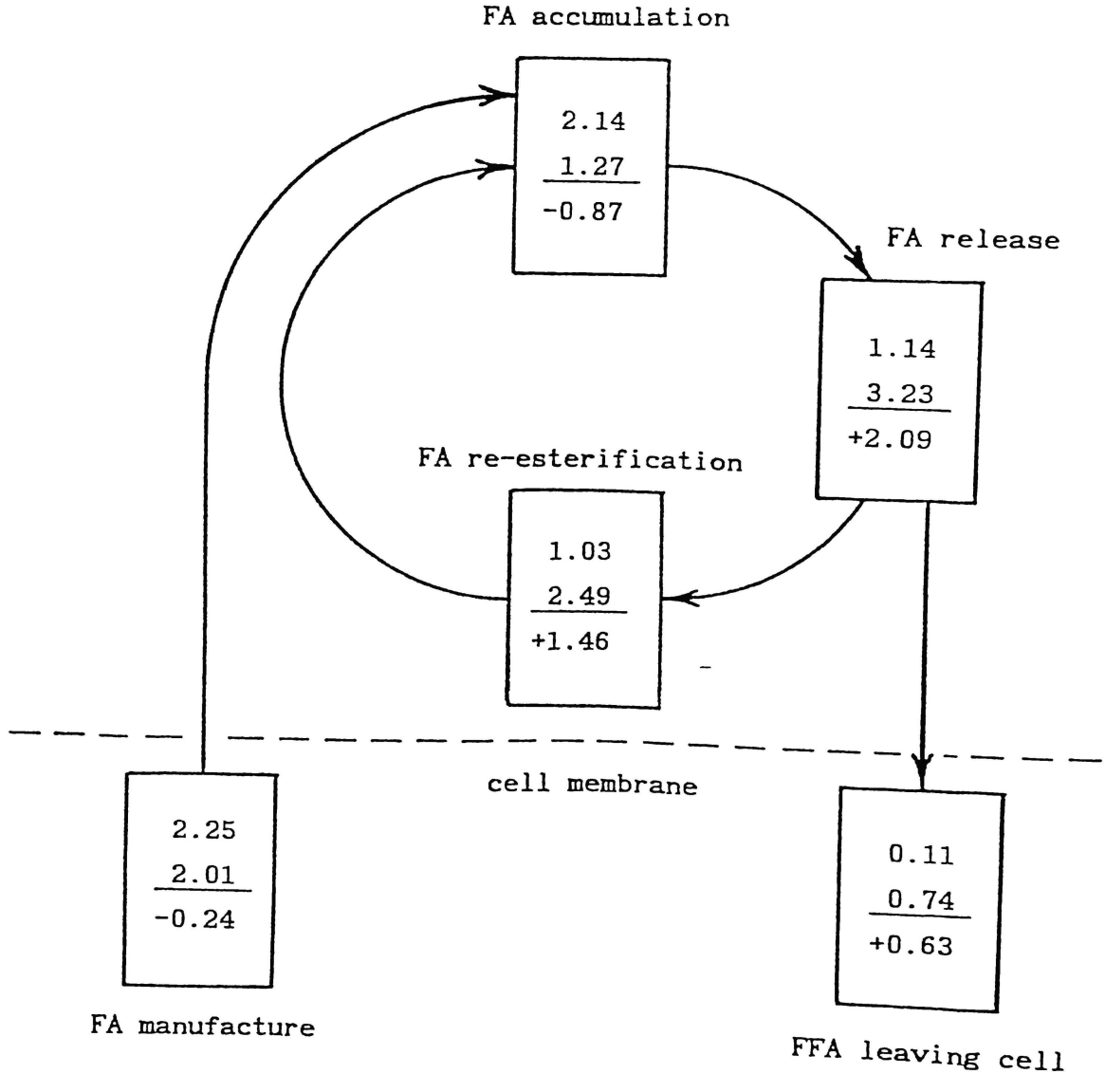


Table 5.1 The effect of adrenalin on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Sheep A

Each rate represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with adrenalin) of ovine adipose tissue slices in Medium 199 with 1% w/v BSA. Adrenalin was present where specified at a concentration of 50 ng/ml.

"FA manufactured" is measured by acetate incorporation into tissue fatty acid.

"FA released" is the fatty acid released by lipolysis within the tissue and was measured by glycerol release into the incubation medium (x3).

"FFA leaving cell" is the amount of fatty acid released into the incubation medium.

"FA re-esterified" is the amount of fatty acid that remained in the tissue after lipolysis and was presumably re-esterified.

"FA accumulation" is the amount of fatty acid that accumulated in the tissue during the time of incubation (6 hours).

"% FA recycling" is the percentage of the fatty acid released which was subsequently re-esterified.

For a description of the method of calculation of each variable, refer to Section 3.16.

The diagram, which can be compared with Figure 3.8, is a summary of the data presented in the Table and represents the mass balance of the fatty acid in the cell and incubation medium. The boxes at each stage in the cycle represent the pools of fatty acid present. The first number in each box is the basal rate (in μmoles fatty acid/6 hours/10^6 cells), the next the rate with adrenalin and the third is the change in the rate which occurred in the presence of adrenalin.

Table 5.2

	μmoles/4 hours/10^6cells			
	basal rate	rate with adrenalin	change in rate (% change)	p
FA manufactured	0.62 ± 0.06	0.68 ± 0.17	+0.06 (+10%)	NS
FA released	1.50 ± 0.04	1.84 ± 0.03	+0.34 (+23%)	***
FFA leaving cell	0.13 ± 0.01	0.18 ± 0.02	+0.05 (+38%)	*
FA re-esterified	1.37 ± 0.04	1.66 ± 0.03	+0.29 (+21%)	**
FA accumulation	0.49 ± 0.06	0.50 ± 0.10	+0.01 (0)	NS
% FA recycling	91%	90%		

*** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$

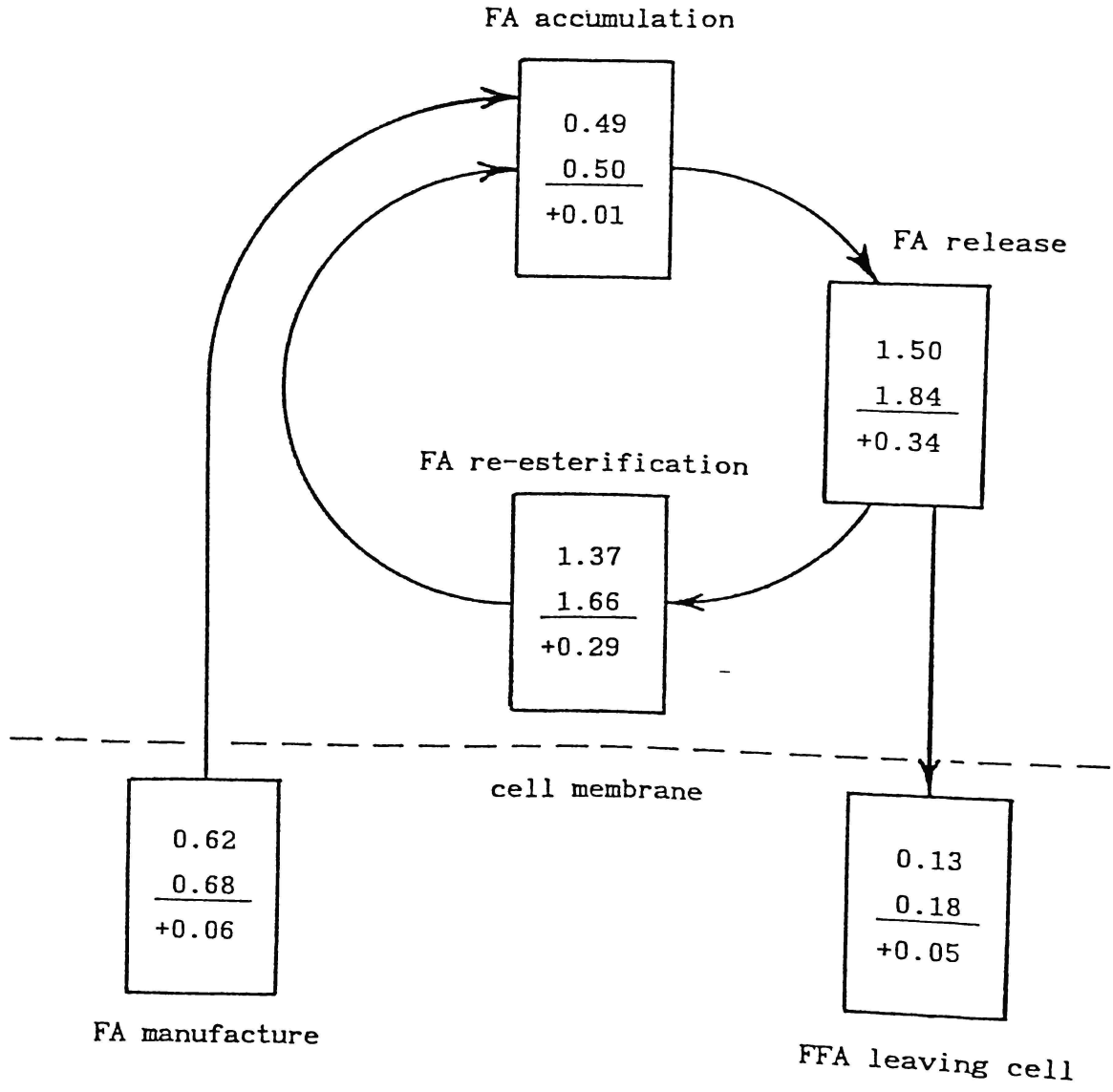
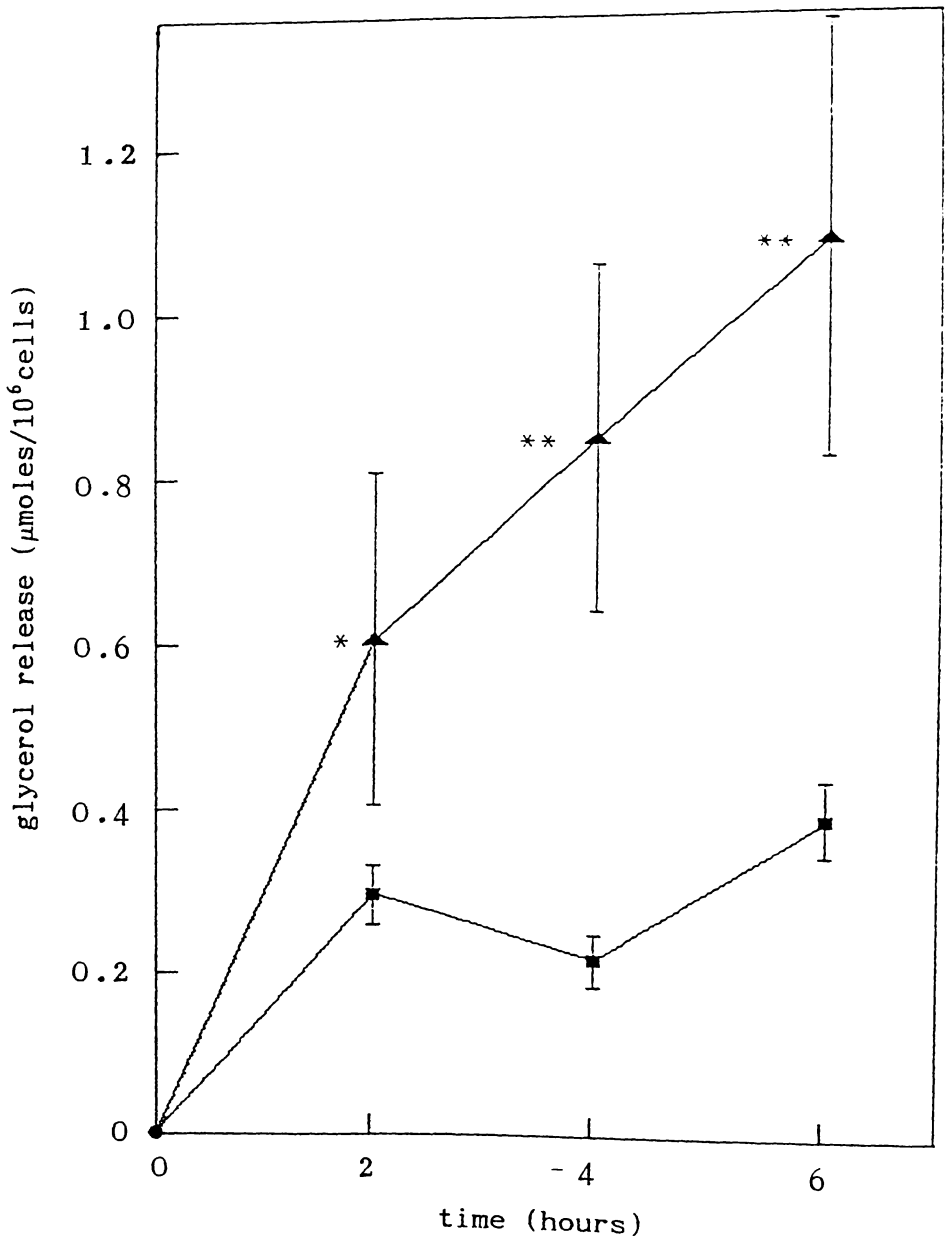


Table 5.2 The effect of adrenalin on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Sheep B

Experimental conditions and the definition of terms used are the same as those given in Table 5.1 except that the incubation time was 4 hours.

Figure 5.1



* $p < 0.05$

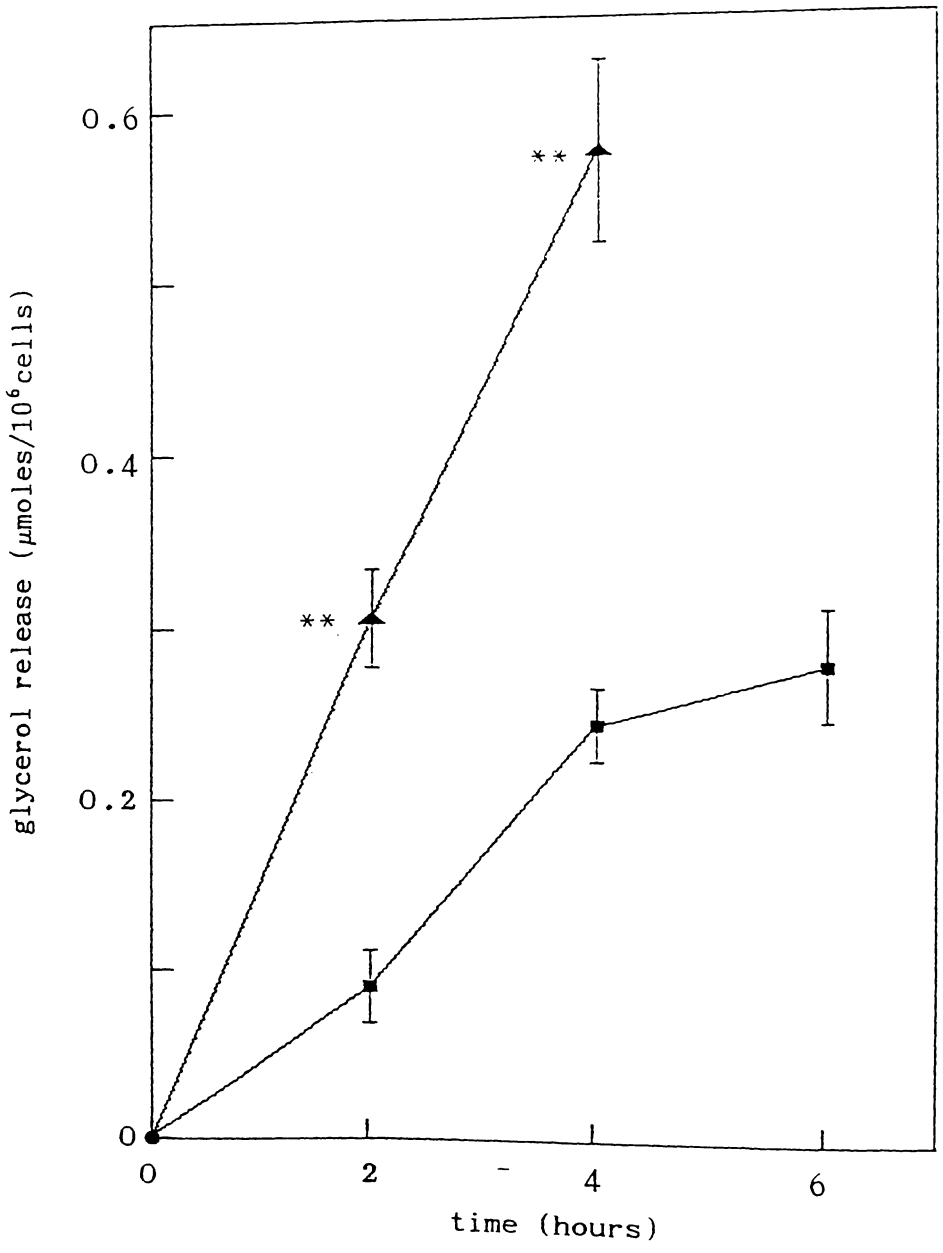
** $p < 0.01$

Figure 5.1 The effect of adrenalin on the rate of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 for adrenalin) of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. Glycerol release (μmoles/10^6cells) is plotted against time (hours).

Glycerol release with no hormone in the medium ■ and in the presence of 50 ng/ml adrenalin ▲ are shown.

Figure 5.2



** $p < 0.01$

Figure 5.2 The effect of adrenalin on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 5.1. Glycerol release (μmoles/10^6cells) is plotted against time (hours).

Glycerol release with no hormone in the medium ■ and in the presence of 50 ng/ml adrenalin ▲ are shown.

Figure 5.3

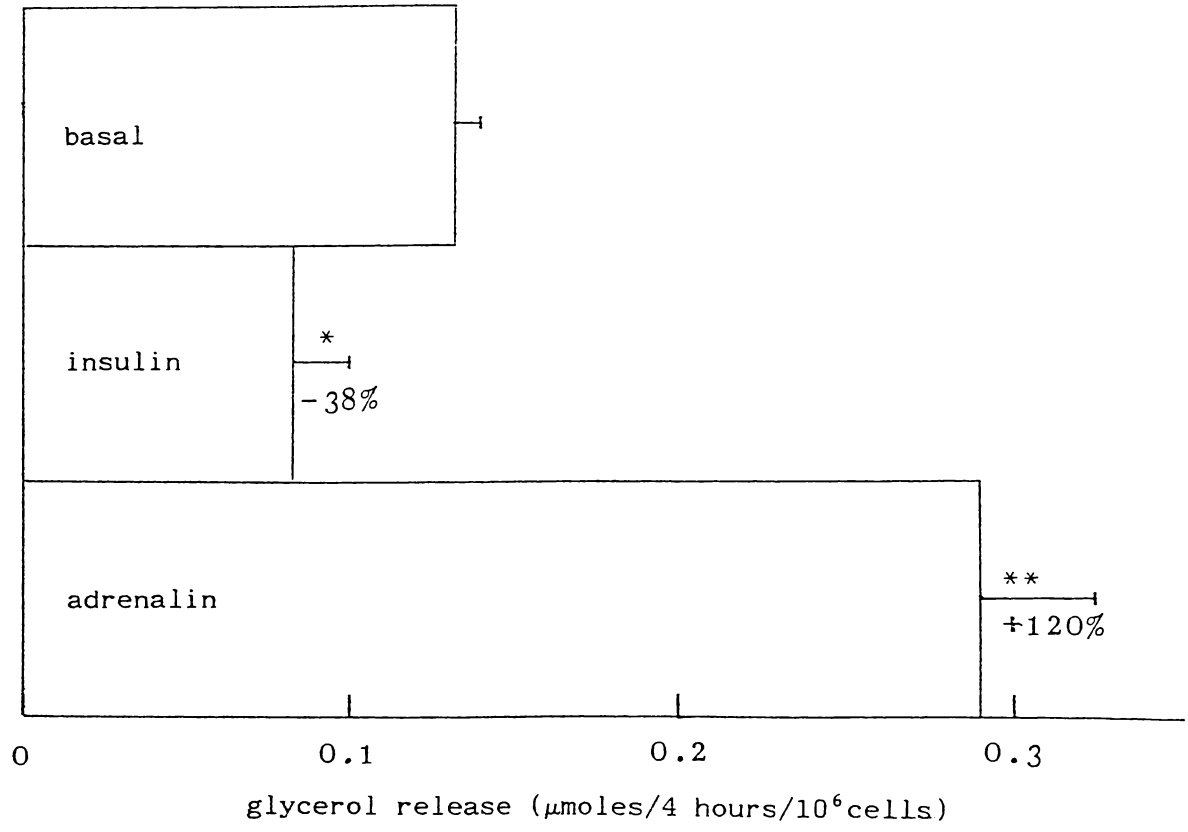
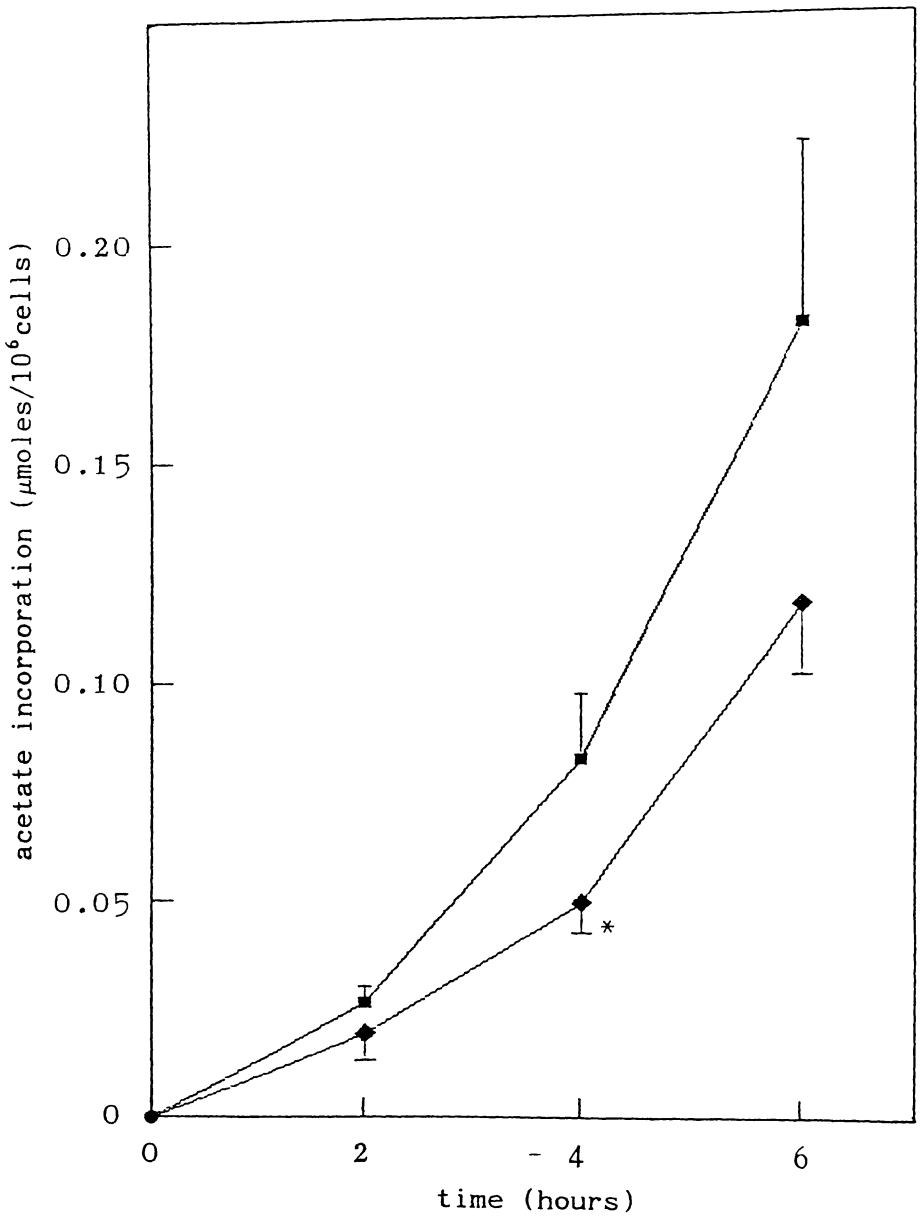

basal
insulin
*
-38%
adrenalin
**
+120%
0
0.1
0.2
0.3
glycerol release (μmoles/4 hours/10⁶cells)

Figure 5.3 A comparison of the effects of adrenalin and insulin on the rate of glycerol release from ovine adipose tissue *in vitro*

Each bar represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices *in vitro* in Medium 199 containing 1% w/v BSA. Insulin was present in the medium, where specified, at a concentration of 2.6 ng/ml and adrenalin at 50 ng/ml. The rate of glycerol release is given in units of μmoles/4 hours/10^6cells.

Figure 5.4



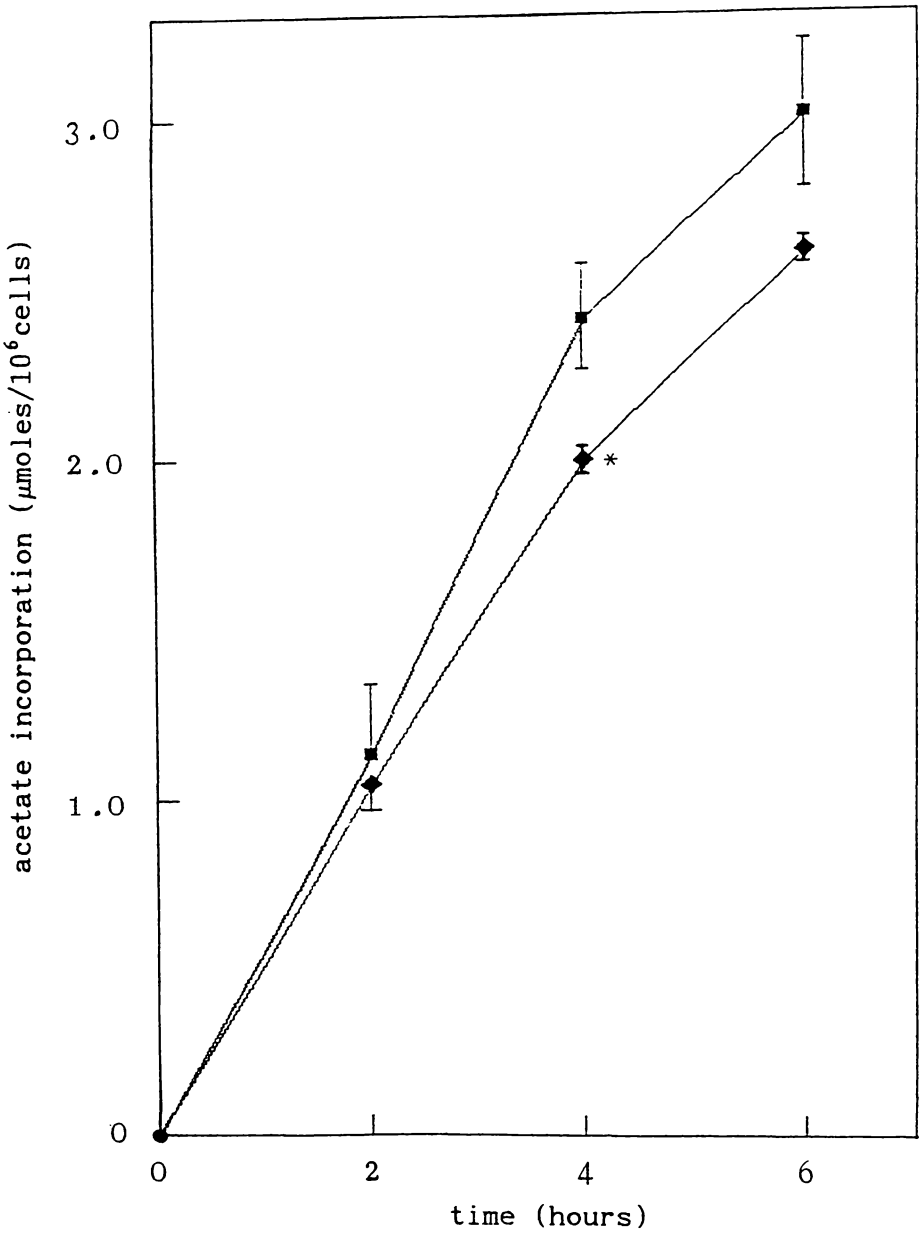
* $p < 0.05$

Figure 5.4 The effect of adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against time (hours).

Acetate incorporation with no hormone in the medium ■ and in the presence of 50 ng/ml adrenalin ◆ are shown.

Figure 5.5



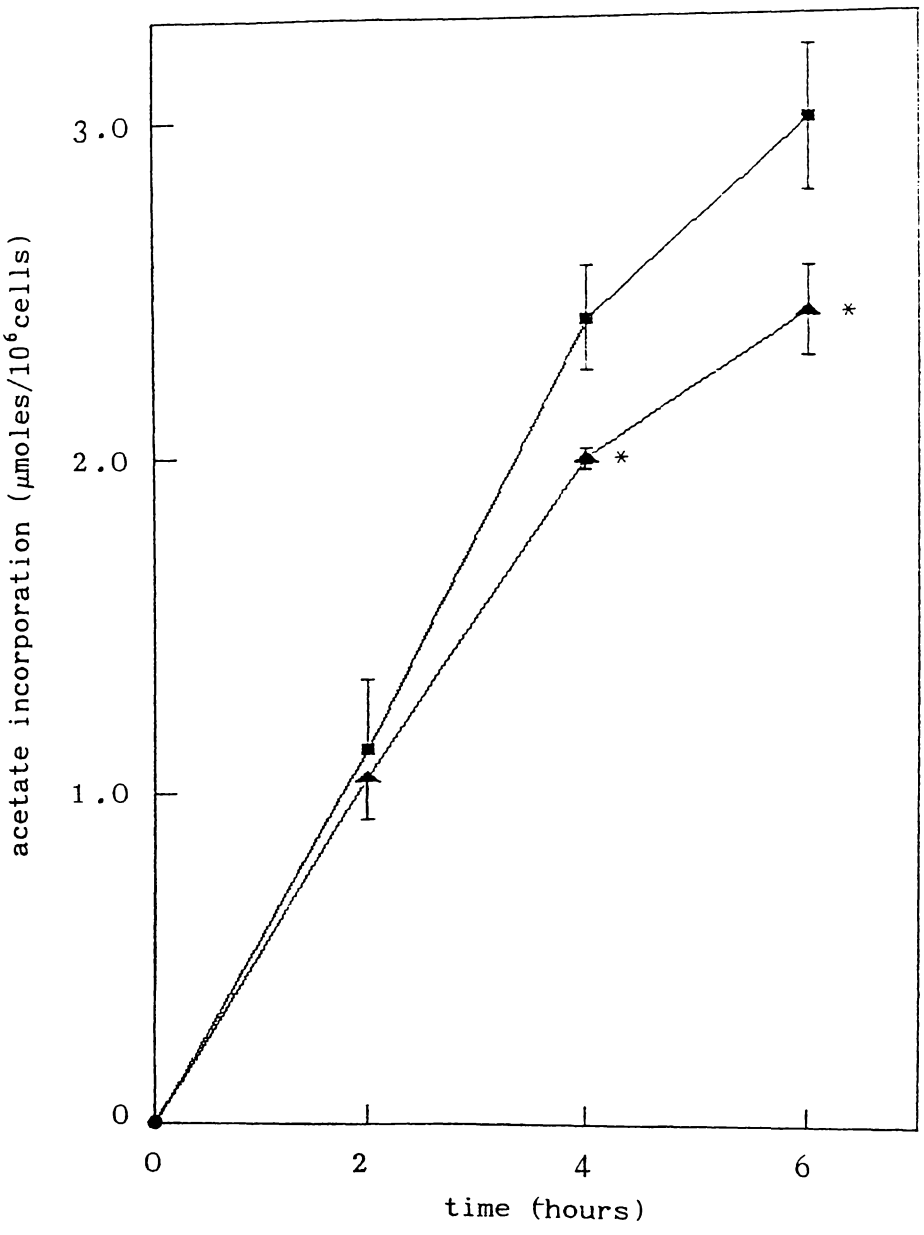
* $p < 0.05$

Figure 5.5 The effect of adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 5.4. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against time (hours).

Acetate incorporation with no hormone in the medium ■ and in the presence of 50 ng/ml adrenalin ◆ are shown.

Figure 5.6



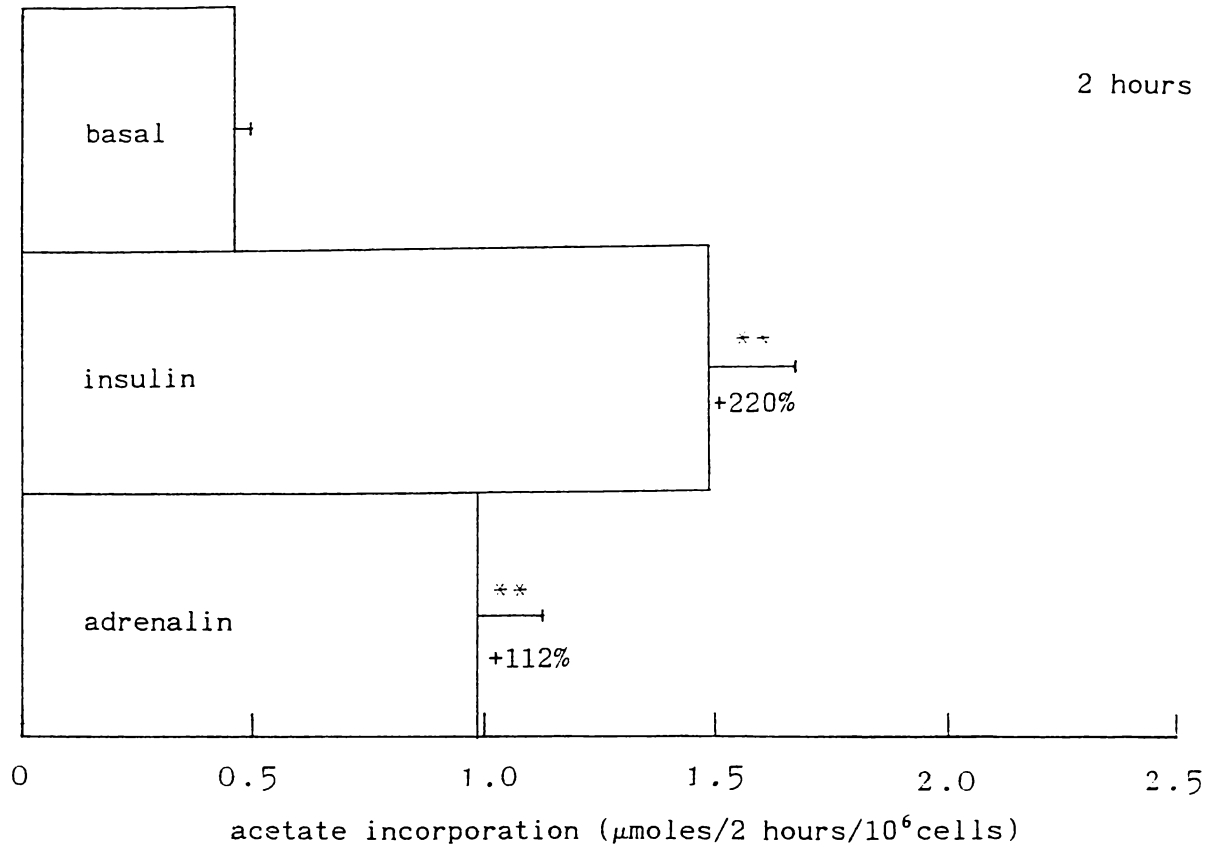
* $p < 0.05$

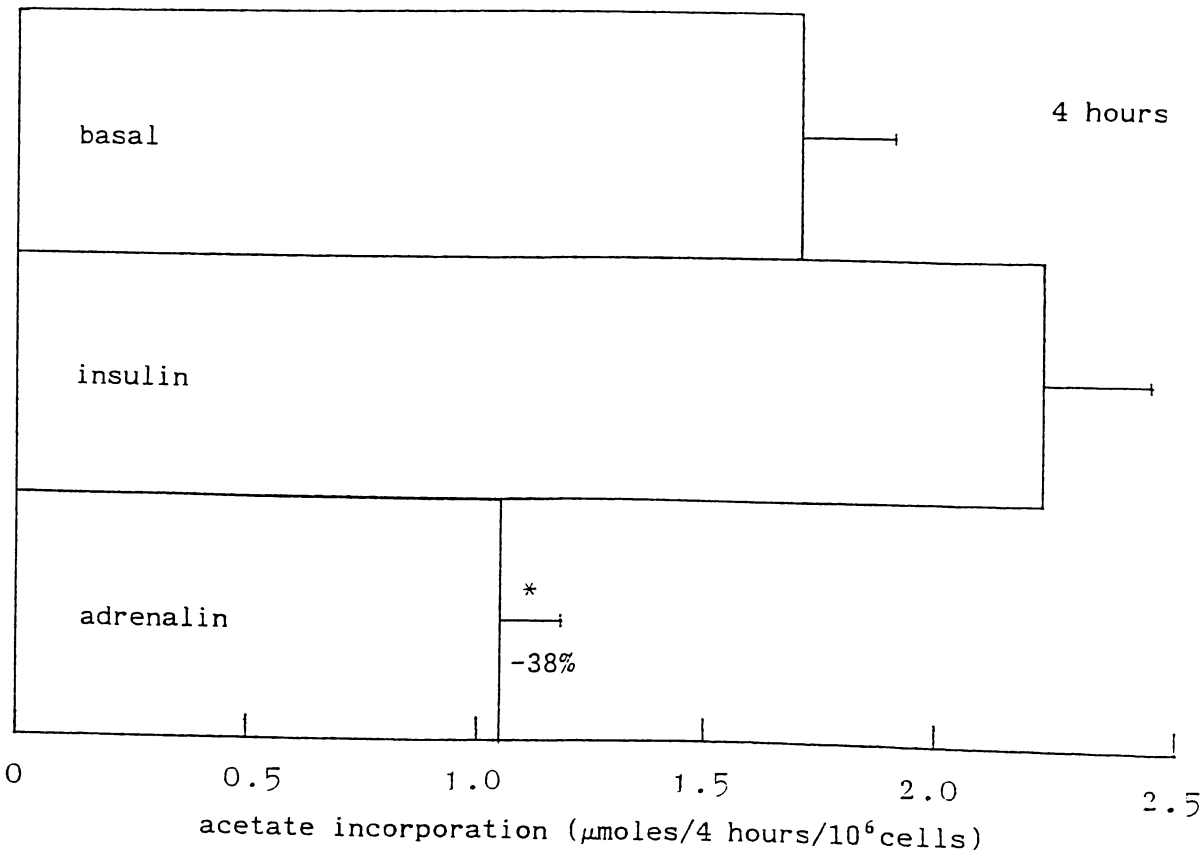
Figure 5.6 The effect of nor-adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 5.4. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against time (hours).

Acetate incorporation with no hormone in the medium ■ and in the presence of 11.7 ng/ml nor-adrenalin ▲ are shown.

Figure 5.7





* $p < 0.05$

** $p < 0.01$

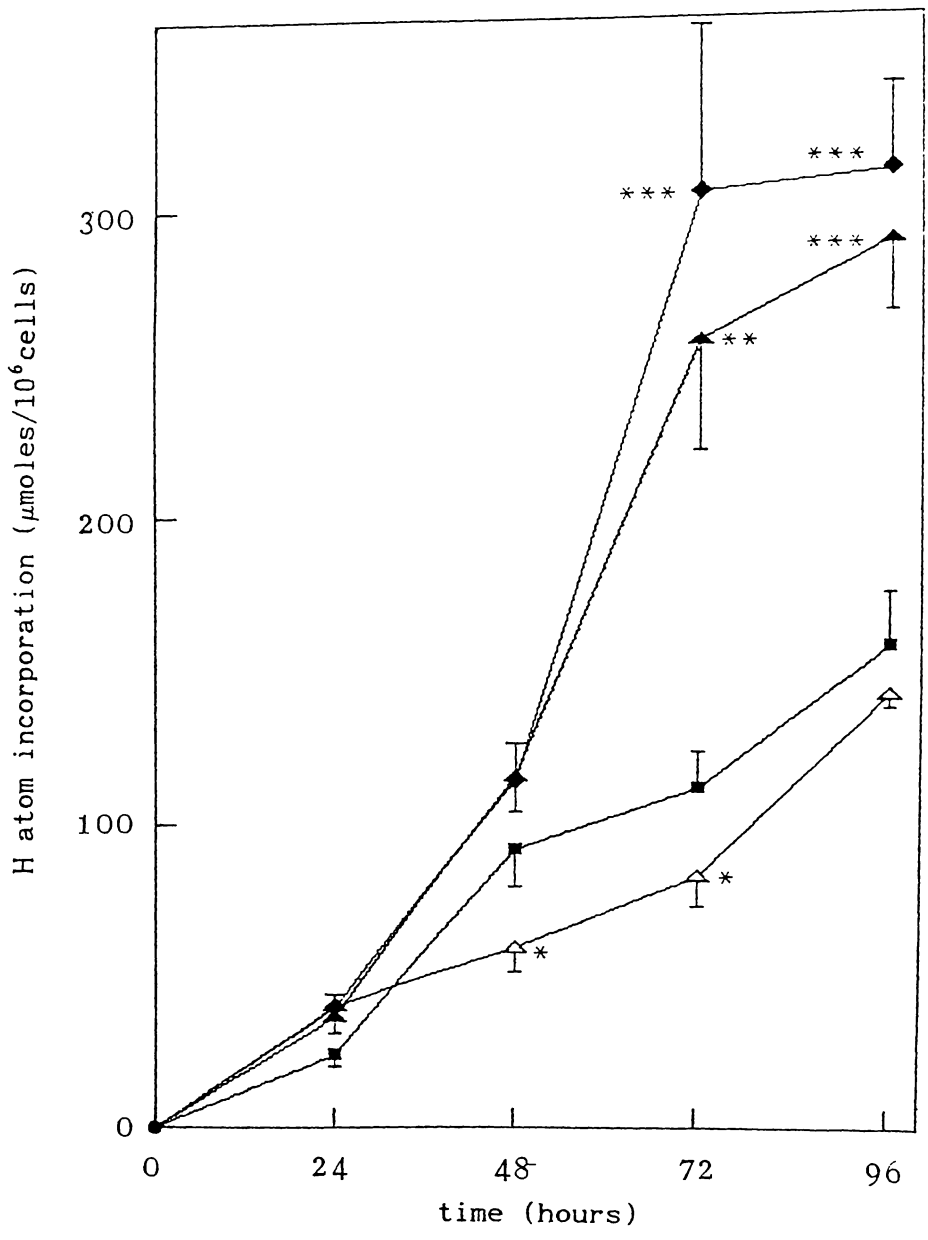
*** $p < 0.001$

Figure 5.7 A comparison of the effects of adrenalin and insulin on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given in Figure 5.3. The rate of acetate incorporation into fatty acid is given in units of μmoles/2 hours/10^6cells in the first histogram and μmoles/4 hours/10^6cells in the second.

The incubations for both histograms were carried out using tissue from one animal.

Figure 5.8



* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Figure 5.8 The effects of insulin and adrenalin on the rate of H atom incorporation into fatty acid *in vitro*

Each bar represents the mean and S.E.M. of six replicate incubations of ovine adipose tissue slices *in vitro* in Medium 199 with no BSA present. H atom incorporation into fatty acid (μmoles/10^6cells) is plotted against time (hours).

H atom incorporation with no hormone in the medium ■ , in the presence of 2.6 ng/ml insulin ◆ , 50 ng/ml adrenalin △ and when insulin (at 2.6 ng/ml) and adrenalin (at 50 ng/ml) were present in the medium together ▲ are shown.

Chapter 6

Growth Hormone

6.1 Introduction

Although the administration of GH to sheep *in vivo* has been shown to reduce the proportion of fat in the carcass (Muir *et al.*, 1983; Johnsson and Hart, 1985; Bass *et al.*, unpublished data, see Chapter 9), a lipolytic response to the hormone in ruminant adipose tissue has not been demonstrated. Using a GH concentration of 1 - 25 μg/ml, Duquette *et al.* (1984) found that there was no lipolytic response to the hormone in ovine adipose tissue slices or isolated adipocytes. However, the concentration of GH used in their study was 10 to 250-fold greater than the highest observed *in vivo*, even following administration of exogenous GH (Muir *et al.*, 1983). It is thought that GH itself may have a regulatory role in the maintenance of GH receptors. The number of GH receptors in cultured human lymphocytes showed a marked decrease in the hours following exposure to hGH (Rosenfeld and Hintz, 1980; Lesniak and Roth, 1976). GH also appeared to down-regulate its own receptors following an initial response in rat diaphragm muscle (Albertsson-Wikland *et al.*, 1980). Since there is therefore a possibility that the high concentrations of hormone used by Duquette *et al.* could have reduced the numbers of GH receptors in the adipose tissue or adipocytes, it was felt that the experiments warranted repeating with more physiologically normal concentrations of GH.

Fain *et al.* (1965) and Goodman (1968) demonstrated that the lipolytic action of GH is enhanced by the action of dexamethasone (glucocorticoid) in rat adipose tissue and isolated adipocytes *in vitro*. However, this question has not been addressed in the present study and dexamethasone was not included in any of the incubations.

Eden *et al.* (1982) reported that a pre-incubation of rat adipocytes in medium without hormone enhanced the ability of the cells to bind and respond to GH. It is not known whether the same refractoriness occurs in ovine adipose tissue or whether it would have any effect on the lipolytic response in the tissue. However, since there was no indication that

Duquette *et al.* (1984) used a pre-incubation period, it is possible that the phenomenon described by Eden *et al.* may also have contributed towards the lack of lipolytic activity observed in this tissue. The experiments were therefore repeated using a pre-incubation period as well.

6.2 The purity and stability of GH preparations.

Radioimmunoassay

Experiments were conducted to test the stability of GH throughout the incubation period.

Method

The method used to test the effects of tissue slices on the stability of GH was similar to that outlined in Section 4.2 for insulin. In this case, samples of medium containing the GH were incubated with ovine adipose tissue slices with no protein in the medium, with 1% w/v BSA and with 1% w/v Polypep^R (a protein digest from Sigma). The GH used was pituitary-extracted bGH purified by Dr I. Hart (ICH/bGH/13); this preparation will be referred to throughout the present study as purified bGH. For this experiment, the concentration of bGH used in each incubation vessel was initially 100 ng/ml, as determined by weight. Samples of medium were removed from the vessel following 4 and 6 hours incubation and the immuno-reactive concentration measured using radioimmunoassay.

Results

The results of the radioimmunoassays, carried out by Ms K. Crossley, are given in Table 6.1.

There was no observed loss of immuno-reactive hormone after 6 hours incubation with tissue slices when BSA was present in the medium. However, there was a loss of approximately half the bGH when the medium contained no protein or when it contained Polypep^R. In the presence of BSA, the initial immuno-reactive concentration of bGH was considerably higher than expected.

Table 6.1 A comparison of the effects of BSA and Polypep^R on GH stability in the presence of ovine adipose tissue slices *in vitro*

Samples of purified bGH at a concentration of 100 ng/ml by weight were made up in Medium 199 with 1% w/v BSA or Polypep^R present where specified. Ovine adipose tissue slices were added to the medium and incubated at 39°C for 6 hours. Samples of medium were removed after 4 and 6 hours of incubation and analysed for GH by radioimmunoassay. The results are expressed in terms of oGH. The percentage loss of GH is compared with the immuno-reactive concentration at the start of the experiment.

Protein in medium	GH by RIA (ng/ml oGH)	% loss of GH	
		4 hours	6 hours
0	63.8	58%	64%
1% BSA	144.4	<1%	<1%
1% Polypep^R	68.0	42%	60%

Electrophoresis

Electrophoresis on polyacrylamide gel was used to investigate the purity of the hormones and to ensure that only those preparations of the highest quality were included in the subsequent *in vitro* analyses of biological activity.

Using isoelectric focusing on polyacrylamide gel, with a pH range of 3.5 to 9.5, the pI values of a number of different GH preparations were investigated and the results are illustrated in Plate 6.1. The three hormones of interest in the present study appeared in tracks 2, 3 and 4 and were compared with two preparations of hGH (tracks 1 and 5) obtained from different sources and not used elsewhere in this study. The purified bGH had 13 bands visible in the range 5.18 to 8.20, with the pI values of the three major bands being 7.60, 7.88 and 8.20. The purified oGH (track 4) showed two major bands with pI values of 7.60 and 7.94. There were a total of five bands visible in the pH range 6.62 to 7.90.

A further sample, run in track 2 of Plate 6.1, was recombinant DNA-derived bGH from Genentec. There were a total of 26 individual bands present between pH 4.70 and 6.34. It was concluded that this preparation contained a continuous array of different protein molecules, very little of which resembled bGH. As a result of this preliminary investigation, the use of hormone from this source was discontinued. All further reference to recombinant DNA-derived bGH is that obtained from Cyanamid.

The purity of most of the hormones used in the present study were investigated by SDS polyacrylamide gel electrophoresis. The results are shown in Plate 6.2 where all samples of GH reduced with 2-mercaptoethanol showed a major component with an estimated molecular weight of approximately 20 kDa. The recombinant DNA-derived bGH from Cyanamid (track 3 reduced; track 10 non-reduced) had a single major band and, in the non-reduced sample, a additional minor band of high molecular weight. The purified bGH (track 2 reduced; track 9 non reduced) contained, in addition to the 20 kDa component, a major component of 19.4 kDa and two minor bands of higher molecular weight. The purified oGH (track 4 reduced; track 11 non-reduced) had at least 5 major bands, two of which had a similar molecular weight to the bGH preparations (20 kDa) and three of which had a lower molecular weight. It is possible that the recombinant DNA-derived bGH had a slightly higher molecular weight than the purified bGH or oGH.

All electrophoresis was carried out by Mr R. McLaren.

Plate 6.1

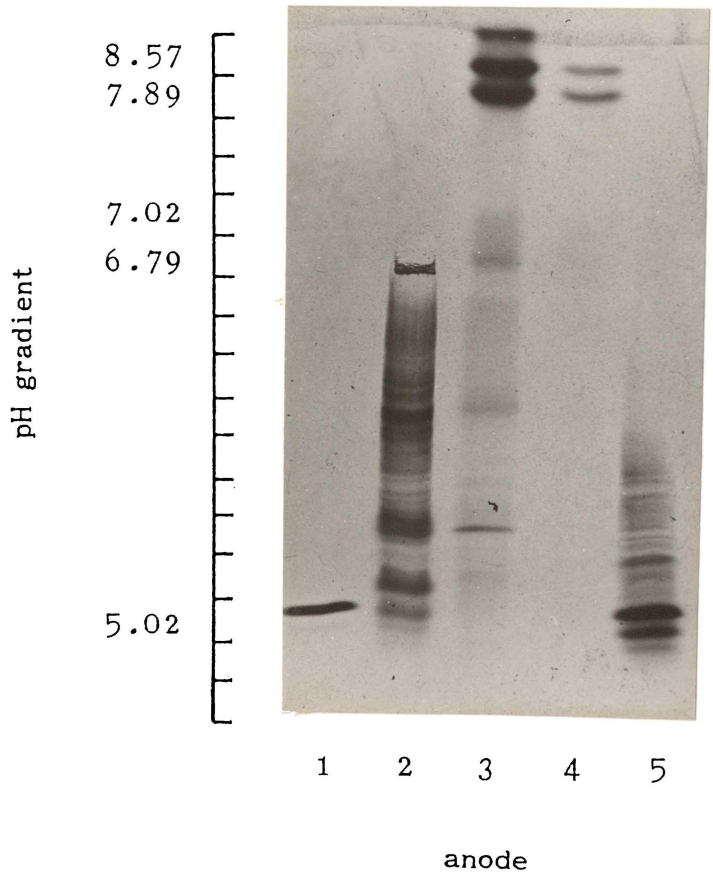


Plate 6.1 Isoelectric focusing on polyacrylamide gel: a comparison of different GH preparations

Isoelectric focusing was performed on the LKB Multiphore apparatus using a prepared polyacrylamide gel plate. The pH gradient was determined by excising 20 gel segments, each 5 mm, in sequence from the cathode to the anode. The individual segments were eluted in 0.5 ml of 0.01 mmol/l KCl for two hours at room temperature. From the pH of these solutions, a grid with lines of equal pH was constructed and the pI of the bands determined.

The sample tracks shown in Plate 6.1 are:

Track no	Hormone	Source
1	recombinant DNA-derived hGH	Genentec
2	recombinant DNA-derived bGH	Genentec
3	purified bGH	Dr I. Hart
4	purified oGH	NIAMDDO
5	purified hGH	Mr S. Hodgkinson

For a more detailed description of the source of the hormones used, see Section 2.7.

Plate 6.2

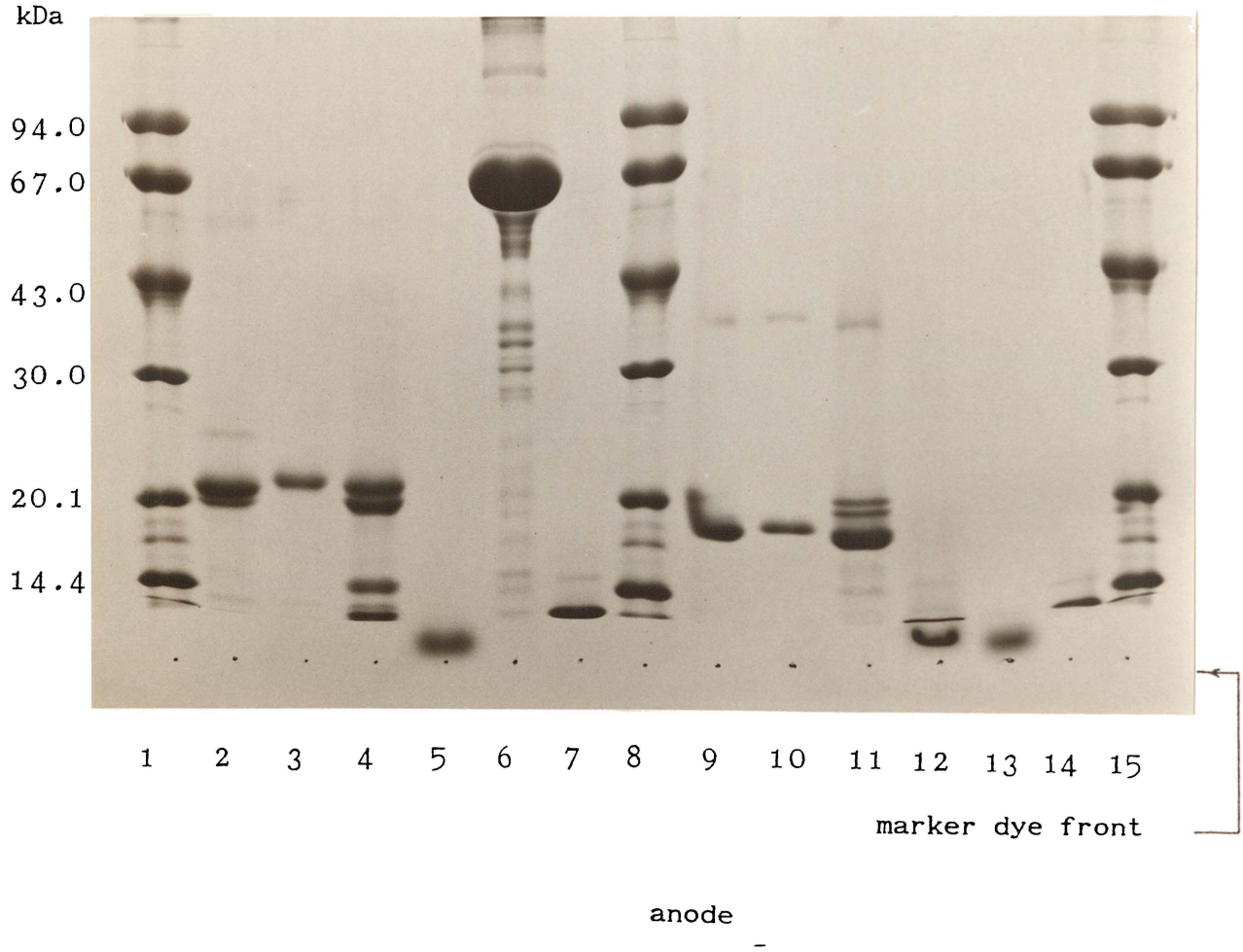

kDa
94.0
67.0
43.0
30.0
20.1
14.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
marker dye front
anode
-

Plate 6.2 SDS polyacrylamide gel electrophoresis: an analysis of GH, insulin, IGF I and BSA

Protein components were analysed using SDS polyacrylamide gel electrophoresis. The Biorad Protean II system was used and the method followed was as described by Laemmli (1970). The separating gel contained 12% monomer acrylamide at pH 8.8. Samples were prepared at concentrations of 1 - 2 mg/ml, where possible, with 10 - 25 μl applied to the gel. All samples were reduced with 0.2 mmol/l 2-mercaptoethanol except those in Track numbers 9 to 14 inclusive.

The sample tracks as shown in Plate 6.2 are:

Track no	Peptide	Source
1	protein standards	
2	purified bGH	Dr I. Hart
3	recombinant DNA-derived bGH	Cyanamid
4	purified oGH	NIAMDDO
5	glucagon	Sigma
6	BSA	Sigma
7	recombinant DNA-derived hIGF I	Dr D. Burleigh
8	protein standards	
9	purified bGH	Dr I. Hart
10	recombinant DNA-derived bGH	Cyanamid
11	purified oGH	NIAMDDO
12	ovine insulin	Sigma
13	glucagon	Sigma
14	recombinant DNA-derived hIGF I	Dr D. Burleigh
15	protein standards	

The main components of the glucagon, insulin and IGF I samples had the same estimated molecular weight but all three hormones migrated to well below the lower limits of the linear range in the standard curve.

For a more detailed description of the source of each hormone, see Section 2.7.

6.3 The effects of growth hormone on the rate of lipogenesis

The action of growth hormone on the rate of lipogenesis was estimated using the rate of acetate incorporation into tissue fatty acids. Purified bGH stimulated the rate of acetate incorporation by 40% ($p < 0.05$) at 2 hours and by 50% ($p < 0.05$) at 4 hours (Figure 6.1). The rate decreased rapidly after 4 hours incubation of this tissue and in the bGH-treated cells, ceased entirely between 4 and 6 hours (point not shown on the graph).

The experiment was repeated and the results are given in Figure 6.2. Here, 10 ng/ml purified bGH had little effect at 2 and 4 hours but stimulated the rate of acetate incorporation into fatty acid by 166% ($p < 0.05$) at 6 hours. In the same experiment, 100 ng/ml of the bGH stimulated the rate by 166% (NS) at 2 hours, by 400% ($p < 0.05$) at 4 hours and by 257% (NS) at 6 hours. Purified oGH, at a concentration of 100 ng/ml, was tested at the same time and there was no significant stimulation of the rate of acetate incorporation above that in tissue with no hormone in the medium throughout the 6 hours of the incubation.

The effects of insulin and bGH on the rate of acetate incorporation into fatty acid were compared and the results are shown in Figure 6.3. After 4 hours of incubation, insulin stimulated the rate by 52% ($p < 0.05$) and bGH by 50% ($p < 0.05$). Thus stimulation of the rate of lipogenesis by bGH was similar to that of insulin.

The effects of GH on the rate of acetate incorporation into fatty acid were investigated in tissue from 10 sheep and a significant stimulation of the rate was observed in 5 of these.

6.4 The effects of growth hormone on the rate of lipolysis

In the following experiments, the rate of lipolysis was estimated using the rate of release of glycerol into the incubation medium. As seen in Figure 6.4, purified bGH stimulated glycerol release from these cells by 256% ($p < 0.01$) at 2 hours and by 99% at 4 hours. It is evident that the effect of bGH on the cells was decreasing with time so that there was no significant stimulation by bGH after 6 hours.

Figure 6.5 shows the effects of bGH on the rate of glycerol release into the incubation medium compared with the effects of insulin and adrenalin. This comparison was made in the same experiment as the determination of the time-course graph shown in Figure 6.4. Thus the responses for bGH at 2 and 4 hours are the same in Figure 6.4 and 6.5. When bGH stimulated the rate by 256% ($p < 0.001$) after 2 hours incubation, insulin had no significant effect while, at the same time, adrenalin stimulated the rate by 313% ($p < 0.01$). After 4 hours incubation, when bGH increased the rate by 99% ($p < 0.01$), adrenalin increased the rate by 120% ($p < 0.01$) and insulin depressed the rate by 38% ($p < 0.05$). In the same experiment, neither insulin, bGH nor adrenalin had any significant effect on the rate of acetate incorporation into fatty acid (results not shown).

Figure 6.6 shows the rate of glycerol release in the presence of 100 ng/ml recombinant DNA-derived bGH compared with that in the presence of 50 ng/ml purified bGH. There was a significant stimulation of the rate of glycerol release by both hormones after 2 hours incubation; the recombinant DNA-derived bGH by 118% ($p < 0.01$) and the purified bGH by 65% ($p < 0.05$). Neither hormone had a significant effect after 4 hours. Owing to an excessive drop in the rate of glycerol release after 4 hours incubation, the results at 6 hours are not shown. There was no significant effect of either hormone on the rate of acetate incorporation into fatty acid during this experiment.

The effects of GH concentration on the rate of glycerol release from the adipose tissue slices (Figures 6.7 and 6.8) were measured at the same time as that for the time-course graph (Figure 6.6). Thus the maximum observed responses shown in Figure 6.7 (for 100 ng/ml recombinant DNA-derived bGH) and Figure 6.8 (for 50 ng/ml purified bGH) are the same as those in Figure 6.6 at 2 hours. As shown in Figure 6.6, these effects

disappeared after 4 hours of incubation. In addition, as illustrated in Figures 6.7 and 6.8, they were significant only at one concentration for each hormone. Neither hormone had any effect on the rate of acetate incorporation into fatty acid at any of the concentrations used in this experiment.

6.5 The effects of growth hormone on the mass balance of fatty acid

Using the techniques described in Section 3.16, the effects of bGH on the mass balance of fatty acids in ovine adipose tissue slices were investigated. It can be seen in Table 6.2 that, although recombinant DNA-derived bGH at 100 ng/ml had no significant effect on the rate of either fatty acid manufacture, fatty acid release within the cells (glycerol release) or the amount of fatty acid re-esterified, bGH stimulated the rate of release of free fatty acid into the incubation medium by 77% ($p < 0.01$) and depressed the rate of fatty acid accumulation by 700% ($p < 0.05$). The rate of recycling of fatty acid decreased from 61% to 42% in the presence of bGH.

The mass balance of fatty acid was measured in tissue from one other animal after 6 hours incubation and in 4 animals after 4 hours incubation. However, bGH had no significant effect on either glycerol or free fatty acid release into the incubation medium or fatty acid accumulation in the tissue in any of these experiments.

The effects of the bGH concentration on the accumulation of fatty acid in the tissue slices are illustrated in Figure 6.9. This is the same experiment as that described in Table 6.2 where 100 ng/ml of the hormone depressed the rate by 700% ($p < 0.05$). As shown in Figure 6.9, bGH was effective only at a concentration of 100 ng/ml in eliciting this response in the tissue.

For a discussion of the results presented in this Chapter, refer to Section 10.4.

Table 6.2

	μmoles/6 hours/10^6cells			
	basal rate	rate with bGH	change in rate (% change)	p
FA manufactured	0.77 ± 0.08	0.78 ± 0.17	+0.01 (0)	NS
FA released	2.22 ± 0.20	2.66 ± 0.11	+0.44 (+ 20%)	NS
FFA leaving cell	0.87 ± 0.08	1.54 ± 0.24	+0.67 (+ 77%)	**
FA re-esterified	1.36 ± 0.32	1.11 ± 0.33	-0.25 (- 18%)	NS
FA accumulation	-0.09 ± 0.16	-0.76 ± 0.13	-0.67 (-700%)	*
% FA recycling	61%	42%		

** $p < 0.01$ * $p < 0.05$

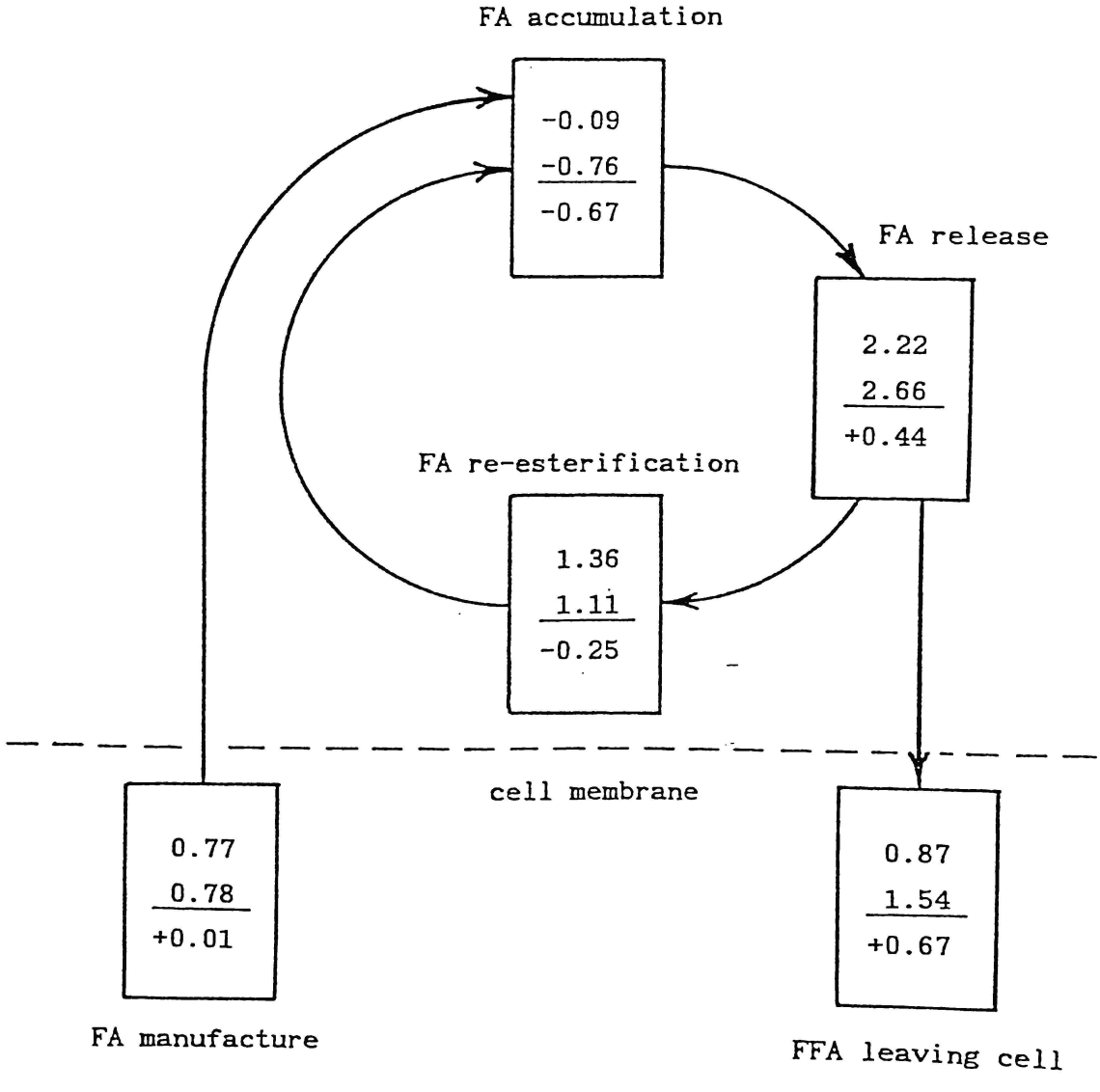


Table 6.2 The effect of bGH on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Each rate represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with bGH) of ovine adipose tissue slices in Medium 199 with 1% w/v BSA present. The bGH, which was a recombinant DNA-derived product from Cyanamid, was present where specified at a concentration of 100 ng/ml.

"FA manufactured" was measured by acetate incorporation into tissue fatty acid.

"FA released" is the fatty acid released by lipolysis within the tissue and was measured by glycerol release into the incubation medium (x3).

"FFA leaving cell" is the amount of fatty acid released into the incubation medium.

"FA re-esterified" is the amount of fatty acid that remained in the tissue after lipolysis and was presumably re-esterified.

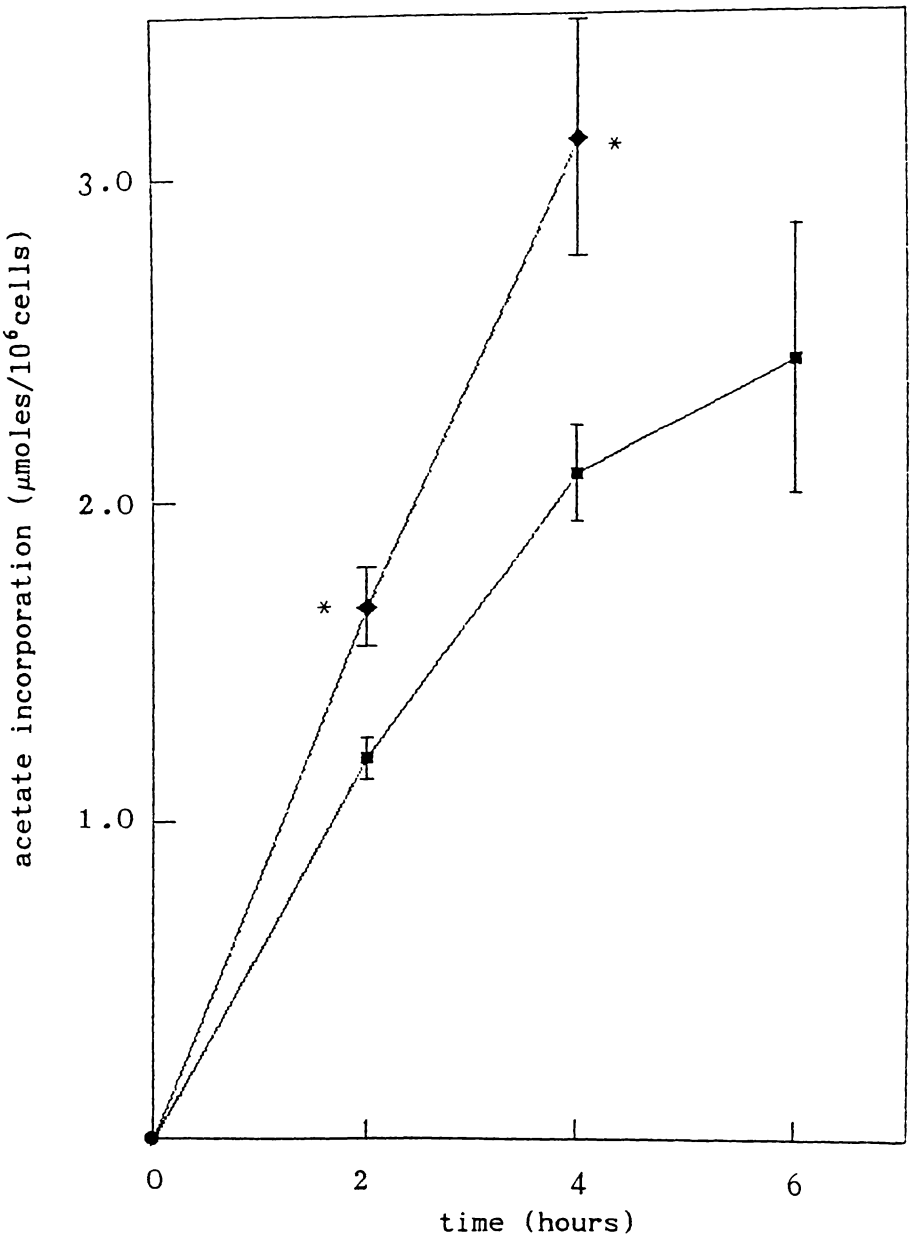
"FA accumulation" is the amount of fatty acid that accumulated in the tissue during the time of incubation (6 hours).

"% FA recycling" is the percentage of the fatty acid released which was subsequently re-esterified.

For a description of the method of calculation of each variable, refer to Section 3.16.

The diagram, which can be compared with Figure 3.8, is a summary of the data presented in the Table and represents the mass balance of the fatty acid in the tissue and incubation medium. The boxes at each stage in the cycle represent the pools of fatty acid present. The first number in each box is the basal rate (in μmoles fatty acid/6 hours/10^6 cells), the next the rate with bGH and the third is the change in the rate which occurred in the presence of bGH.

Figure 6.1



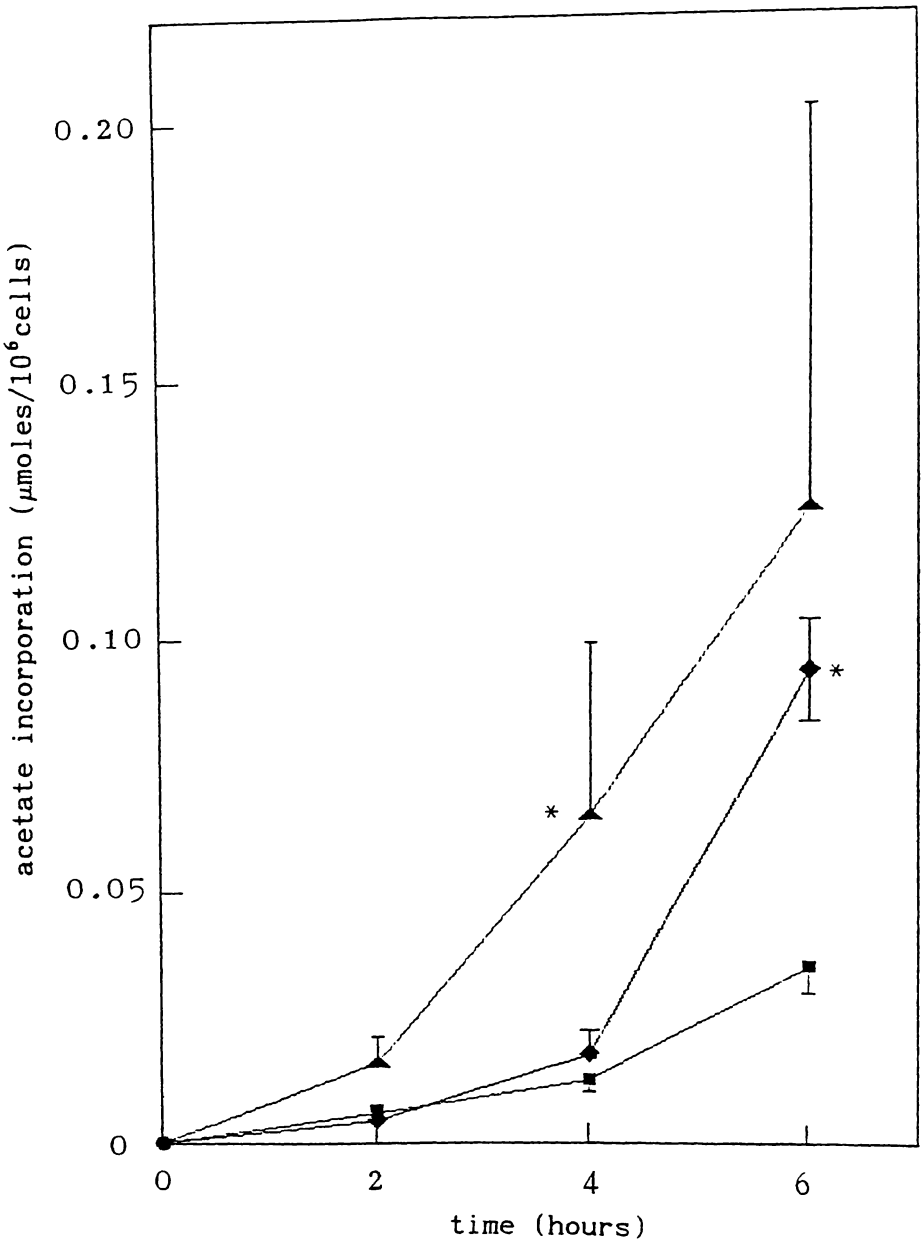
* $p < 0.05$

Figure 6.1 The effect of bGH on the rate of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The tissue slices were pre-incubated in medium without hormone for three hours before the start of the experiment. The hormone used was a pituitary-extracted preparation purified by Dr I. Hart. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ in the presence of 100 ng/ml bGH ◆ are shown.

Figure 6.2



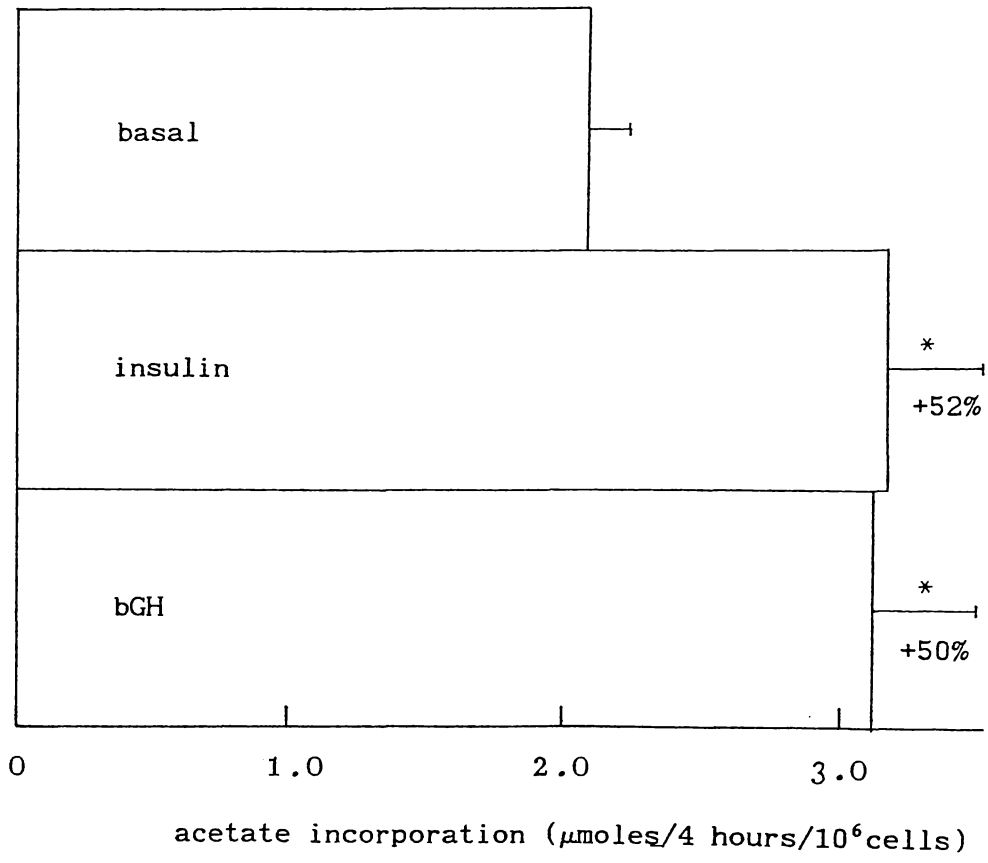
* p < 0.05

Figure 6.2 The effect of bGH on the rate of acetate incorporation into fatty acid *in vitro*

Experimental conditions were as given for Figure 6.1. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation into fatty acid with no hormone in the medium ■ , in the presence of 10 ng/ml bGH ◆ and 100 ng/ml bGH ▲ are shown.

Figure 6.3



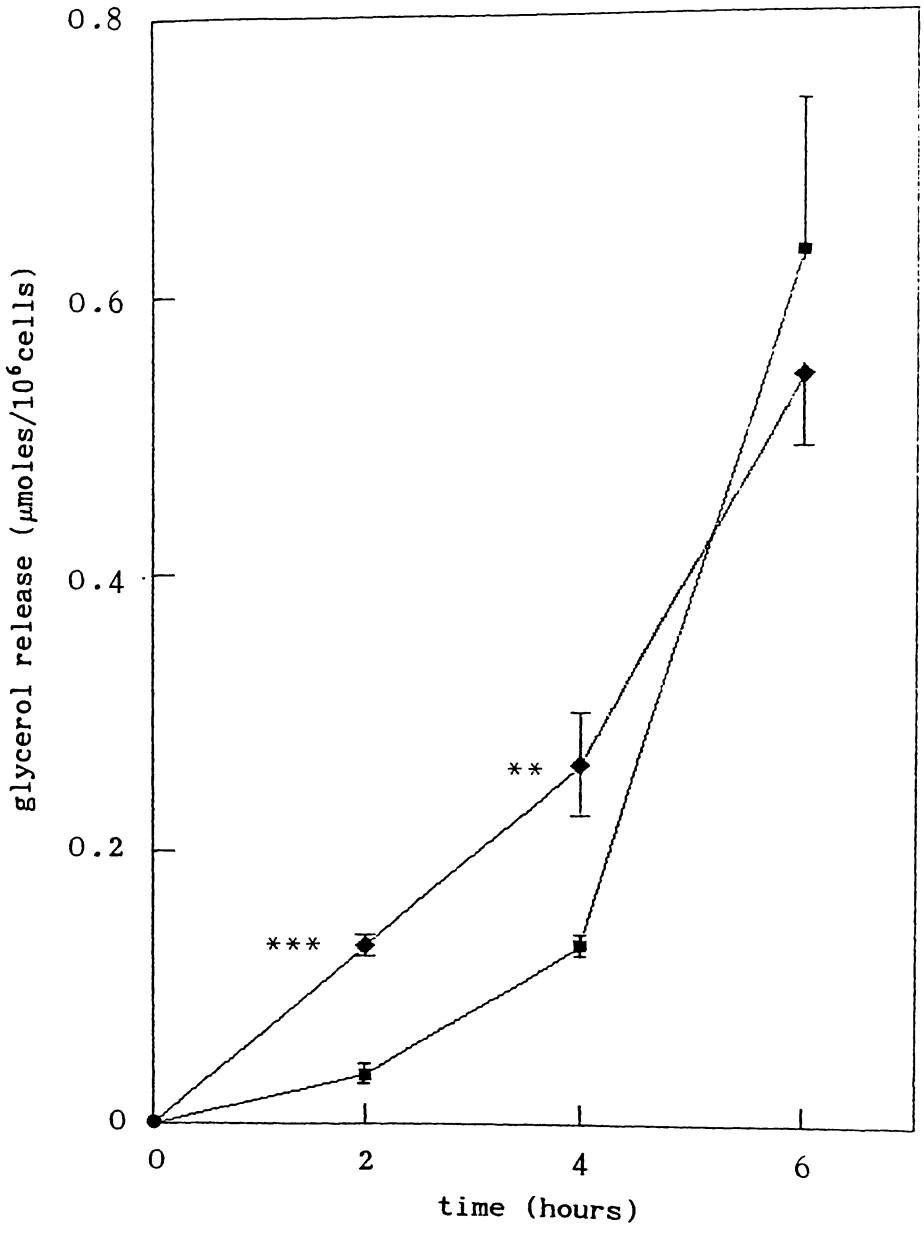
* $p < 0.05$

Figure 6.3 A comparison of the action of bGH and insulin on the rate of acetate incorporation into fatty acid *in vitro*

The bars represent the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The tissue slices were pre-incubated for 2 hours in Medium 199 without hormone before the start of the experiment. The purified bGH was present at a concentration of 100 ng/ml while insulin was at 2.6 ng/ml.

The rate of acetate incorporation into fatty acid (μmoles/4 hours/10^6cells) is shown for each hormone.

Figure 6.4



** p < 0.01

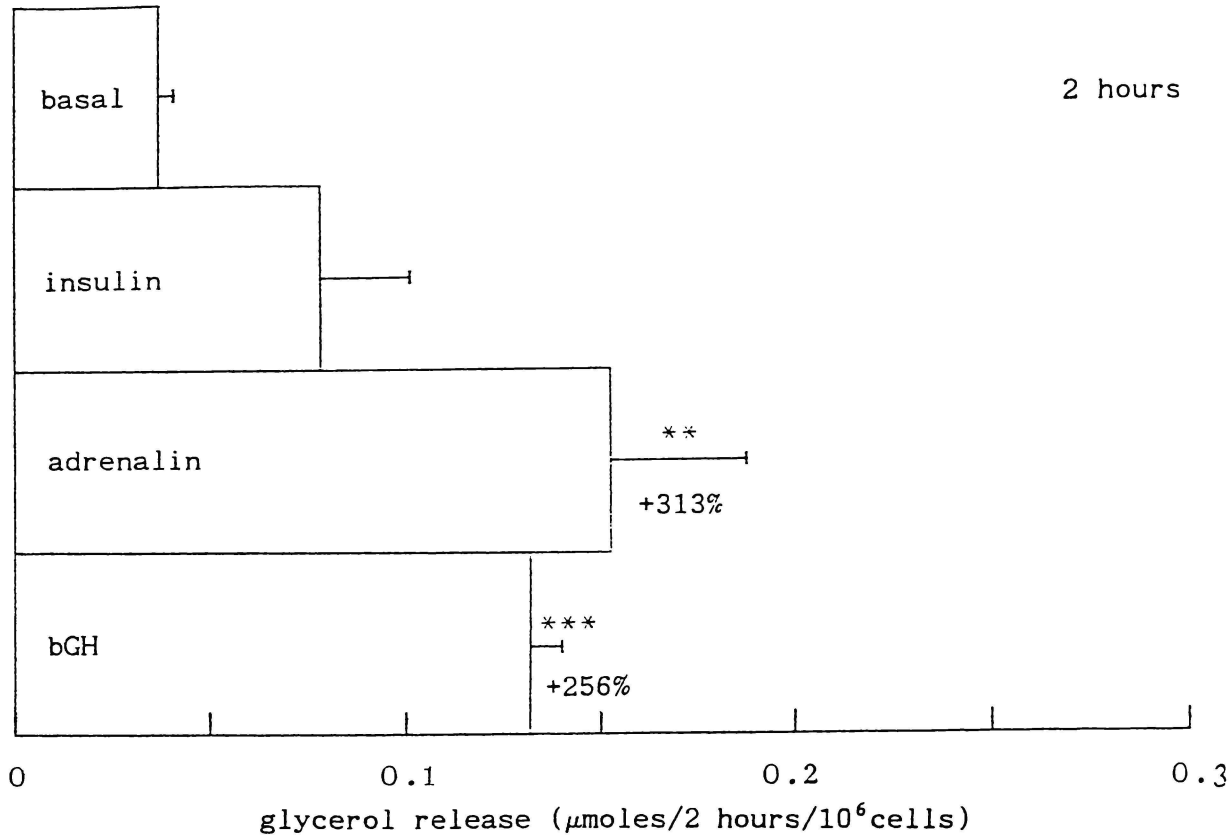
*** p < 0.001

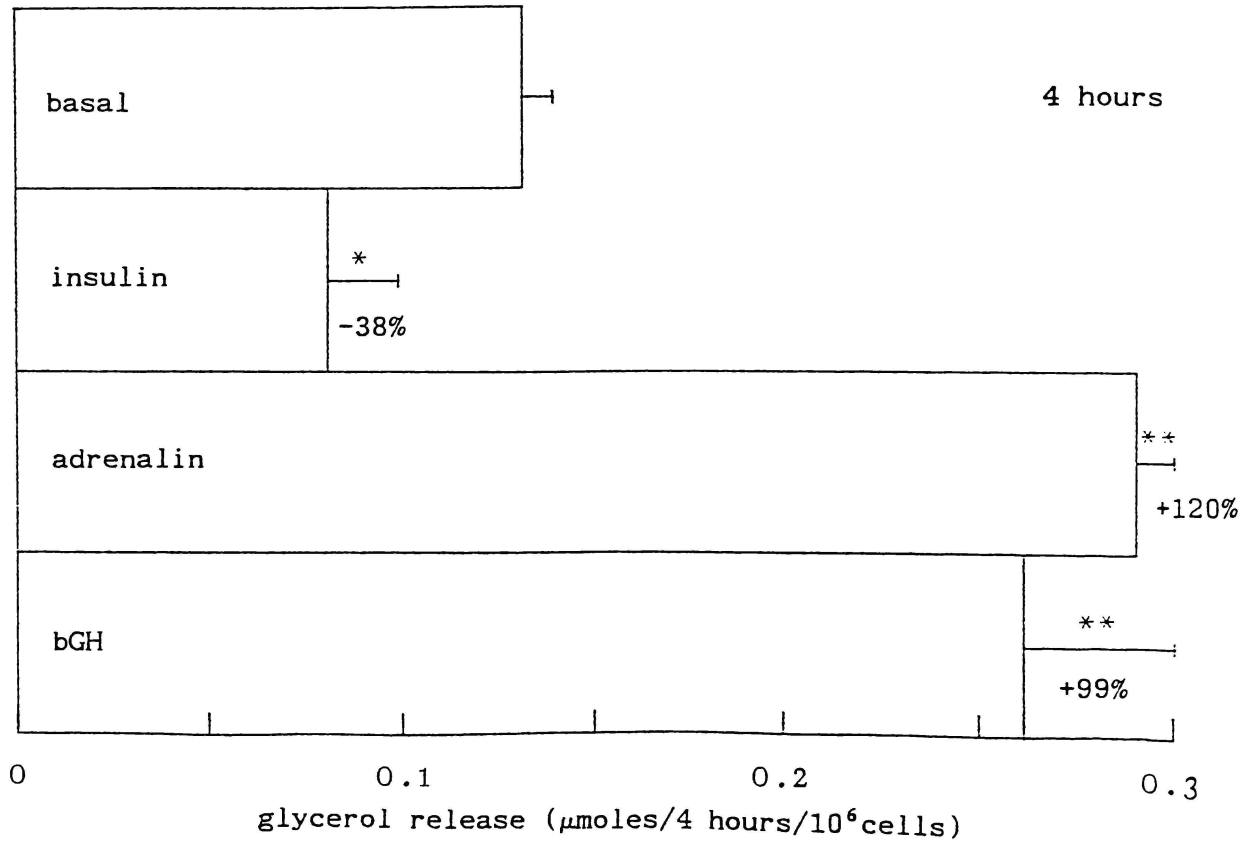
Figure 6.4 The effect of bGH on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 6.1. Glycerol release from the tissue (μmoles/10^6cells) is plotted against incubation time (hours).

Glycerol release with no hormone in the medium ■ and in the presence of 100 ng/ml bGH ◆ are shown.

Figure 6.5





* $p < 0.05$

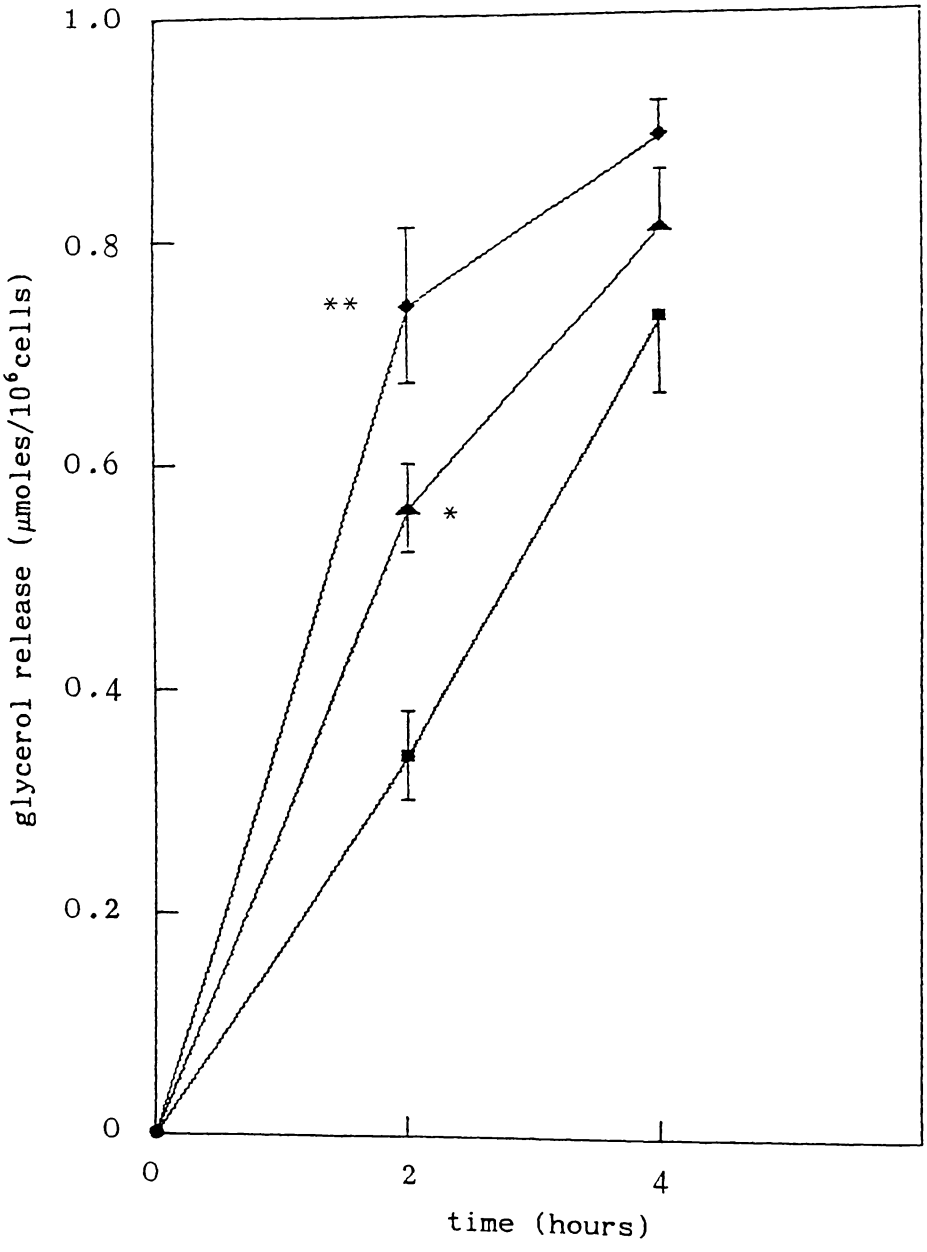
** $p < 0.01$

*** $p < 0.001$

Figure 6.5 A comparison of the effects of insulin, adrenalin and bGH on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 6.3. The purified bGH was present, where specified, at a concentration of 100 ng/ml, the insulin at 2.6 ng/ml and the adrenalin at 50 ng/ml. The rate of glycerol release has units of μmoles/2 hours/10^6cells in the first histogram and μmoles/4 hours/10^6cells in the second.

Figure 6.6



* p < 0.05

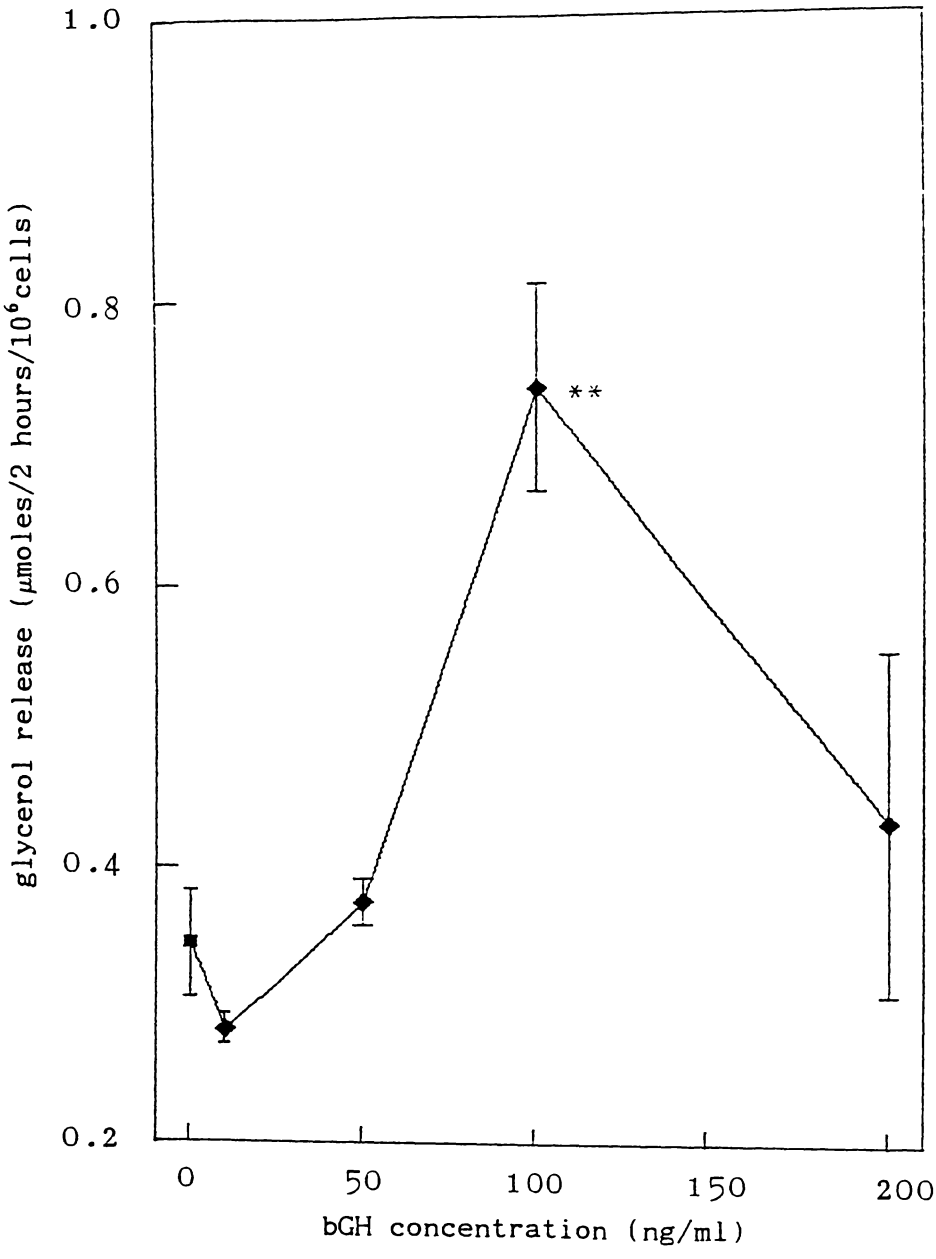
** p < 0.01

Figure 6.6 The effect of bGH on the rate of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with bGH) of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The tissue slices were pre-incubated in medium without hormone for 3 hours before the start of the experiment. Glycerol release (μmoles/10^6cells) is plotted against incubation time (hours).

Glycerol release with no hormone in the medium ■ , in the presence of 100 ng/ml recombinant DNA-derived bGH from Cyanamid ◆ and 50 ng/ml of purified bGH from Dr I. Hart ▲ are shown.

Figure 6.7



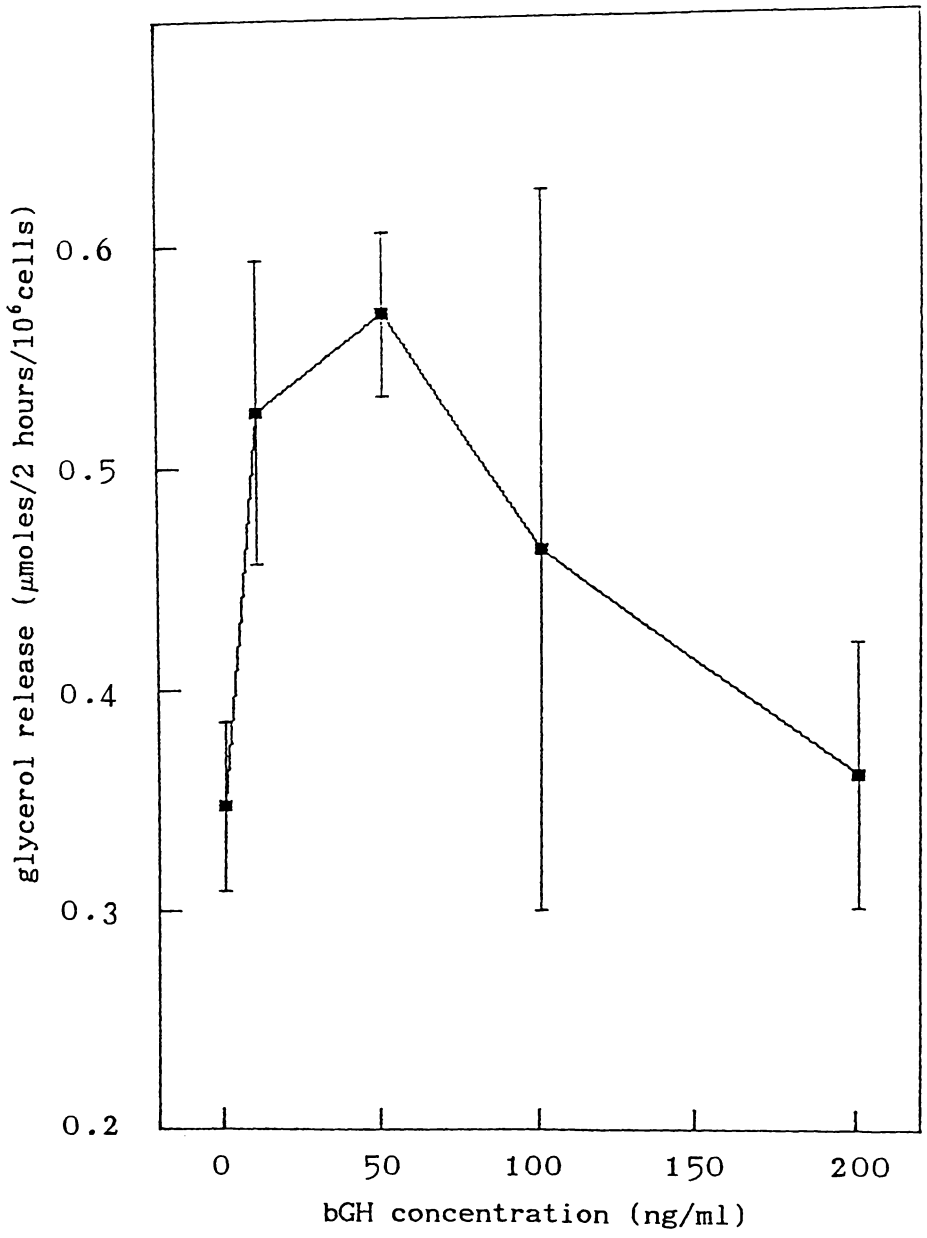
** p < 0.01

Figure 6.7 The effect of bGH concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 6.6. The hormone used was recombinant DNA-derived bGH from Cyanamid. The rate of glycerol release (μmoles/2 hours/10^6cells) is plotted against bGH concentration (ng/ml).

The basal rate of glycerol release ■ and that in the presence of 100 ng/ml bGH ◆ are shown.

Figure 6.8

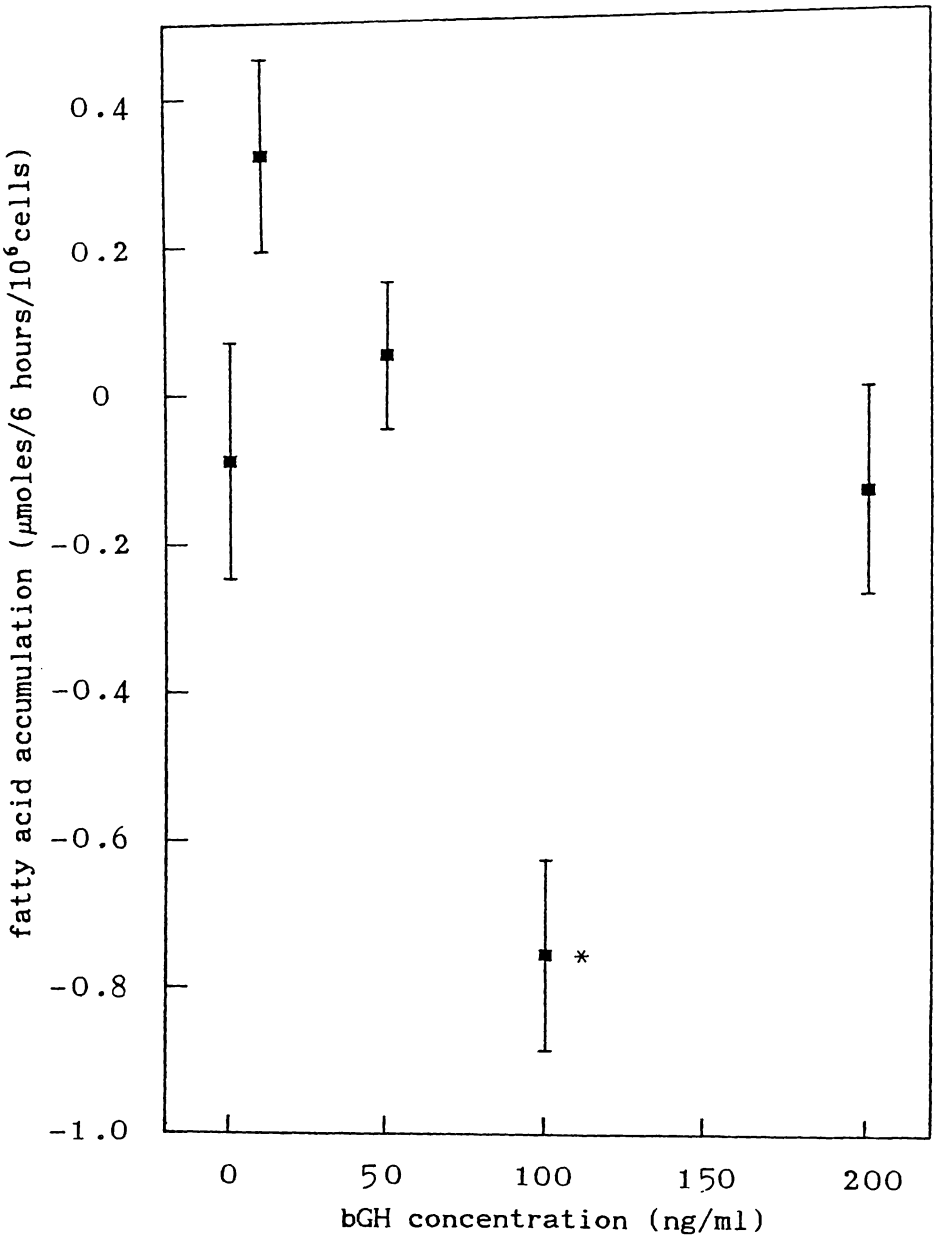


* $p < 0.05$

Figure 6.8 The effect of bGH concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

The experimental conditions were the same as those given for Figure 6.6 but the hormone used in this case was purified bGH from Dr I. Hart. The rate of glycerol release (μmoles/4 hours/10^6cells) is plotted against bGH concentration (ng/ml).

Figure 6.9



* $p < 0.05$

Figure 6.9 The effect of bGH concentration on fatty acid accumulation in ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 6.6. The hormone used was recombinant DNA-derived bGH from Cyanamid. The rate of fatty acid accumulation (μmoles/6 hours/10^6cells) is plotted against bGH concentration (ng/ml).

Chapter 7

Insulin-like Growth Factors

7.1 Introduction

The IGFs are polypeptide hormones with structures similar to that of pro-insulin (Rinderknecht and Humbel, 1978a, 1978b). Growth hormone is thought to be the dominant controlling factor for the release of IGF I and, to a lesser extent, IGF II *in vivo*.

Previously reported studies of the actions of IGFs *in vitro* using rat adipocytes have shown that both hormones elicit classical insulin-like effects. IGF I at 300 ng/ml and IGF II at 100 ng/ml stimulated glucose uptake and net gas exchange in rat adipose tissue (Zapf *et al.*, 1978). In the same study, both IGFs at similar concentrations stimulated the rate of incorporation of ^{14}C-glucose into total lipids and inhibited the rate of lipolysis. The IGFs were found to be 1 - 20% as potent in exerting these effects as insulin. It has been suggested that the IGFs interact with the insulin receptor to cause these insulin-like responses (Zapf *et al.*, 1981a). IGF I, at a concentration of 30 - 500 ng/ml, has also been shown to increase the rate of acetate incorporation into lipid in bovine adipose tissue slices *in vitro* (Etherton and Evock, 1986).

Both IGF I and IGF II stimulated ^{3}H-thymidine incorporation into DNA at concentrations of 10 - 100 ng/ml (Zapf *et al.*, 1978). In mouse soleus muscle *in vitro*, the IGFs stimulated glucose transport, glycolysis and protein synthesis and were 5 - 10% as potent as insulin when mimicking these insulin-like effects (Poggi *et al.*, 1979).

It is apparent that, at concentrations similar to those normally seen in serum, IGFs are capable of exerting insulin-like effects in both adipose tissue and muscle *in vitro*. However, IGF I and IGF II are present in blood bound to specific binding proteins which may restrict their

biological activity (Zapf *et al.*, 1979). This could be interpreted to mean that, at the cell membrane, the effective concentration of hormone may be considerably less than the concentration of IGF normally seen in serum. In the present study, therefore, the effects of the IGFs at lower concentrations were also investigated.

7.2 The purity and stability of IGF I and IGF II

The immuno-reactive concentration of the IGF I used was determined by radioimmunoassay and expressed in terms of recombinant DNA-derived hIGF I from Dr D. Burleigh, IMCC, USA. A 70 ng/ml solution of hIGF I from Amgen in Medium 199 had an effective immuno-reactive concentration of 40 ng/ml. The radio-receptor assay for detection of IGF II was not available and the immuno-reactive concentration of the IGF II and MSA used in this study is not known.

An experiment was carried out to determine the stability of IGF I during a six hour incubation with ovine adipose tissue slices. The experimental design was as described for a similar experiment with insulin in Section 4.2. The IGF I at a concentration of 70 ng/ml by weight and BSA at 1% w/v were present in the incubation medium. Samples of the medium were removed after 2, 4 and 6 hours incubation with tissue slices and the immuno-reactive concentration of IGF I measured in duplicate at each time interval using radioimmunoassay carried out by Ms K. Crossley.

When compared to the immuno-reactive concentration of IGF I present in the medium at the start of the experiment, there was no detectable loss of IGF I at the 95% confidence level throughout the 6 hours of incubation.

Because of the extremely small quantities of IGFs available for the present study, it was not possible to carry out extensive electrophoresis on either hormone. An SDS polyacrylamide gel electrophoresis system, as developed for growth hormone, was found to be not suitable for the IGF molecules (Plate 6.2). Insulin and IGFs have molecular weights which are well below the lower limits of the linear range of the standard curve determined for Plate 6.2.

Some information regarding the structure of the molecules was given by the suppliers:

(1) recombinant DNA-derived hIGF I from IMCC.
The molecule has a methionine at position 59 and has arginine instead of threonine at position 56. Using amino acid analysis, it was determined that 67.5% by weight is unmodified IGF I; the remainder has possible de-amination or metabolic oxidation products and includes ammonium bicarbonate to stabilize the freeze-dried preparation.

(2) recombinant DNA-derived hIGF I from Amgen
The molecule has a threonine instead of a methionine at position 59 in the amino acid sequence. It is at least 95% pure, based on analysis by liquid chromotography and polyacrylamide gel electrophoresis.

(3) biologically-derived MSA from Professor J.R. Florini
The material was essentially one component on HPLC and SDS gel electrophoresis.

(4) manually constructed hIGF II from Professor C.H. Li
This manually synthesized product is considered to be 65% pure, with the rest being ammonium bicarbonate.

It is not known if the amino acid substitutions in these molecules affected their biological activity. The hormone concentrations were initially determined by weight and adjusted using the purity values given by the suppliers. However, in the case of IGF I from Amgen, the immuno-reactive concentration in terms of IMCC IGF I was about 60%. Therefore weights of the Amgen product have been expressed in terms of the IMCC IGF I by using this factor. Thus all concentrations given are as near as possible to being the concentrations of pure IGF I.

7.3 The effects of IGF on the rate of lipogenesis

Initially, experiments were carried out to determine whether the IGFs, when present at high concentrations, could stimulate the rate of acetate incorporation into fatty acid in ovine adipose tissue. This would be equivalent to the other insulin-like effects previously reported. A concentration of 40 ng/ml was chosen to simulate the concentration of IGF in sheep serum. Supplies of IGF were not sufficient to investigate the effects of concentrations higher than this.

IGF I, when present at a concentration of 40 ng/ml, was shown to stimulate the rate of acetate incorporation into fatty acid by 86% ($p < 0.05$) at 2 hours, by 500% ($p < 0.001$) at 4 hours and by 357% ($p < 0.001$) at 6 hours (Figure 7.1). Thus IGF I had its maximal effect after four hours of incubation. In the same experiment, the effect of IGF I on the rate of acetate incorporation into fatty acid at 4 hours was compared with that of insulin and adrenalin (Figure 7.2). Both insulin and IGF I significantly stimulated the rate of lipogenesis while adrenalin had no detectable effect. The difference between the responses to insulin and IGF I were not statistically significant.

Figure 7.3 shows the results of an experiment carried out on tissue from a different animal. The IGF I and MSA were present in the incubation medium at a concentration of 40 ng/ml. The hIGF I from Amgen stimulated the rate of acetate incorporation into fatty acid by 122% ($p < 0.01$), MSA by 53% ($p < 0.05$) and insulin by 85% ($p < 0.05$). Neither IGF I from IMCC nor adrenalin had a statistically significant effect on the rate.

The IGFs, when present at a concentration of 40 ng/ml, were shown to enhance the rate of acetate incorporation into fatty acid in tissue from half of the animals tested ($n = 8$). There was no indication that the ability of the tissue to respond to IGFs was linked to its ability to respond to insulin in this regard. However, more data would be needed before a definite conclusion could be reached. At concentrations of IGF lower than 40 ng/ml (0.04 - 4 ng/ml) neither IGF I nor IGF II was shown to have any significant effect on the rate of acetate incorporation into fatty acid in tissue from any of the sheep tested ($n = 6$).

7.4 The effects of IGF on the rate of lipolysis

In the following experiments, the effects of IGFs on the rate of lipolysis, as measured by glycerol release, were investigated using lower concentrations of hormones than those used in previous studies (see Section 7.1). In the time-course graphs, the effects of IGF I, MSA and IGF II on the rate of lipolysis are shown when each hormone was present in the medium at a concentration which gave a maximal response. As seen in the dose response curves given at the end of this section, the optimal concentration varied between experiments.

In Figure 7.4, the effects of 0.4 ng/ml IGF I are shown. The rate of glycerol release between 4 and 6 hours decreased rapidly in most incubations in this experiment and therefore the data for 6 hours was not used. There was no significant difference between the effects of IGF I from Amgen and that from IMCC in this experiment. At 2 hours, both had stimulated the rate by 151 - 156% ($p < 0.01$) while adrenalin increased the rate by 248% ($p < 0.01$). At 4 hours, both the IGF I preparations stimulated the rate by 60 - 64% ($p < 0.01$) while adrenalin increased the rate by 137% ($p < 0.01$). In the same experiment (see Figure 7.5), MSA, at a concentration of 0.4 ng/ml, had the effect of increasing the rate of glycerol release by 224% ($p < 0.01$) at 2 hours and by 87% ($p < 0.01$) at 4 hours. There was no significant difference between the effects of adrenalin and MSA in this experiment.

A comparison of the actions of IGF I and MSA with those of adrenalin and insulin in this experiment is shown in Figure 7.6. Whereas insulin had no significant effect on the rate of glycerol released from the cells, IGF I from Amgen stimulated the rate by 60% ($p < 0.01$), IGF I from IMCC by 64% ($p < 0.01$), MSA by 87% ($p < 0.01$) and adrenalin by 137% ($p < 0.01$), all after 4 hours incubation. There was a significant difference between the effects of the IGF I preparations and that of adrenalin (IGF I from Amgen $p < 0.05$; IGF I from IMCC $p < 0.01$). There was no significant difference between the effects of MSA and adrenalin, nor between the effects of MSA and IGF I.

In a different experiment (see Figure 7.7), IGF I (IMCC), at a concentration of 0.4 ng/ml, stimulated the rate of glycerol release by 150% at 4 hours ($p < 0.05$) and also at 6 hours ($p < 0.01$). The effect of this hormone at 2 hours was not statistically significant. At the same

time, IGF II, at a concentration of 0.4 pg/ml, stimulated the rate by 176% at 2 hours ($p < 0.01$), by 260% at 4 hours ($p < 0.01$) and by 181% at 6 hours ($p < 0.01$). The effects of IGF I and IGF II for the same experiment at 4 hours were compared with that of insulin and adrenalin (see Figure 7.8). Insulin had no significant effect on the rate of glycerol release from the tissue, IGF I stimulated the rate by 150% ($p < 0.05$), IGF II by 260% ($p < 0.01$) and adrenalin by 300% ($p < 0.01$). In their effects on the rate of glycerol release, neither IGF I nor IGF II were significantly different from adrenalin.

A further two experiments were carried out to investigate the effects of IGF I and IGF II on glycerol release. Both hormones were tested only at 0.4 ng/ml. In the first experiment, IGF I (IMCC) stimulated the rate of glycerol release by 77% ($p < 0.05$) and IGF II had no significant effect. In the second experiment, IGF I (IMCC) stimulated the rate by 215% ($p < 0.01$) and IGF II by 161% ($p < 0.05$).

The effects of different concentrations of IGF I and IGF II were also investigated. As seen in Figure 7.9, the rate of glycerol release was highest when IGF I (IMCC) was present at 4.0 ng/ml. At this concentration, the rate was stimulated by 96% ($p < 0.01$). In another experiment, all concentrations of IGF I tested (0.04 - 40 ng/ml) were effective in stimulating the rate of glycerol release (Figure 7.10). At a concentration of 0.4 ng/ml, IGF I stimulated the rate of glycerol release by 156% ($p < 0.01$).

In Figure 7.11, MSA enhanced the rate of glycerol release at all concentrations tested (0.04 - 40 ng/ml) The maximum response was obtained at a concentration of 0.4 ng/ml when MSA stimulated the rate by 224% ($p < 0.01$). In this initial experiment with MSA, it appeared that the hormone may possibly be active at concentrations lower than 0.04 ng/ml. Thus, when supplies of IGF II became available, the hormone was tested at a lower range of concentrations. The results of this experiment are presented in Figure 7.12. IGF II appears to be more lipolytic at 0.4 pg/ml than at 0.4 ng/ml. At a concentration of 0.4 pg/ml, IGF II stimulated the rate of glycerol release by 260% ($p < 0.01$) and by 210% ($p < 0.01$) at 4.0 pg/ml. The lipolytic effects of IGF II at concentrations higher than 0.4 ng/ml were not statistically significant.

7.5 The effects of IGF on the mass balance of fatty acid

The mass balance of fatty acid in the adipose tissue slices was investigated. In Table 7.1, the results are given for ovine adipose tissue slices incubated in 0.4 ng/ml of IGF I (IMCC). Although there was no significant increase in the rate of fatty acid manufactured during the 6 hour incubation with IGF I, the rate of lipolysis increased by 150% ($p < 0.01$). The rate of FFA efflux from the tissue increased by 318% ($p < 0.01$) while the rate of fatty acid re-esterification increased by 130% ($p < 0.01$). Overall, there was no significant effect of IGF I on the amount of fatty acid accumulated in the cell during the 6 hour incubation. The percentage of fatty acid recycled decreased from 90% to 84% during the 6 hour incubation.

In Table 7.2, the effects of 0.4 ng/ml IGF II on the mass balance of fatty acid in tissue slices from the same animal as that shown in Table 7.1 are given. As with IGF I, IGF II had no significant effect on the rate of fatty acid manufacture at this concentration but significantly increased the rate of lipolysis (+128%, $p < 0.05$). The rate of FFA efflux from the tissue increased by 336% ($p < 0.01$) as has the rate of fatty acid re-esterification (+106%, $p < 0.05$). Overall, there was no significant effect of IGF II on the rate of fatty acid accumulation in the tissue. The percentage of fatty acid recycling in the tissue decreased from 90% to 82% in the presence of IGF II.

The effects of IGF I and IGF II on the mass balance of fatty acid was measured in 4 sheep and, although the hormones were shown to stimulate the release of glycerol from this tissue, the same slight non-significant decrease in the rate of fatty acid accumulation was seen in all experiments.

Table 7.1

	μmoles/6 hours/10^6cells			
	basal rate	rate with IGF I	change in rate (% change)	p
FA manufactured	2.25 ± 0.09	2.29 ± 0.18	+0.04 (0)	NS
FA released	1.15 ± 0.14	2.85 ± 0.36	+1.70 (+150%)	**
FFA leaving cell	0.11 ± 0.08	0.46 ± 0.10	+0.35 (+318%)	**
FA re-esterified	1.04 ± 0.12	2.39 ± 0.31	+1.35 (+130%)	**
FA accumulation	2.14 ± 0.11	1.83 ± 0.19	-0.31 (- 14%)	NS
% FA recycling	90%	84%		

** $p < 0.01$

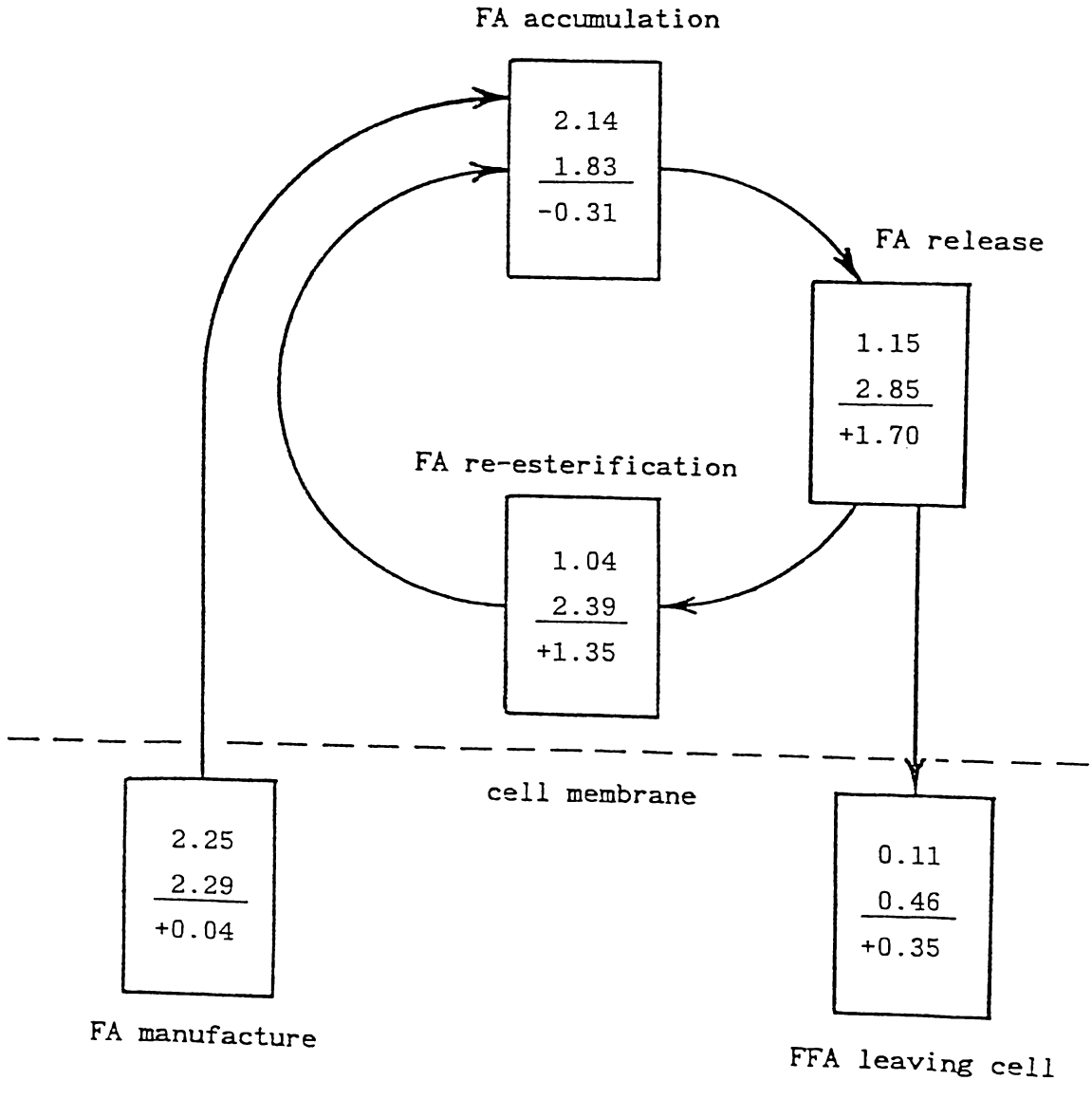


Table 7.1 The effect of IGF I on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Each rate represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with IGF I) of ovine adipose tissue slices in Medium 199 with 1% w/v BSA present. IGF I, which was recombinant DNA-derived hIGF I from IMCC, was present where specified at a concentration of 0.4 ng/ml.

"FA manufactured" was measured by acetate incorporation into tissue fatty acid.

"FA released" is the fatty acid released by lipolysis within the tissue and was measured by glycerol release into the incubation medium (x3).

"FFA leaving cell" is the amount of fatty acid released into the incubation medium.

"FA re-esterified" is the amount of fatty acid that remained in the tissue after lipolysis and was presumably re-esterified.

"FA accumulation" is the amount of fatty acid that accumulated in the tissue during the time of incubation (6 hours).

"% FA recycling" is the percentage of the fatty acid released which was subsequently re-esterified.

For a description of the method of calculation of each variable, refer to Section 3.16.

The diagram, which can be compared with Figure 3.8, is a summary of the data presented in the Table and represents the mass balance of the fatty acid in the tissue and incubation medium. The boxes at each stage in the cycle represent the pools of fatty acid present. The first number in each box is the basal rate (in μmoles fatty acid/6 hours/10^6 cells), the next the rate with IGF I and the third is the change in the rate which occurred in the presence of IGF I.

Table 7.2

	μmoles/6 hours/10^6cells			
	basal rate	rate with IGF II	change in rate (% change)	p
FA manufactured	2.25 ± 0.09	2.25 ± 0.21	0 (0)	NS
FA released	1.15 ± 0.14	2.62 ± 0.51	+1.47 (+128%)	*
FFA leaving cell	0.11 ± 0.08	0.48 ± 0.10	+0.37 (+336%)	**
FA re-esterified	1.04 ± 0.12	2.14 ± 0.49	+1.10 (+106%)	*
FA accumulation	2.14 ± 0.11	1.76 ± 0.15	-0.38 (- 18%)	NS
% FA recycling	90%	82%		

* $p < 0.05$ ** $p < 0.01$

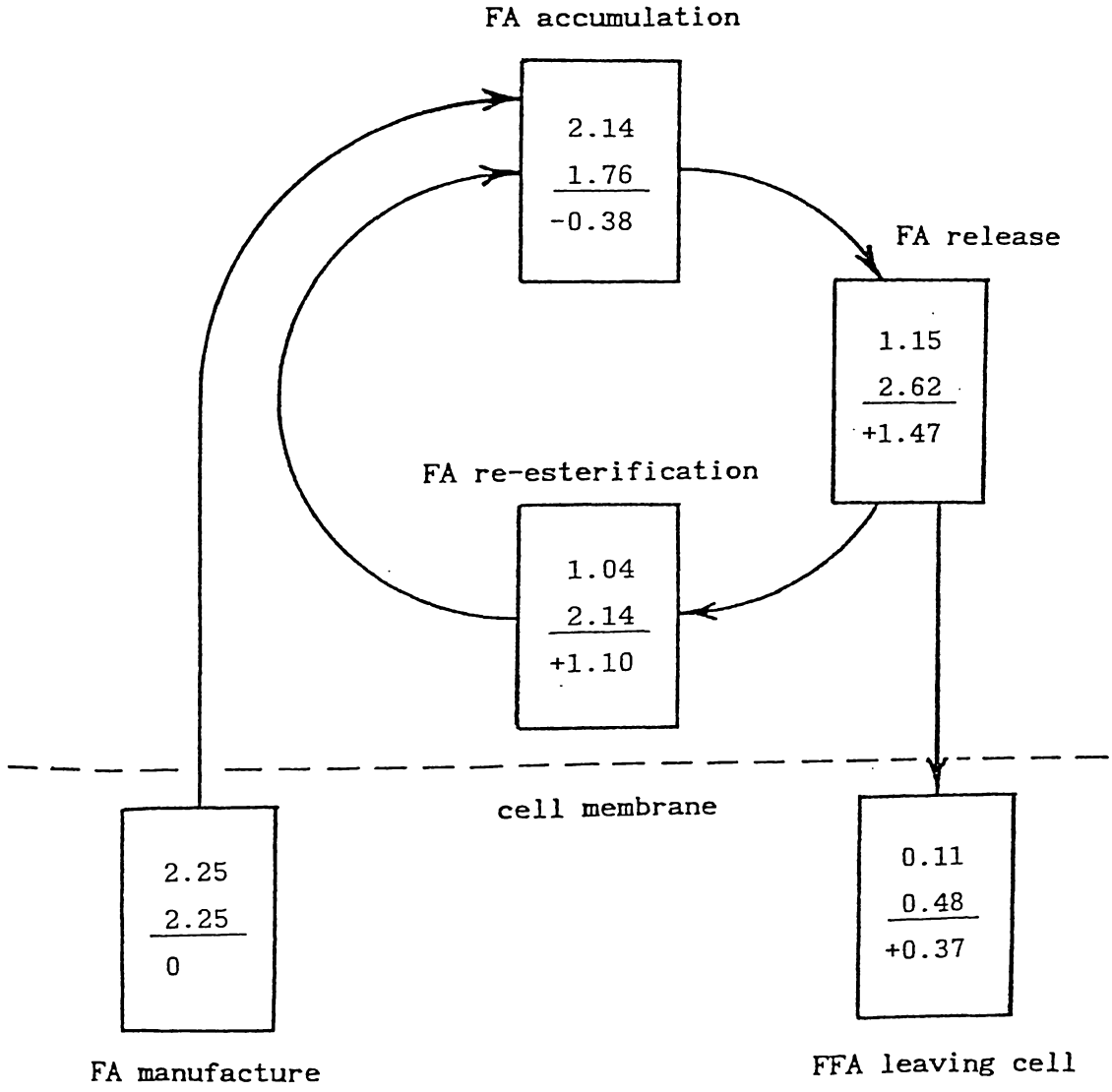
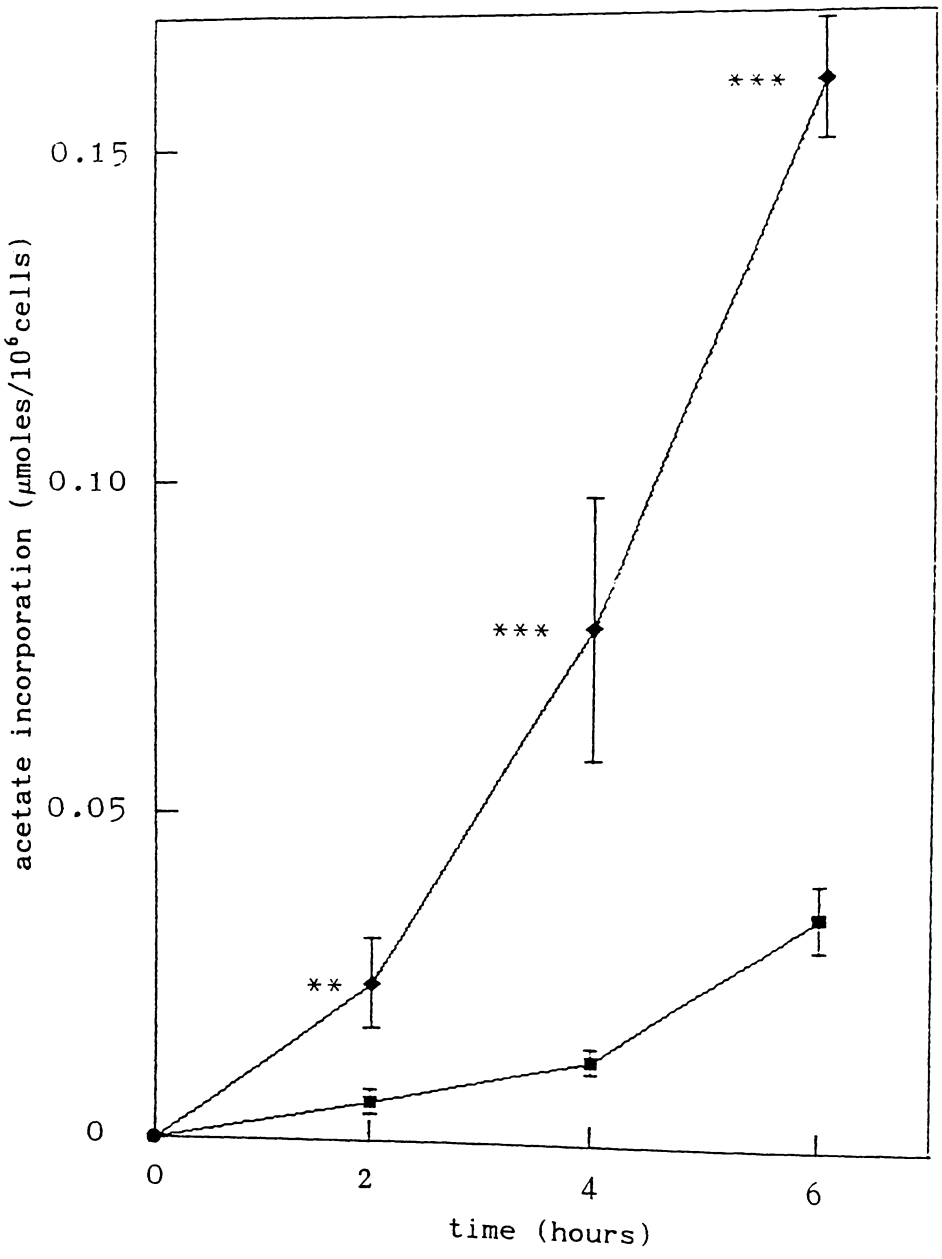


Table 7.2 The effect of IGF II on the mass balance of fatty acid in ovine adipose tissue *in vitro*

Each rate represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with IGF II) of ovine adipose tissue slices in Medium 199 with 1% w/v BSA present. The incubation time was 6 hours. IGF II, which was a manually constructed hIGF II from Professor C.H. Li, was present where specified at a concentration of 0.4 ng/ml.

The definition of terms is the same as those given in Table 7.1

Figure 7.1



** p < 0.01

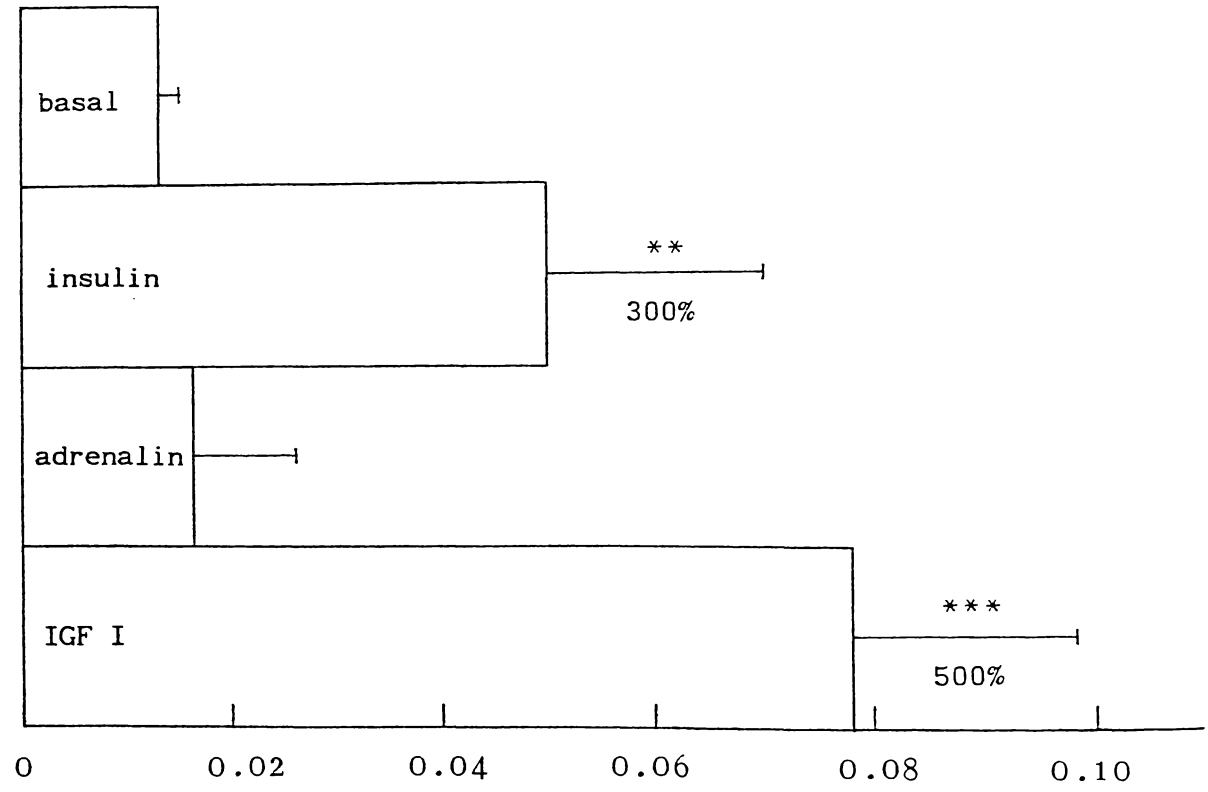
*** p < 0.001

Figure 7.1 The effect of IGF I on the rate of acetate incorporation into fatty acid *in vitro*

Each point represents the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. Acetate incorporation into fatty acid (μmoles/10^6cells) is plotted against incubation time (hours).

Acetate incorporation with no hormone in the medium ■ and in the presence of 40 ng/ml recombinant DNA-derived hIGF I (from Amgen) ◆ are shown.

Figure 7.2



** p < 0.01

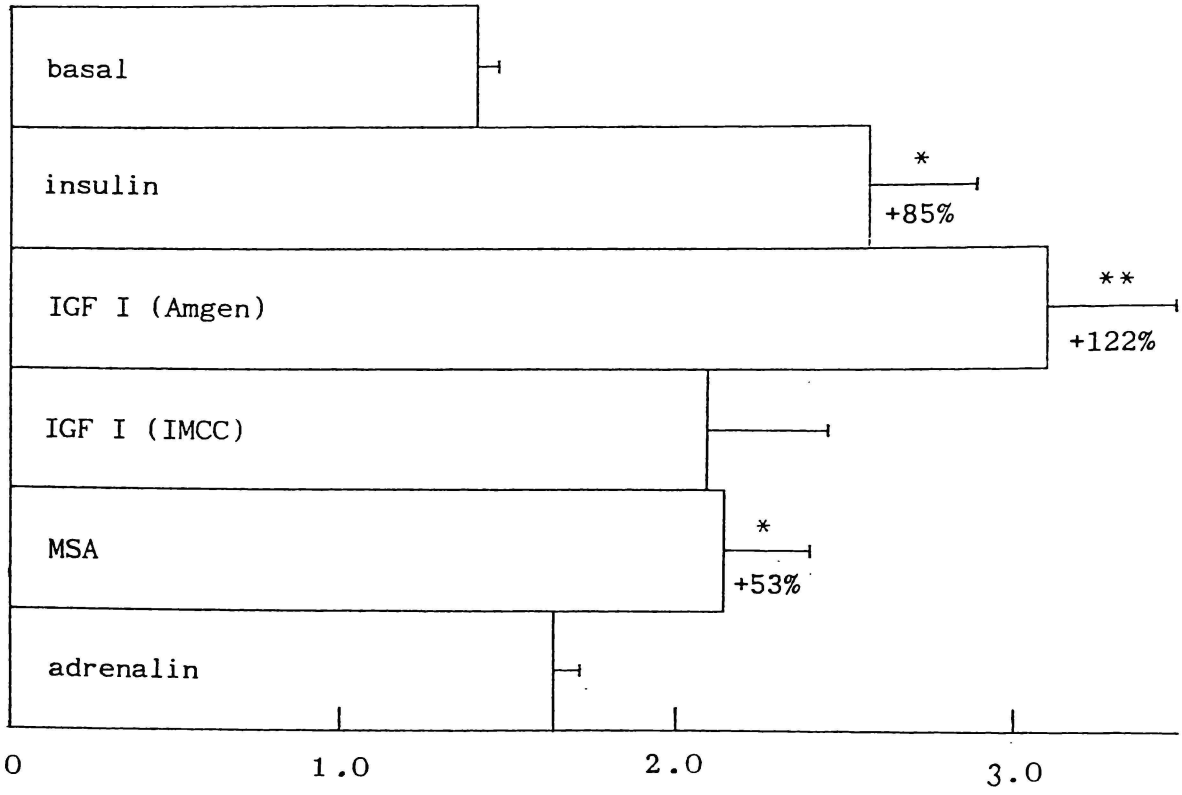
*** p < 0.001

Figure 7.2 A comparison of the actions of IGF I, insulin and adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

The bars represent the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The recombinant DNA-derived hIGF I (from Amgen) was present where specified at a concentration of 40 ng/ml, insulin at 2.6 ng/ml and adrenalin at 50 ng/ml.

The effect of each hormone on the rate of acetate incorporation into fatty acid (μmoles/4 hours/10^6cells) is shown.

Figure 7.3



* $p < 0.05$

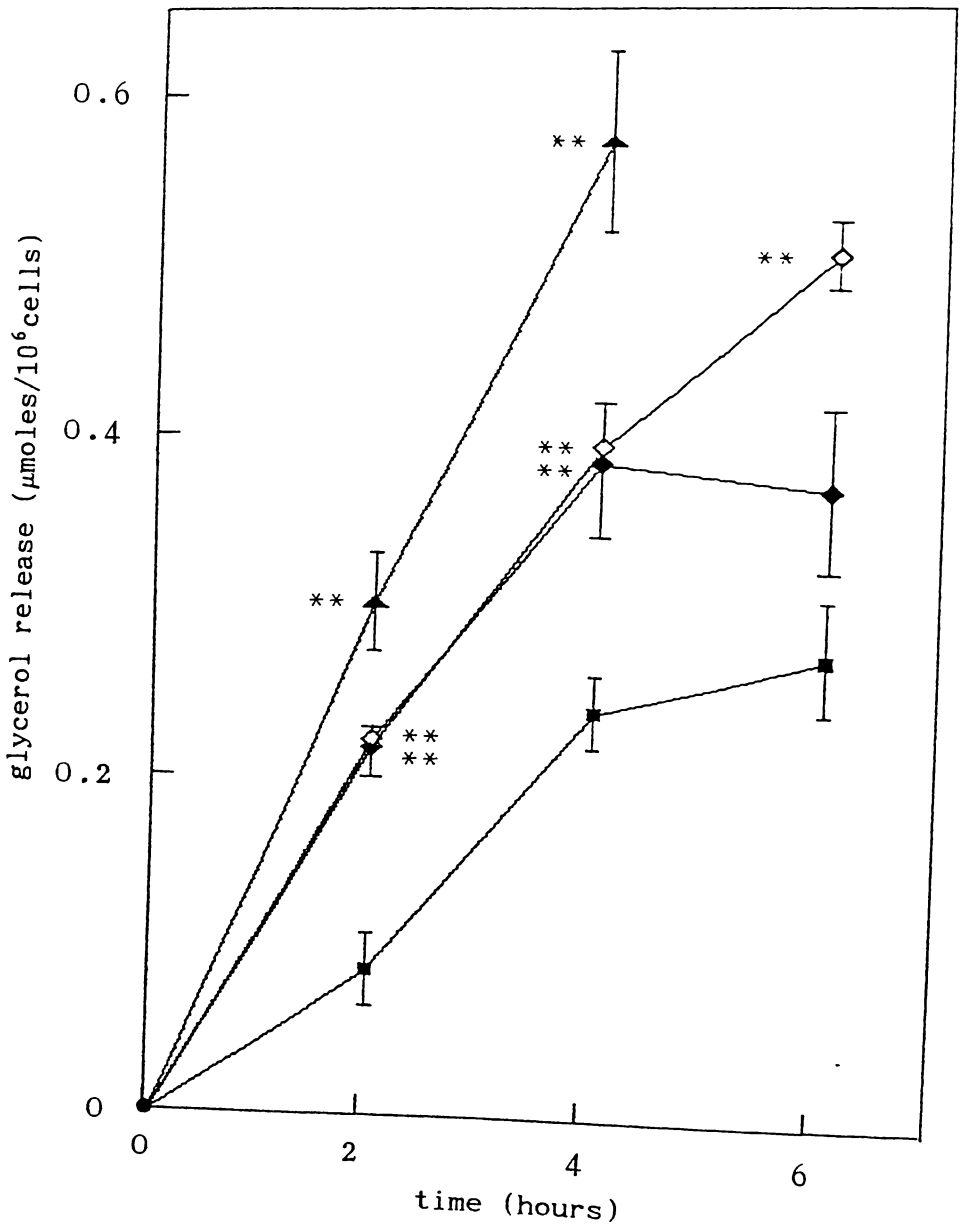
** $p < 0.01$

Figure 7.3 A comparison of the actions of IGF I, MSA, insulin and adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

The experimental conditions were as given in Figure 7.2. MSA was present at a concentration of 40 ng/ml.

The effect of each hormone on the rate of acetate incorporation into fatty acid (μmoles/6 hours/10^6cells) is shown.

Figure 7.4



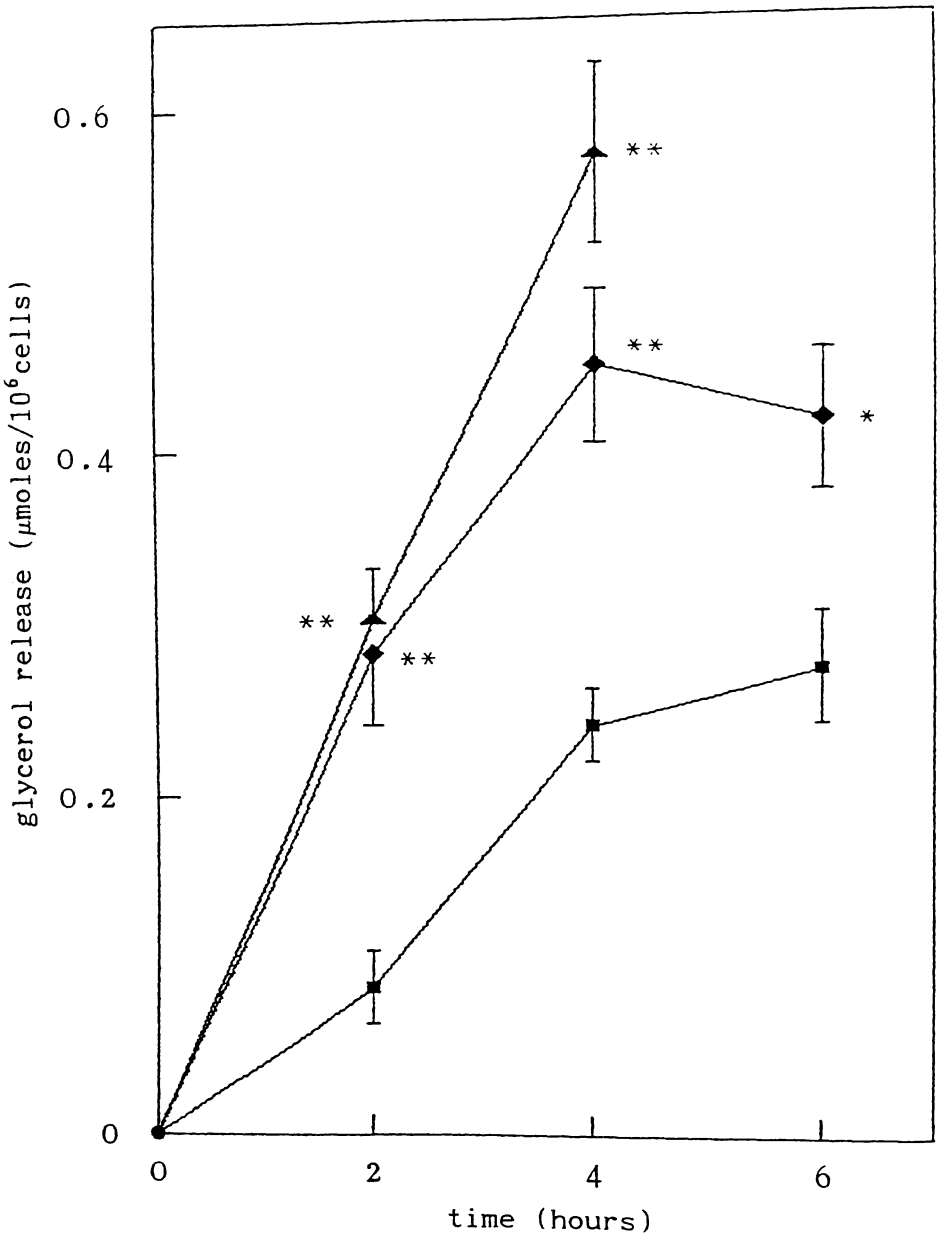
** p < 0.01

Figure 7.4 The effect of IGF I on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.1. Glycerol release (μmoles/10^6cells) is plotted against incubation time (hours).

Glycerol release from the tissue with no hormone in the medium ■ , together with that in the presence of 0.4 ng/ml hIGF I from Amgen ◆ and IMCC ◇ are shown. Glycerol release with 50 ng/ml adrenalin in the medium ▲ is also shown.

Figure 7.5



* p < 0.05

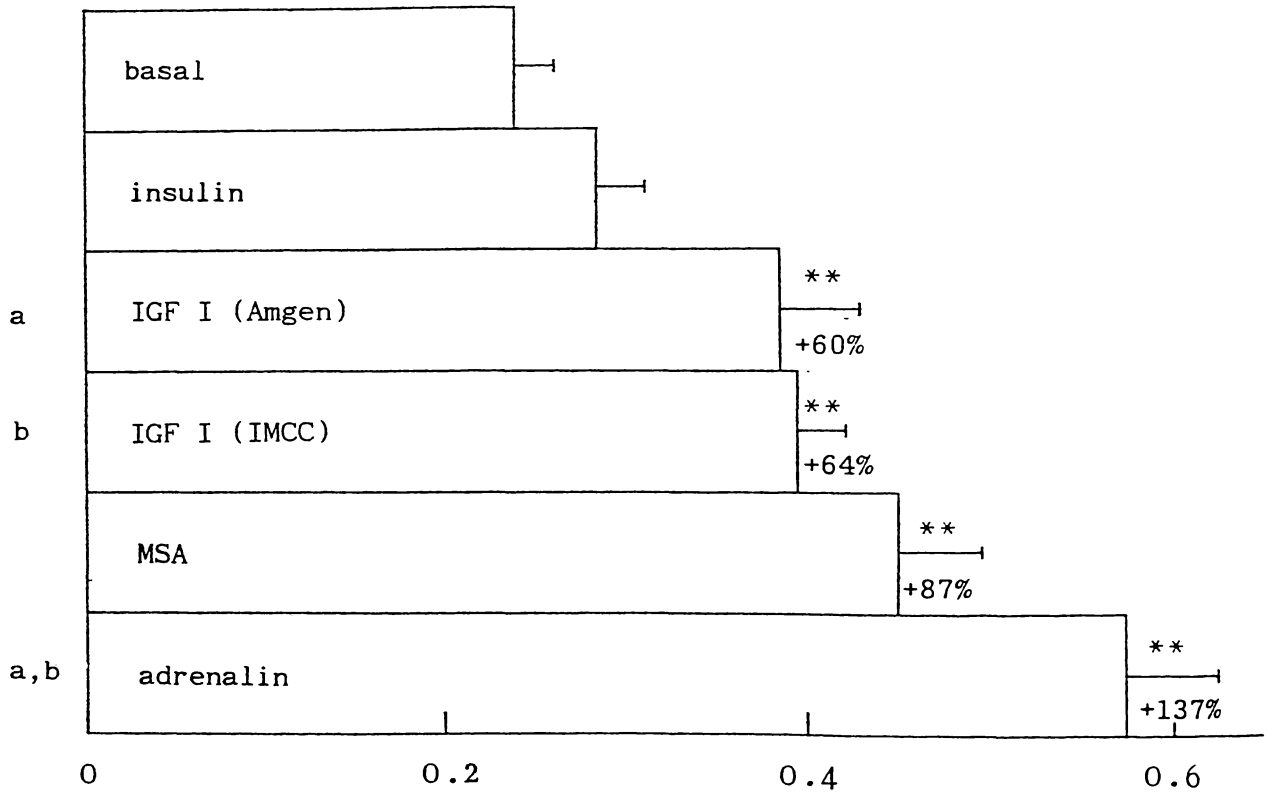
** p < 0.01

Figure 7.5 The effect of MSA on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.1. Glycerol release (μmoles/10^6cells) is plotted against incubation time (hours).

Glycerol release with no hormone in the medium ■ , together with that in the presence of 0.4 ng/ml MSA ◆ and 50 ng/ml adrenalin ▲ are shown.

Figure 7.6



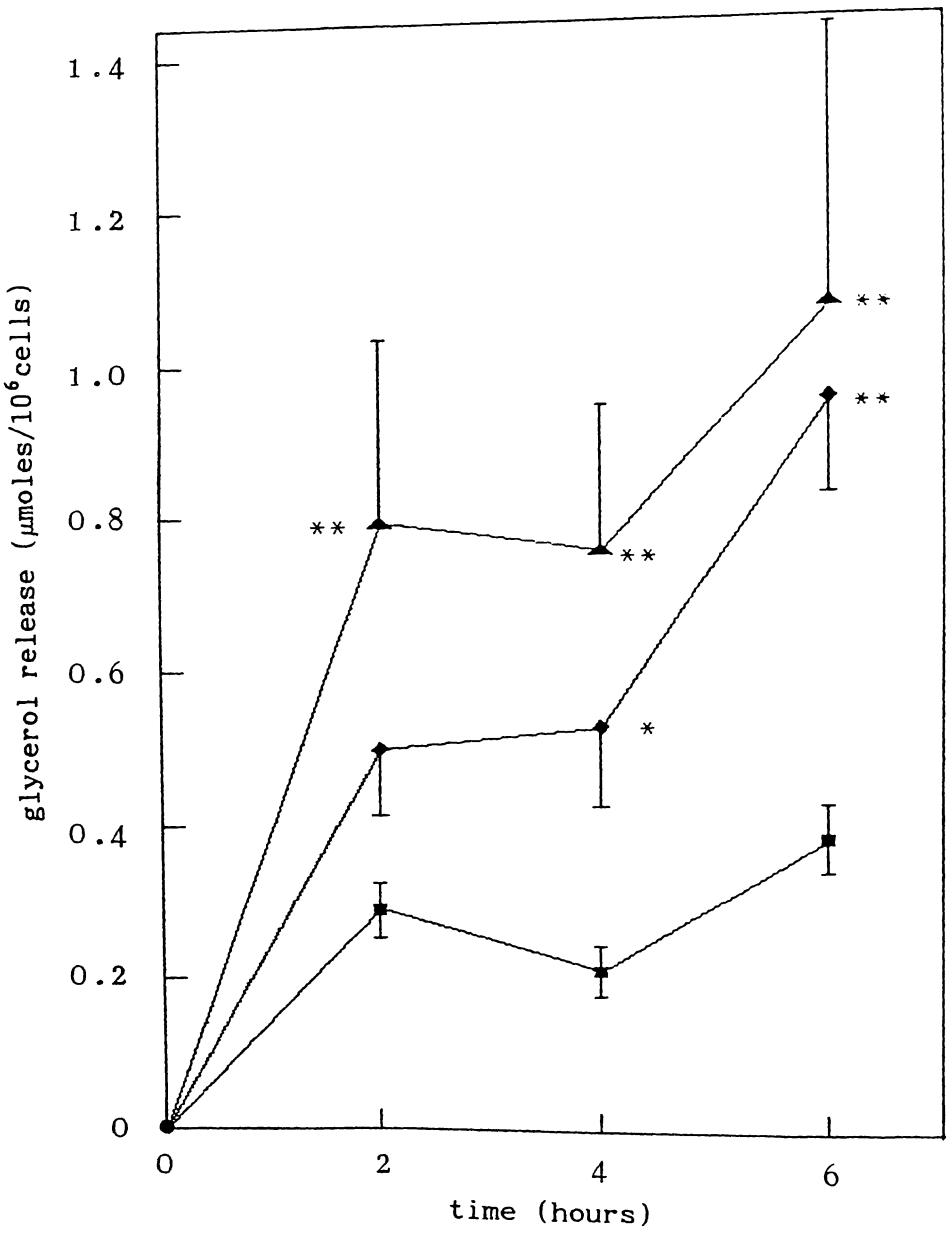
* p < 0.05
** p < 0.01
a p < 0.05
b p < 0.01

Figure 7.6 A comparison of the actions of IGF I, MSA, insulin and adrenalin on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.2. Insulin, IGF I and MSA were present where specified at a concentration of 0.4 ng/ml and adrenalin was at 50 ng/ml.

The effect of each hormone on the rate of glycerol release (μmoles/4 hours/10^6cells) is shown.

Figure 7.7



* p < 0.05

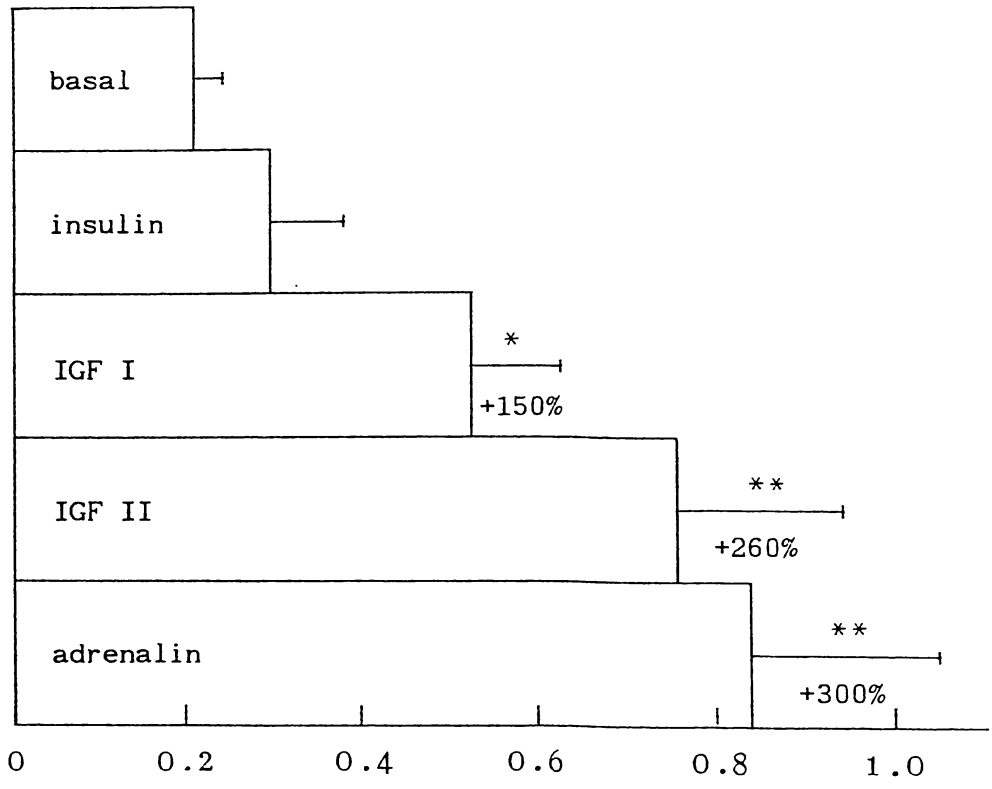
** p < 0.01

Figure 7.7 The effects of IGF I and IGF II on the rate of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with IGF) of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The rate of glycerol release (μmoles/10^6cells) is plotted against incubation time (hours).

Glycerol release with no hormone in the medium ■ , together with that in the presence of 0.4 ng/ml IGF I (IMCC) ◆ and 0.4 pg/ml IGF II ▲ are shown.

Figure 7.8



* $p < 0.05$

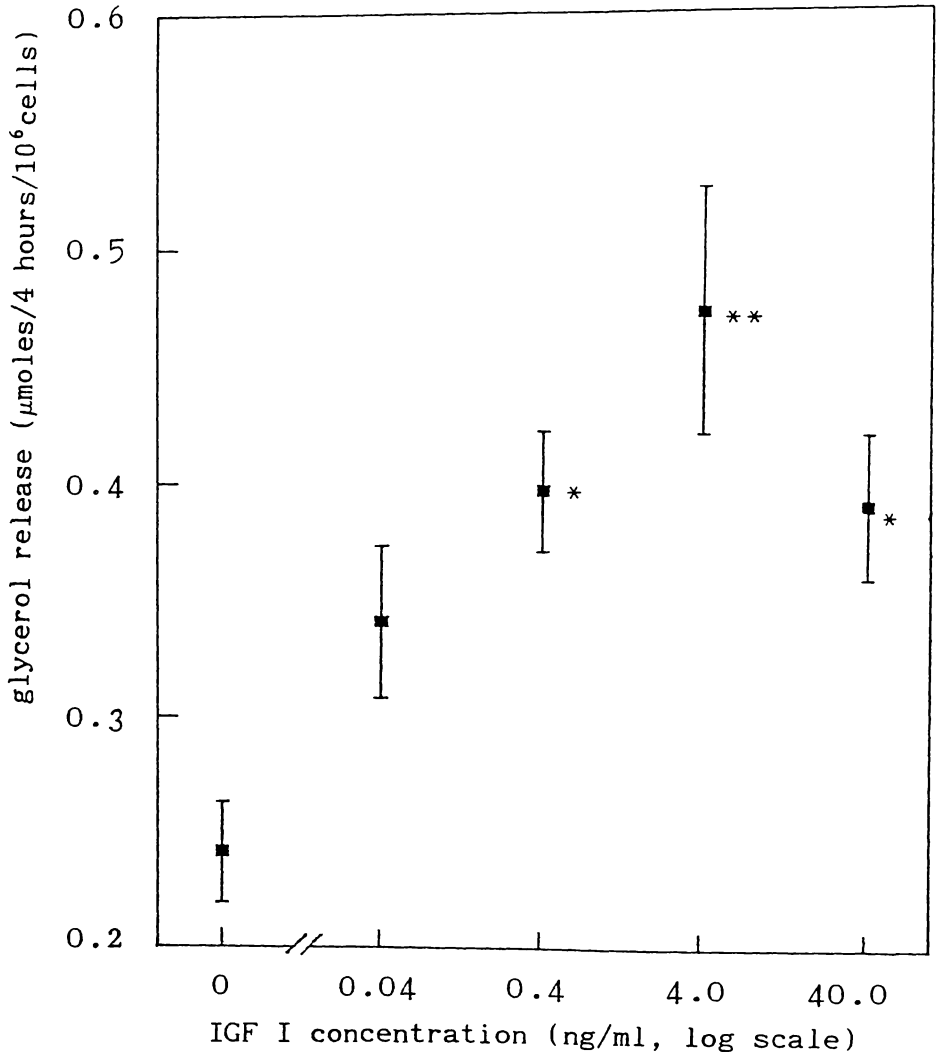
** $p < 0.01$

Figure 7.8 A comparison of the actions of IGF I, IGF II, insulin and adrenalin on the rate of glycerol release from ovine adipose tissue *in vitro*

The bars represent the mean and S.E.M. of replicate incubations (n = 6 for basal; n = 3 for each hormone) of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. IGF I was present where specified at a concentration of 0.4 ng/ml, IGF II at 0.4 pg/ml, insulin at 2.6 ng/ml and adrenalin at 50 ng/ml.

The effect of each hormone on the rate of glycerol release (μmoles/4 hours/10^6cells) is shown.

Figure 7.9



* $p < 0.05$

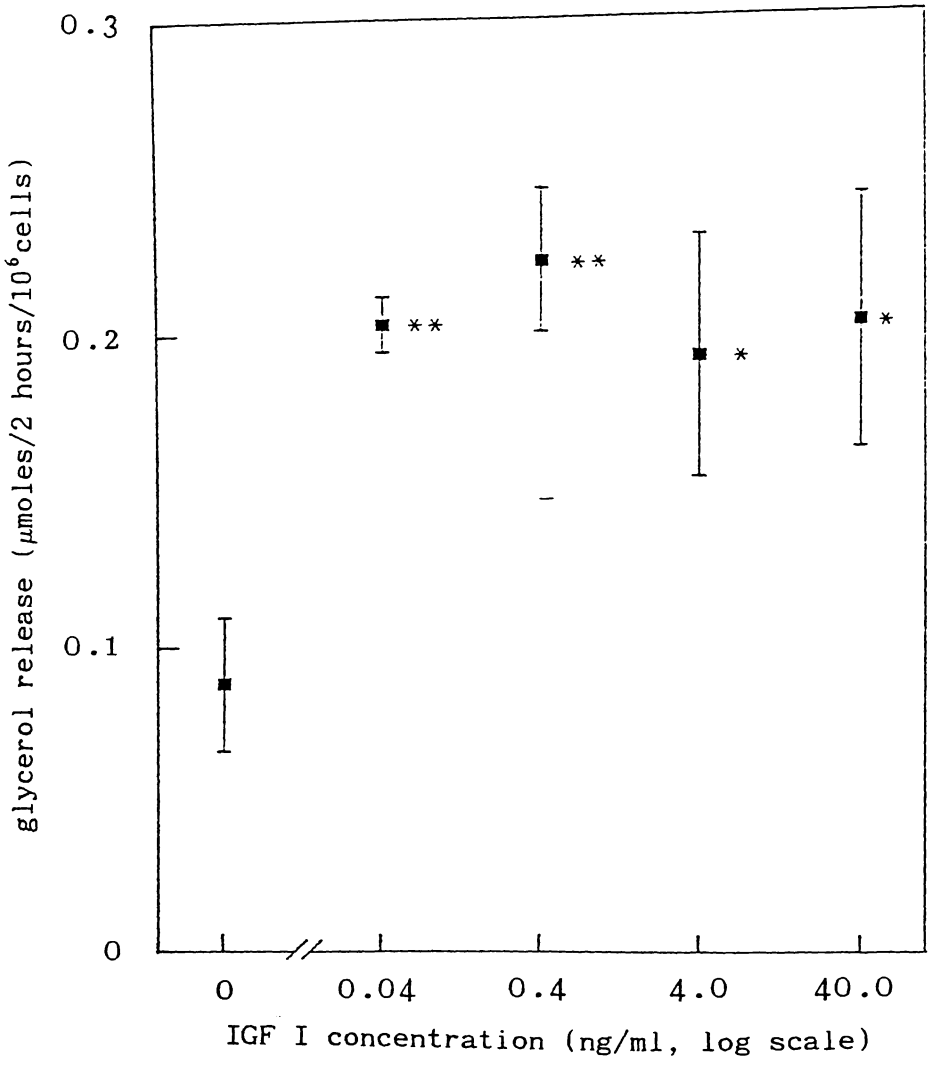
** $p < 0.01$

Figure 7.9 The effect of IGF I concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.1. The hormone used was recombinant DNA-derived hIGF I from IMCC.

The rate of glycerol release (μmoles/4 hours/10^6cells) is plotted against IGF I concentration (ng/ml).

Figure 7.10



* p < 0.05

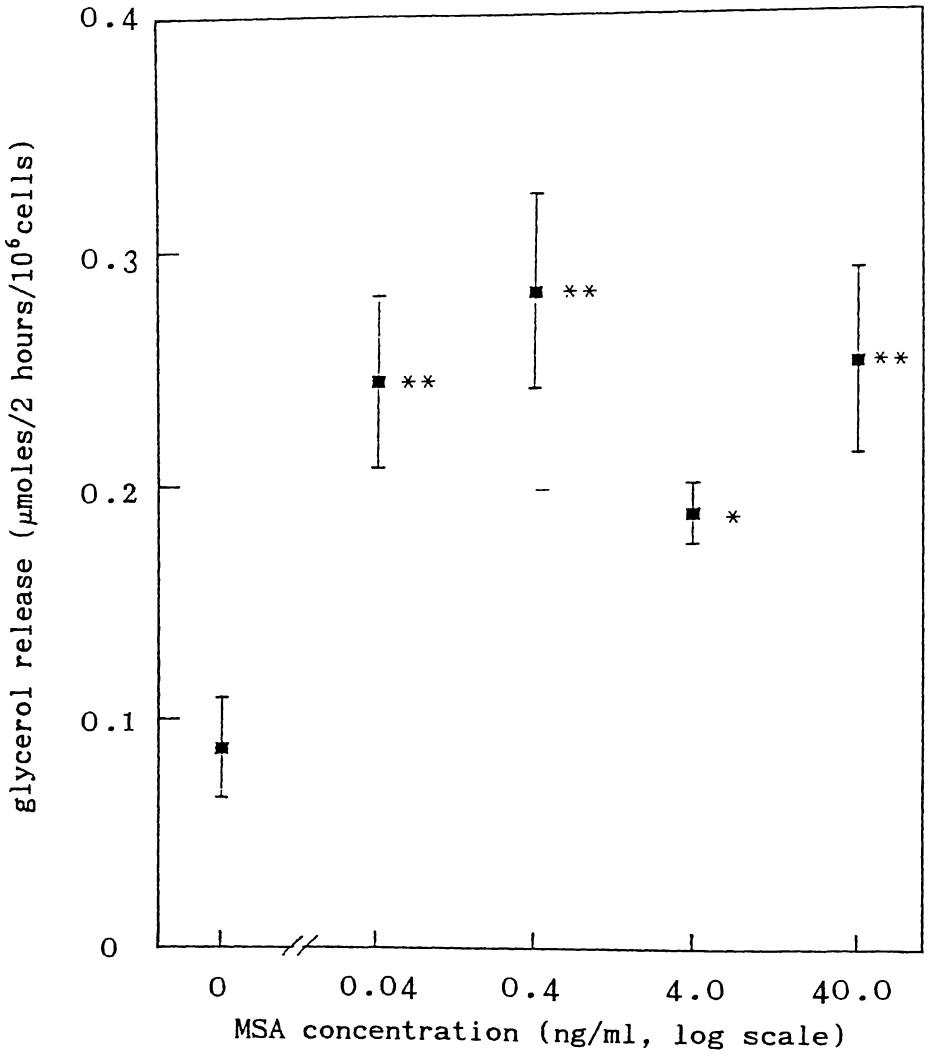
** p < 0.01

Figure 7.10 The effect of IGF I concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.1. The hormone used was recombinant DNA-derived hIGF I from Amgen.

The rate of glycerol release (μmoles/2 hours/10^6cells) is plotted against IGF I concentration (ng/ml).

Figure 7.11



* $p < 0.05$

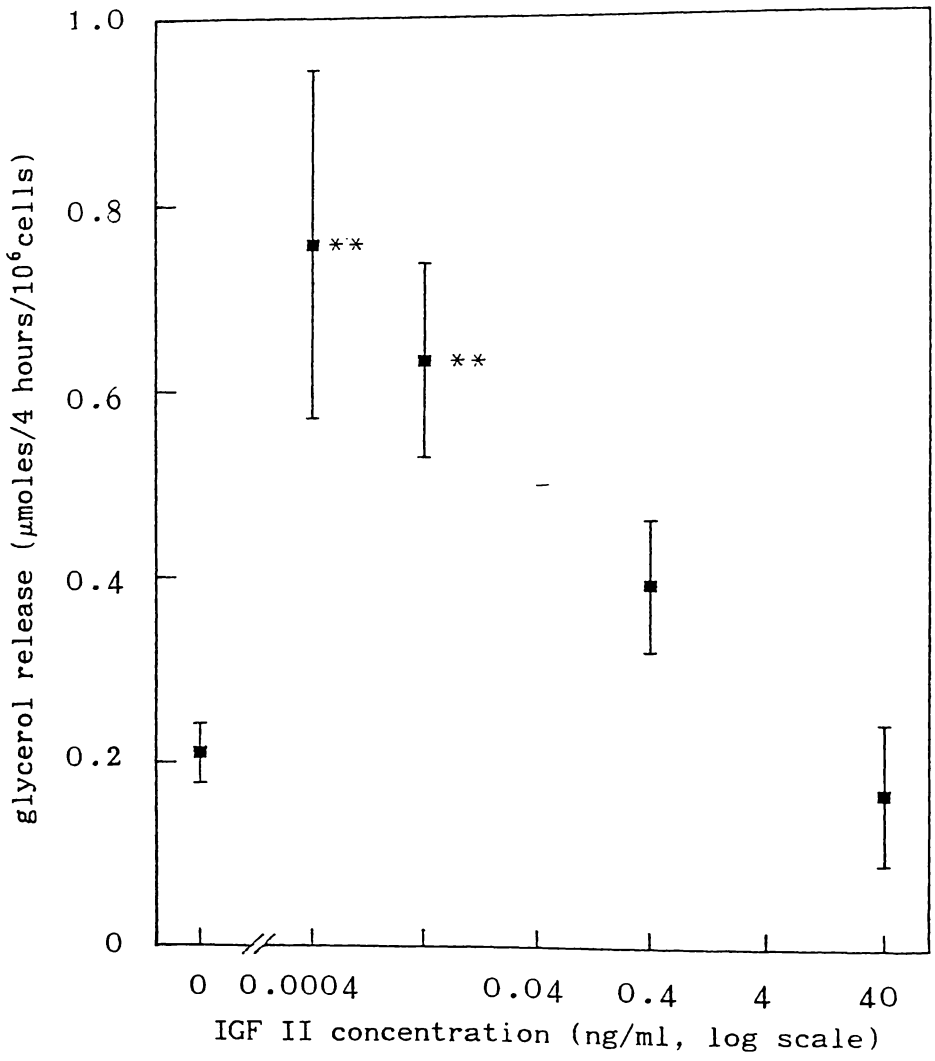
** $p < 0.01$

Figure 7.11 The effect of MSA concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given in Figure 7.1. The hormone used was biologically-derived MSA from Professor J.R. Florini.

The rate of glycerol release (μmoles/2 hours/10^6cells) is plotted against MSA concentration (ng/ml).

Figure 7.12



** p < 0.01

Figure 7.12 The effect of IGF II concentration on the rate of glycerol release from ovine adipose tissue *in vitro*

Each point represents the mean and S.E.M. of replicate incubations (n = 6 for basal, n = 3 with IGF II) of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. The hormone used was manually constructed hIGF II from Professor C.H. Li.

The rate of glycerol release (μmoles/4 hours/10^6cells) is plotted against IGF II concentration (ng/ml).

Chapter 8

A Comparison of Growth Hormone and Insulin-like Growth Factors

8.1 Introduction

Exogenous administration of GH to sheep has been found to decrease total fat deposition and increase the weight of protein depots in the carcass (Muir *et al.*, 1983; Johnsson and Hart, 1985). At the same time, levels of plasma NEFA rise (Hart *et al.*, 1984b). Plasma concentrations of IGFs are known to be GH dependent (see Section 1.10 and Chapter 9) and it is therefore possible that the resulting lipolytic activity in the adipose tissue could be due either to the actions of GH, IGF I, IGF II or another lipolytic agent under the control of any of these hormones.

It was therefore of interest to compare the actions of GH and IGF in ovine adipose tissue. In several experiments in the present study, both GH and IGF were tested simultaneously in separate incubations of tissue from the same animal. Thus the effects of the hormones on the rates of lipid metabolism could be directly compared.

8.2 The effects of growth hormone and insulin-like growth factors on the rate of lipogenesis

In the following experiments, the responses for only one concentration of GH and IGF are shown. However, in each case, other concentrations of the hormones were tested (see preceding chapters) and only the maximum response for each experiment is shown in this section. The concentration at which the hormones elicited their maximum response varied between experiments. Insulin was used at 2.6 ng/ml, which is less than its optimal concentration, because this was considered to be closer to the physiologically normal concentration. The responses to insulin at 2.6 ng/ml, as seen in Figure 4.6, are probably half-maximal.

To compare the effects of the hormones on the rate of lipogenesis, as discussed in Section 7.3, it was necessary to use a high concentration of IGF. The effects of insulin, GH and IGF I on the rate of acetate

incorporation into fatty acid are compared in Figure 8.1. Insulin, when present at a concentration of 2.6 ng/ml, stimulated the rate by 300% ($p < 0.05$) while IGF I, at a concentration of 40 ng/ml, stimulated the rate by 500% ($p < 0.001$). At the same time, 100 ng/ml of purified bGH stimulated the rate by 585% ($p < 0.01$) although 10 ng/ml of the same hormone had no effect. There was no significant difference between the lipogenic responses in the tissue to insulin, bGH (at 100 ng/ml) and IGF I in this experiment.

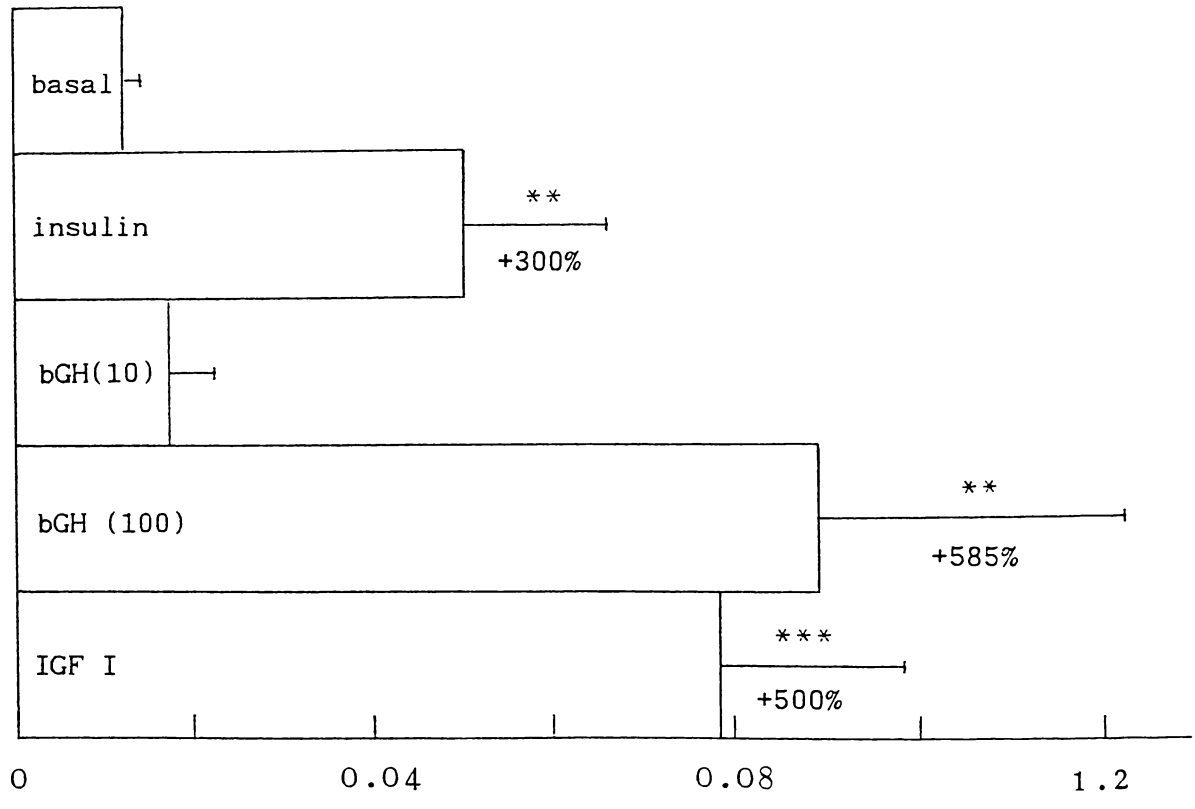
To illustrate the different responses to IGF I at different concentrations, a similar experiment is shown where the effects of the hormone on lipogenesis at lower concentrations were tested (Figure 8.2). IGF I was present at 0.4 ng/ml and had no effect on the rate of acetate incorporation into fatty acid, whereas recombinant DNA-derived bGH at 100 ng/ml stimulated the rate by 40% ($p < 0.05$). Insulin stimulated the rate by 91% ($p < 0.01$) while adrenalin had no effect. There was no significant difference between the lipogenic responses to insulin and GH in this experiment.

8.3 The effects of growth hormone and insulin-like growth factors on the rate of lipolysis

A comparison of the actions of insulin, IGF I, bGH and adrenalin on the rate of lipolysis were made and the results can be seen in Figure 8.3. In this experiment, IGF I was present at 0.4 ng/ml and stimulated the rate of glycerol release by 46% ($p < 0.05$) while adrenalin stimulated the rate by 38% ($p < 0.05$). There was no significant difference between the actions of IGF I and adrenalin. Neither insulin nor recombinant DNA-derived bGH had any effect on the rate of glycerol release.

As illustrated in Figure 8.4, a comparison was made between the effects of insulin, GH, IGF I and IGF II on the rate of glycerol release from ovine adipose tissue *in vitro*. Recombinant DNA-derived bGH, at a concentration of 100 ng/ml, stimulated the rate by 114% ($p < 0.01$) while purified bGH, at 50 ng/ml, stimulated the rate by 64% ($p < 0.01$). (See Figures 6.6, 6.7 and 6.8 which are the same experiment.) At the same time, IGF I stimulated the rate by 118% ($p < 0.01$) and IGF II by 85% ($p < 0.05$). There was no significant difference between the responses to either GH preparations, IGF I and IGF II in this experiment. Insulin had no significant effect on the rate of glycerol release.

Figure 8.1



** p < 0.01

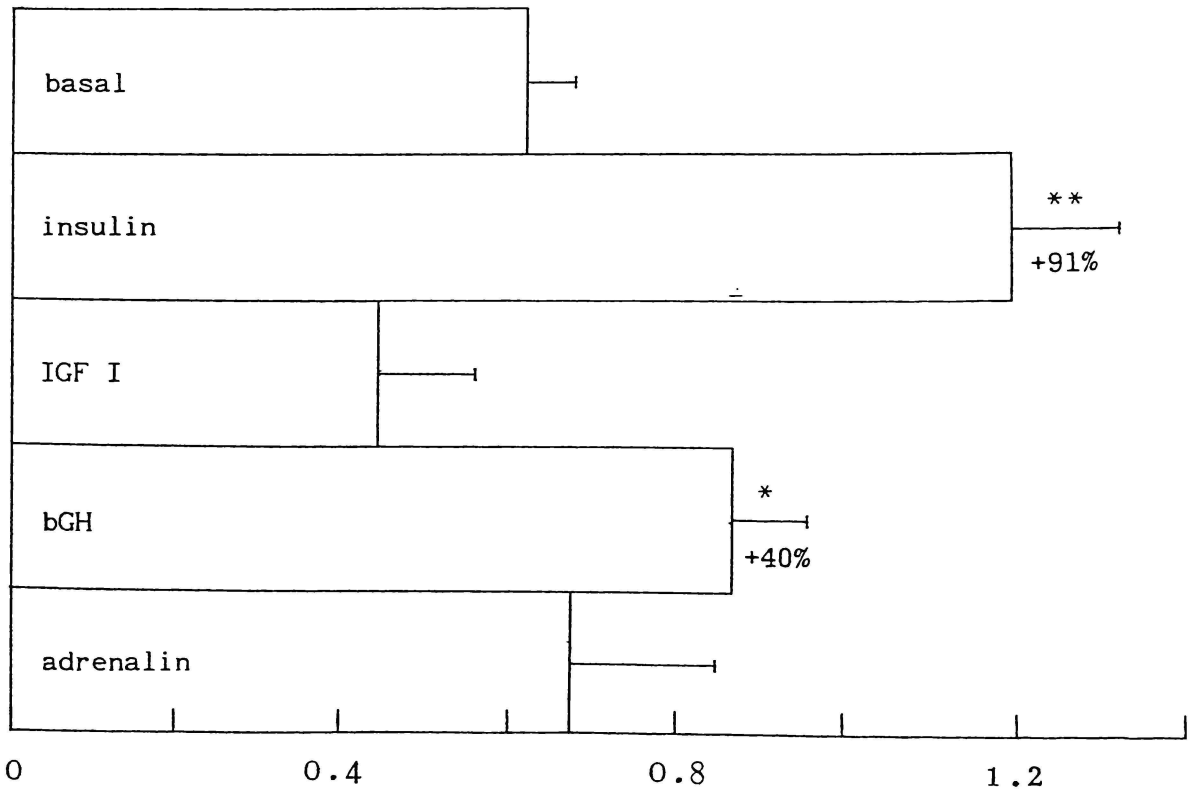
*** p < 0.001

Figure 8.1 A comparison of the actions of insulin, IGF I and bGH on the rate of acetate incorporation into fatty acid *in vitro*

The bars represent the mean and S.E.M. of triplicate incubations of ovine adipose tissue slices in Medium 199 containing 1% w/v BSA. Insulin was present where specified at 2.6 ng/ml, purified bGH at 10 and 100 ng/ml and recombinant DNA-derived hIGF I from Amgen at 40 ng/ml.

The effect of each hormone on the rate of acetate incorporation into fatty acid (μmoles/4 hours/10^6cells) is shown.

Figure 8.2



* $p < 0.05$

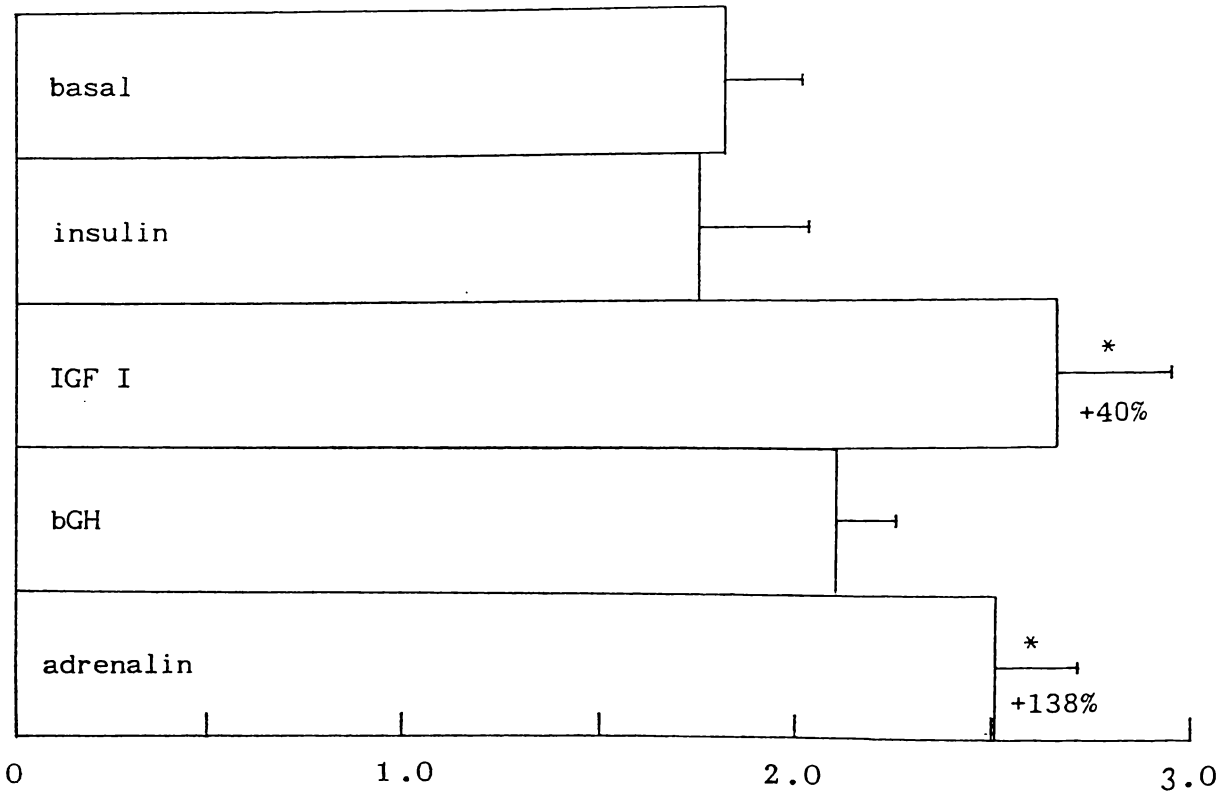
** $p < 0.01$

Figure 8.2 A comparison of the actions of insulin, IGF I, bGH and adrenalin on the rate of acetate incorporation into fatty acid *in vitro*

The experimental conditions were as given in Figure 8.1 but the bars represent the mean and S.E.M. of replicate incubations (n = 6 for basal; n = 3 with each hormone). Insulin was present where specified at 2.6 ng/ml, recombinant DNA-derived hIGF I from IMCC at 0.4 ng/ml, recombinant DNA-derived bGH from Cyanamid at 100 ng/ml and adrenalin at 50 ng/ml.

The effect of each hormone on the rate of acetate incorporation into fatty acid (μmoles/4 hours/10^6cells) is shown.

Figure 8.3



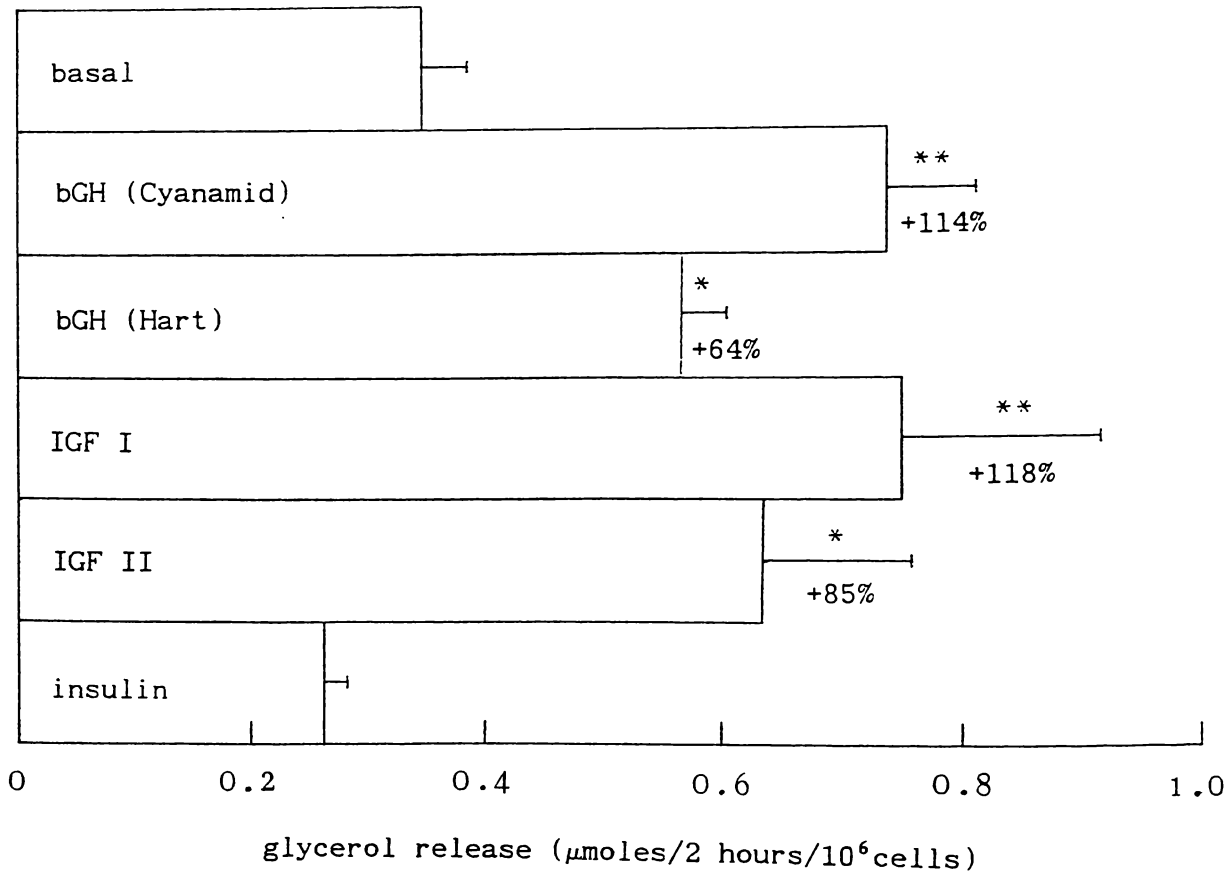
* $p < 0.05$

Figure 8.3 A comparison of the actions of IGF I, bGH, insulin and adrenalin on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given for Figure 8.2. Insulin was present where specified at a concentration of 2.6 ng/ml, recombinant DNA-derived hIGF I (IMCC) at 0.4 ng/ml, purified bGH at 100 ng/ml and adrenalin at 50 ng/ml.

The effect of each hormone on the rate of glycerol release (μmoles/4 hours/10^6cells) is shown.

Figure 8.4



* $p < 0.05$

** $p < 0.01$

Figure 8.4 A comparison of the actions of IGF I, IGF II, bGH and insulin on the rate of glycerol release from ovine adipose tissue *in vitro*

Experimental conditions were as given for Figure 8.2. Recombinant DNA-derived hIGF I (IMCC) and manually synthesized IGF II were present where specified at a concentration of 0.4 ng/ml, recombinant DNA-derived bGH from Cyanamid at 100 ng/ml, purified bGH from Dr I. Hart at 50 ng/ml and insulin at 2.6 ng/ml.

The effect of each hormone on the rate of glycerol release (μmoles/2 hours/10^6cells) is shown.

Chapter 9

Events In Vivo: Subsequent Effects In Vitro

9.1 Introduction

A number of studies where exogenous GH was administered to sheep have been reported. Wagner and Veenhuizen (1978) demonstrated significant growth-promoting effects following administration of GH to lambs. although this was not seen by others (Muir *et al.*, 1983). Both studies showed a decrease in carcass fat in the GH-dosed animals. On the other hand, Johnsson *et al.* (1985) have reported that administration of GH increased live-weight with little effect on carcass weight. However they observed a significant increase in the weight of lean tissue and bone and some decrease in the amount of fat in the carcass. The latter study supports the view that administration of GH increases the percentage of protein and decreases fat deposition in the carcass, as measured by chemical analysis.

The stimulation of endogenous insulin secretion in animals with circulating exogenous GH has been observed in several previous experiments (Wallace and Bassett, 1966; Davis *et al.*, 1970; Johnsson *et al.*, 1985). It has also been reported that exogenous bGH can increase the mean concentration of plasma prolactin in lactating cows (Bines *et al.*, 1980) and lambs (Johnsson *et al.*, 1985). GH is thought to be the dominant controlling factor for the release of IGFs and a marked rise in plasma levels of IGF following administration of GH has been demonstrated in sheep (Wien *et al.*, 1983), lactating cows (Davis *et al.*, 1984), pigs (Chung *et al.*, 1985) and humans (Copeland *et al.*, 1980).

Thus the evidence is that administration of GH *in vivo* causes marked physiological changes in both the rate of fatty acid metabolism in the adipose tissue and the hormones to which the adipose tissue is being exposed. The following experiment was designed with a view to investigating the effects of two months administration of GH *in vivo* on the basal rates of lipid metabolism in adipose tissue *in vitro*. In

addition, an investigation was carried out to determine whether there had been any alteration in the sensitivity of the tissue to bGH, insulin and IGF I as a result of the physiological changes *in vivo*.

9.2 Methods

Trials were being conducted by J Bass, W. Carter, P. Fowke and P. Gluckman to determine the effects of exogenous administration of recombinant DNA-derived bGH (from Cyanamid) on growth, carcass composition and plasma hormone concentrations in 40 wether lambs. It was these lambs which were used at slaughter for the experiments *in vitro* described in this chapter.

The lambs were Coopworth/Suffolk cross, aged 7 to 8 months, and weighed 37 - 48 kg at slaughter. They were allowed unrestricted access to pasture during the trial. There were 8 control animals which were not treated and 4 experimental groups of 8 animals with each experimental group receiving different fixed daily doses of bGH for a period of 2 months. Only one of the experimental groups was used for the *in vitro* experiment: those receiving a dose of 0.25 mg/kg/day bGH each. Tissue samples were taken at slaughter from 4 representative animals (2 with live-weights above the mean and 2 with live-weights below the mean) from the control group (referred to as Lambs K, L, M and N) and 4 similarly chosen from those receiving 0.25 mg/kg/day of bGH (referred to as Lambs P, Q, R and S).

During the *in vivo* part of the trial and at slaughter, the measurements made included the following:

(1) live-weight changes
(2) carcass composition using GR (see Section 3.2), the weights of internal depots of adipose tissue and analysis of chemical composition.
(3) hormone assays for plasma concentrations of GH, insulin and IGF I during the trial.
(4) levels of NADPH-generating enzymes in the adipose tissue at slaughter.

The animals were all slaughtered at the same time, using electrical stunning. Samples of subcutaneous adipose tissue were removed from the animals chosen for the *in vitro* experiment and placed immediately in Medium 199 at 39°C. The tissue from each animal was held in separate identifiable containers so that each could be incubated in isolation from tissue from the other animals. All tissue was collected within 15 minutes and the containers for each animal were treated identically during all experimental procedures.

Tissue preparation and incubation were as previously described (see Chapter 3). Replicate incubations (n = 6 for basal; n = 3 for each hormone) were carried out on tissue from each animal to determine their individual basal rates of lipid metabolism and the response to physiological concentrations of bGH (recombinant DNA-derived bGH from Cyanamid; the same hormone which the experimental group of animals had received *in vivo*), insulin and IGF I (recombinant DNA-derived hIGF I from IMCC) in the incubation medium. The tissue used in all incubations was pre-incubated in medium without added hormone for 3 hours prior to the start of the experiment.

9.3 Exogenous administration of bGH: effects *in vivo*

The results of the following investigations are given for the group of eight control animals and the eight animals which received 0.25 mg/kg/day bGH. The S.E.D. is the pooled standard error of the difference for the entire group of 40 animals. All data has been adjusted to a constant initial live-weight. A team of staff from Ruakura Agricultural Research Centre, who were under the guidance of Dr J. Bass and Ms W. Carter, were responsible for measuring the weights of the animals, carcasses and tissue depots.

Live-weight changes

The live-weights, determined as the animals were taken off pasture, increased from 40.58 kg in the control group to 44.11 kg in the bGH-dosed group (S.E.D. = 1.06; $p < 0.01$). The effects of bGH on the live-weight were not evident until the second month of the trial.

Hot carcass weight

The hot carcass weight increased from 19.0 kg in the control group to 20.4 kg in the bGH-dosed group (S.E.D. = 0.61; $p < 0.05$).

GR

The average GR measurement for the control group was 11.3 and for the bGH-dosed group was 7.6 (S.E.D. = 1.20). This was an indication of a significant decrease ($p < 0.01$) in subcutaneous adipose tissue in the bGH-dosed group of animals.

Perirenal adipose tissue

The average weight of this adipose tissue depot was 336 g in the control group and 283 g in the bGH-dosed group (S.E.D. = 35.5 g; NS).

Omental adipose tissue

The average weight of the omental adipose tissue depot decreased from 754 g in the control group to 450 g in the bGH-dosed animals (S.E.D. = 90.9; $p < 0.01$).

Mesenteric adipose tissue

The average weight of the mesenteric adipose tissue was 402 g in the control group and 360 g in the bGH-dosed animals (S.E.D. = 35.5; NS).

Table 9.1 The effects of exogenous bGH on the chemical composition of the carcass

The bGH-dosed group of animals received 0.25 mg/kg/day exogenous bGH for 2 months prior to slaughter and the control group did not. The chemical composition of a sample of a minced half-carcass from each animal is given.

	Control group (n = 8)	bGH-dosed group (n = 8)	S.E.D.	p
% fat	23.6	16.6	1.06	< 0.001
% protein	16.6	17.8	0.33	< 0.01
% water	55.6	61.3	0.83	< 0.001
% ash	4.3	4.3	0.08	NS

Chemical composition of the carcass

The results for the chemical analysis of a sample of the minced half-carcass are given in Table 9.1. These assays were carried out by Ms K. Wentworth under the guidance of Ms B. Crane using the method described in Section 2.6.

The presence of exogenenous bGH has increased the percentage protein ($p < 0.01$) and water ($p < 0.001$) in the carcass while decreasing the percentage fat ($p < 0.001$). The percentage ash remained unchanged.

Enzymes present in the subcutaneous adipose tissue

The maximum activities of the enzymes involved in the production of NADPH in the subcutaneous adipose tissue were measured under optimised conditions and the results are given in Table 9.2. The presence of exogenous bGH in these animals decreased the activities of all NADPH-generating enzymes measured, with the exception of ICDH. The decrease for 6-PGDH and MDH was significant ($p < 0.01$) although it was not significant for G-6-PDH. These assays were carried out by Ms R. Ratcliff under the guidance of Dr G. Upreti using the method described in Section 2.7.

Table 9.2 The effects of exogenous bGH on the amount of NADPH-generating enzymes present in the adipose tissue

The means and S.E.M of the activities of NADPH-generating enzymes in the subcutaneous adipose tissue of the control group and those which received 0.25 mg/kg/day exogenous bGH are given. The enzymes measured were glucose-6-phosphate dehydrogenase (G-6-PDH); 6-phosphogluconate dehydrogenase (6-PGDH); NADP-malate dehydrogenase (decarboxylating) (MDH); isocitrate dehydrogenase (decarboxylating) (ICDH). All enzyme activities are expressed in terms of enzyme units per g of lipid in the sample. The probablity that there is a difference between the two groups is also given.

	Control group (n = 8)	bGH-dosed group (n = 8)	p
G-6-PDH	0.25 ± 0.05	0.16 ± 0.01	NS
6-PGDH	0.34 ± 0.03	0.23 ± 0.01	< 0.01
MDH	0.020 ± 0.002	0.014 ± 0.001	< 0.01
ICDH	0.36 ± 0.02	0.37 ± 0.01	NS

Hormone assays

In the control group, the baseline plasma GH concentration was 2 - 3 ng/ml while, in the group receiving exogenous bGH, the GH concentration in the plasma reached a maximum of 500 ng/ml 2 hours after administration and returned to 30 - 40 ng/ml 15 hours after administration.

Plasma insulin levels in the control group averaged 0.59 ng/ml (n = 8) while, in the bGH-dosed group, they averaged 0.98 ng/ml (n = 8). The pooled S.E.M. was 0.06 ng/ml and the difference between the two groups was significant ($p < 0.001$).

In the control group, the plasma IGF I concentration varied from 30 - 40 ng/ml (S.E.M. = 5 ng/ml; n = 8) while in the group receiving exogenous bGH, the concentration varied from 100 - 140 ng/ml (S.E.M. = 15 ng/ml; n = 8). This represents a highly significant increase in the bGH-dosed group ($p < 0.001$).

Administration of exogenous bGH to the lambs therefore resulted in increased plasma levels of GH, insulin and IGF I.

Radioimmunoassays for insulin were carried out by Mr T. Braggins and for IGF I and GH by Ms K. Crossley under the guidance of Professor P. Gluckman using the methods described in Section 2.4.

9.4 Administration of bGH *in vivo*: effects *in vitro*

Although each parameter of lipid metabolism presented in the following section was determined by separate incubations of tissue for each animal studied, the results for the four animals in each group were pooled to determine, by Student's t test, the overall significance of the differences caused by the presence of exogenous bGH *in vivo*. When hormones were added to the incubation medium, the change in rate of metabolic activity as a result of the action of the hormone was pooled for each group of animals and compared. This is referred to as the response to the hormone. The change in the rate due to the action of the hormone was also expressed as a percentage response.

Administration of bGH *in vivo*: effects on basal rates of fatty acid metabolism

Adipose tissue slices from 4 control animals (Lambs K, L, M and N) and 4 which received 0.25 mg/kg/day exogenous bGH (Lambs P, Q, R and S) were incubated separately in Medium 199 with no added hormone to determine the basal rate of lipid metabolism *in vitro*. The rate of lipogenesis in tissue from these lambs was estimated using the rate of incorporation of acetate into the tissue fatty acids. The results are presented in Figure 9.1 and show an overall significant decrease of the rate of lipogenesis in the tissue from the bGH-dosed animals as compared with the control animals ($p < 0.001$).

The rate of lipolysis in tissue from these animals was estimated using the rate of glycerol release from the tissue. The results are given in Table 9.3 and indicate that there is little difference in the average rate of lipolysis in tissue from the two groups of animals. However, in the tissue from one of the bGH-dosed animals, there was a marked decrease in the rate of lipolysis (Lamb S).

Table 9.3 The effect of exogenous bGH *in vivo* on the rate of lipolysis *in vitro*

Following administration of exogenous bGH to lambs in vivo, *subcutaneous adipose tissue from each of the four animals (Lambs P, Q, R and S) was incubated* in vitro *and the rate of glycerol release into the medium measured. The rate of glycerol release was compared to that from tissue taken from 4 control animals (Lambs K, L, M and M).*

Lamb	rate of glycerol release (μmoles/4 hours/10^6cells)	S.E.M. n = 6
M	0.88	0.03
K	0.65	0.05
N	0.61	0.07
L	0.50	0.01
Average K L M N	0.66 ± 0.03	
P	0.69	0.60
R	0.51	0.03
Q	0.36	0.02
S	0.14	0.01
Average P Q R S	0.44 ± 0.04	

There was no significant difference between the two groups in the amount of fatty acid being re-esterified or the amount of fatty acid leaving the cell (results not shown). There was a small, non-significant decrease in the latter in the bGH-dosed animals as compared with the control group. This was one of the factors which lead to a significant reduction ($p < 0.001$) in the rate of fatty acid accumulation in the tissue from the animals which received bGH *in vivo* (Figure 9.2).

The percentage of recycling of fatty acid in the tissue was also estimated and the results are presented in Figure 9.3. There is some evidence that the administration of exogenous bGH to the animals resulted in a slight but significant decrease in fatty acid recycling in the adipose tissue *in vitro* ($p < 0.05$). The rate decreased from an average of 93.4% in the control group to an average of 84.1% in the bGH-dosed group.

Administration of bGH *in vivo*: effects on insulin, bGH and IGF I action *in vitro*

The effects of the presence of exogenous GH in lambs on the subsequent action of insulin, bGH and IGF I in the adipose tissue *in vitro* were also investigated. Tissue from the control animals and from those given exogenous bGH were incubated separately to determine the response to 2.6 ng/ml insulin, 100 ng/ml bGH or 0.7 ng/ml IGF I in the medium. The presence of exogenous bGH *in vivo* did not appear to alter the responses to IGF I or bGH *in vitro* although considerable variation between responses in tissue from different animals may have masked a real change (results not shown). Thus more experiments with larger groups of animals will have to be carried out before any conclusions can be made.

On the other hand, exogenous bGH interfered with the ability of insulin to stimulate the rate of fatty acid manufacture in the adipose tissue (Figure 9.4). The effects of insulin on the actual amounts of fatty acid manufactured in tissue from each animal are given in Table 9.4. When the results for each group were pooled, the response to insulin in the tissue from the bGH-dosed group, in terms of the amount of lipogenesis, was significantly decreased ($p < 0.001$). However, the percentage stimulation of the rate of lipogenesis by insulin for each group was the same, with the average stimulation being approximately 60% in each case (Table 9.4).

The presence of the exogenous bGH *in vivo* also affected the ability of insulin to enhance the rate of fatty acid accumulation (Figure 9.5): when the results were pooled, the response in the bGH-dosed group was significantly lower than in the control group ($p < 0.05$). There is some evidence of a differences between the two groups in the percentage change in the rate as a result of the action of insulin (Table 9.5). Insulin increased the rate of fatty acid accumulation in tissue from three out of four of the animals in the control group by more than 300% and yet in tissue from three out of four of the bGH-dosed group, the response was less than 100%. On the other hand, as with bGH and IGF I, the variation between responses in tissue from different animals is such that no conclusions can be made until more animals are tested.

Figure 9.1

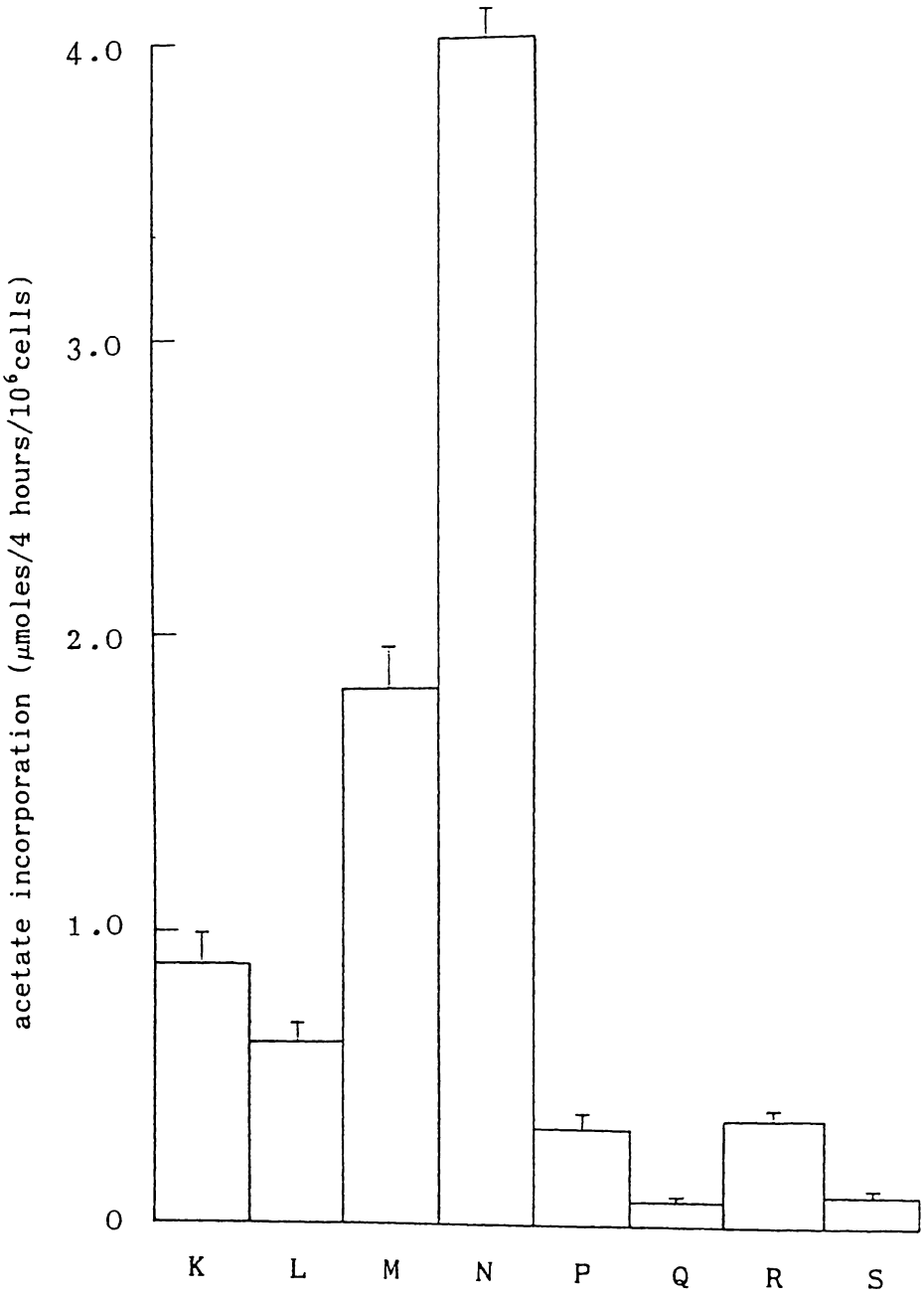

4.0
3.0
2.0
1.0
0
acetate incorporation (μmoles/4 hours/10^6cells)
K
L
M
N
P
Q
R
S

Figure 9.1 The effect of exogenous bGH *in vivo* on the basal rate of acetate incorporation into fatty acid *in vitro*

Each bar represents the mean and S.E.M. of 6 replicate incubations of adipose tissue slices from separate lambs carried out at the same time. The tissue, which was incubated in Medium 199 containing 1% w/v BSA and no added hormone, was pre-incubated for 3 hours prior to the start of the experiment. The rate of acetate incorporation into tissue fatty acids (μmoles/4 hours/10^6cells) is given.

The letters at the bottom of the histogram give the identity of each lamb. Lambs K, L, M and N were control animals while Lambs P, Q, R and S received 0.25 mg/kg/day exogenous bGH for two months prior to slaughter for this experiment.

Figure 9.2

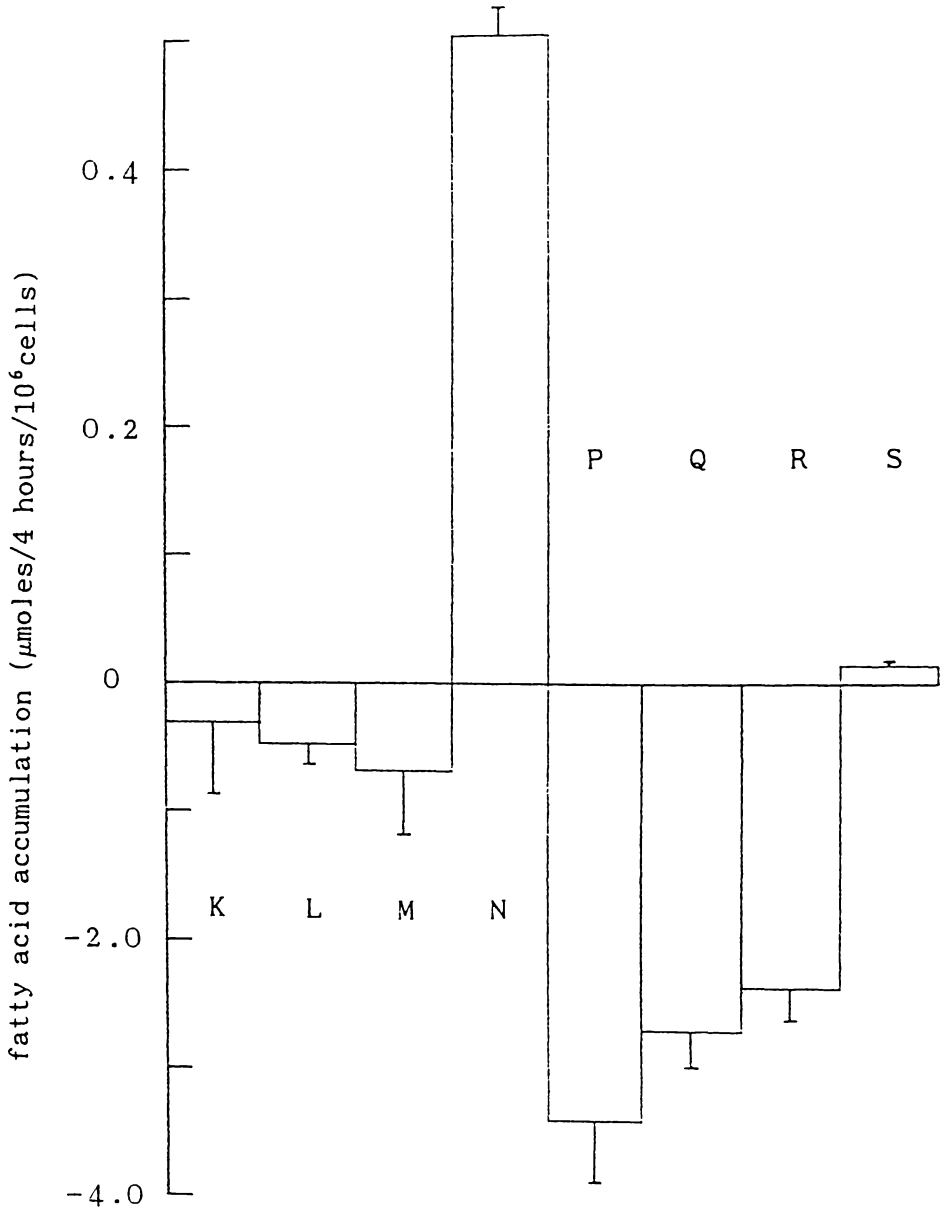

fatty acid accumulation (μmoles/4 hours/10⁶cells)
0.4
0.2
0
-2.0
-4.0
K
L
M
N
P
Q
R
S

Figure 9.2 The effect of exogenous bGH *in vivo* on the basal rate of fatty acid accumulation *in vitro*

The experimental conditions were as given in Figure 9.1. The method for calculation of fatty acid accumulation is detailed in Section 3.16. The rate of fatty acid accumulation is given in units μmoles/4 hours/10^6cells.

Lambs K, L, M and N were the control animals and P, Q, R and S received exogenous bGH *in vivo*.

Figure 9.3

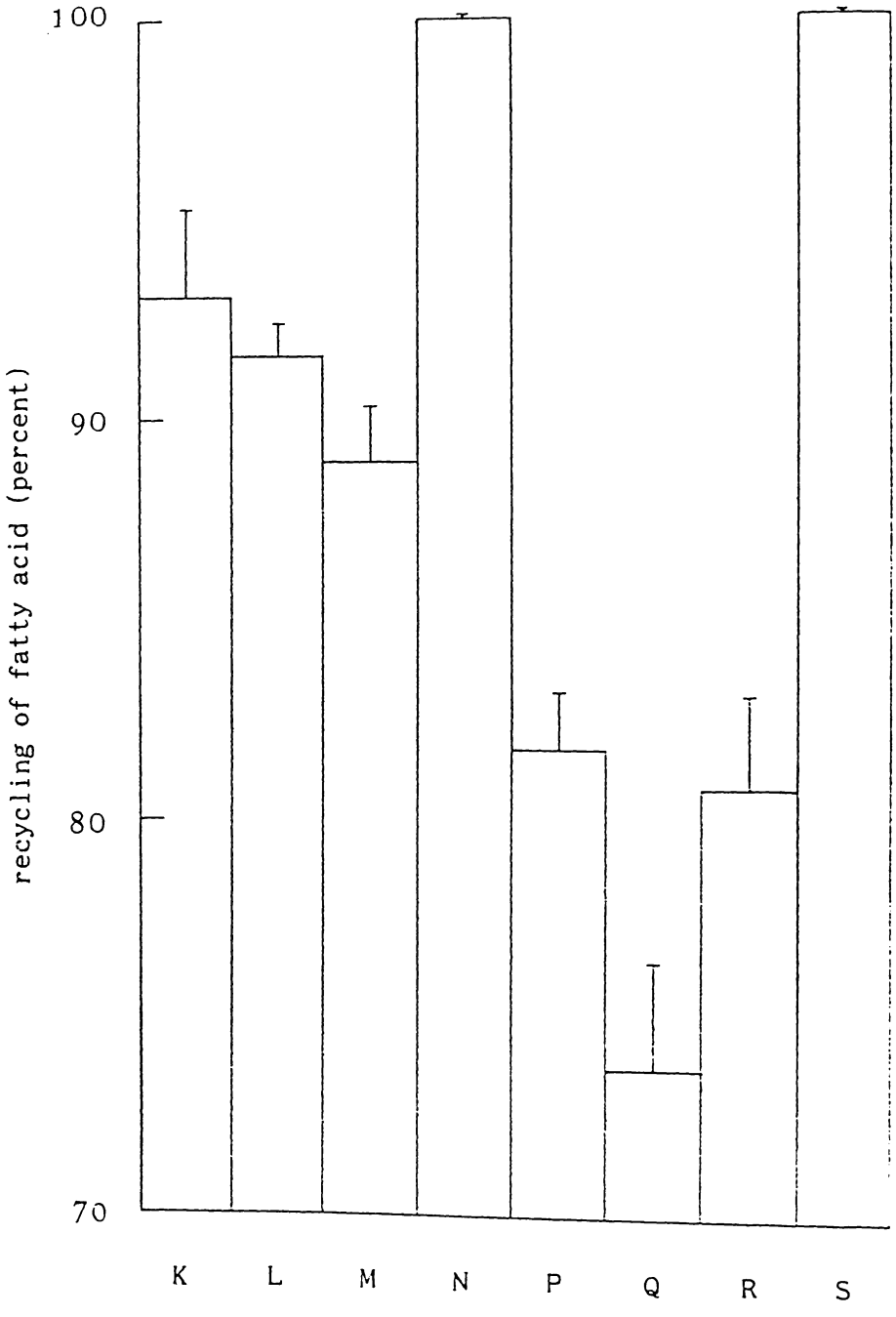


Figure 9.3 The effect of exogenous bGH *in vivo* on the rate of fatty acid recycling *in vitro*

The experimental conditions were as given in Figure 9.1. Each bar represents the percentage of fatty acid recycling in the adipose tissue *in vitro*. The method for calculation of this parameter is detailed in Section 3.16.

Lambs K, L, M and N were control animals while P, Q, R and S received exogenous bGH *in vivo*.

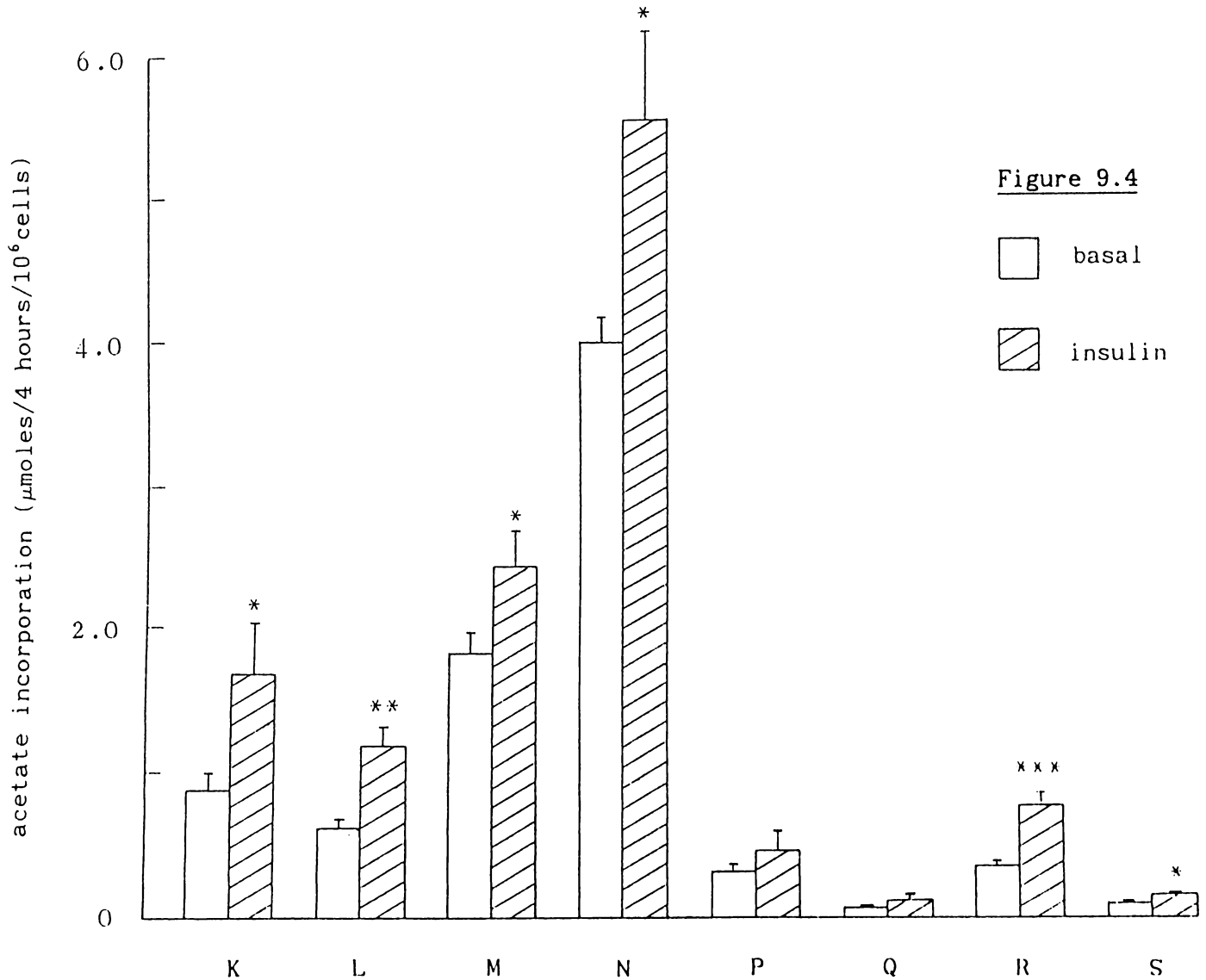

6.0
4.0
2.0
0
acetate incorporation (μmoles/4 hours/10^6cells)
basal
insulin
K
L
M
N
P
Q
R
S
* p < 0.05
** p < 0.01
*** p < 0.001

Figure 9.4

Figure 9.4 The effect of exogenous bGH *in vivo* on the ability of insulin to alter the rate of lipogenesis *in vitro*

The experimental conditions were as given in Figure 9.1 except that the effects of 2.6 ng/ml insulin in the incubation medium were also determined.

Each bar represents the mean and S.E.M. of the basal rate of acetate incorporation into fatty acid (n = 6) and the rate in the presence of insulin (n = 3). The rates are given in units of μmoles/4 hours/10^6cells. The percentage change with insulin and the probability that the difference between the rates for each lamb was significant are given in Table 9.4.

Lambs K, L, M and N were control animals and P, Q, R and S received exogenous bGH *in vivo*.

Table 9.4 The effect of exogenous bGH *in vivo* on the ability of insulin to alter the rate of lipogenesis *in vitro*

The experimental conditions were as given in Figure 9.1 except that the effects of 2.6 ng/ml of insulin in the incubation medium were also determined. The additional acetate incorporated into fatty acid as a result of the action of insulin in vitro *is given, together with percentage stimulation compared with the basal rate and the probability that the stimulation by insulin was significant for tissue from each animal. The actual rates, both the basal (n = 6) and in the presence of insulin (n = 3) are given in Figure 9.4, as are the identities of the lambs.*

Lamb	additional lipogenesis (μmoles/4 hours/10^6cells)	change with insulin	p
K	0.79 ± 0.37	+ 89%	<0.05
L	0.57 ± 0.14	+ 91%	<0.01
M	0.62 ± 0.29	+ 34%	<0.05
N	1.55 ± 0.63	+ 38%	<0.05
P	0.14 ± 0.14	+ 42%	NS
Q	0.05 ± 0.04	+ 56%	NS
R	0.41 ± 0.08	+111%	<0.001
S	0.05 ± 0.01	+ 46%	<0.05

Table 9.5 The effect of exogenous bGH *in vivo* on the ability of insulin to alter the rate of fatty acid accumulation *in vitro*

The experimental conditions were as given in Figure 9.1 except that the effects of 2.6 ng/ml of insulin in the incubation medium were also determined. The additional fatty acid accumulated in the tissue as a result of the action of insulin in vitro *is given, together with percentage change compared with the basal rate and the probability that the change was significant for tissue from each animal. The actual rates, both for the basal (n = 6) and in the presence of insulin (n = 3) are given in Figure 9.5, as are the identities of the lambs.*

Lamb	additional accumulation (μmoles/4 hours/10^6cells)	change with insulin	p
K	0.19 ± 0.08	+613%	NS
L	0.15 ± 0.03	+306%	<0.001
M	0.23 ± 0.09	+327%	<0.05
N	0.19 ± 0.08	+ 39%	<0.05
P	0.15 ± 0.10	+446%	NS
Q	-0.01 ± 0.08	- 4%	NS
R	0.16 ± 0.06	+ 70%	<0.05
S	0.01 ± 0.002	+ 49%	<0.001

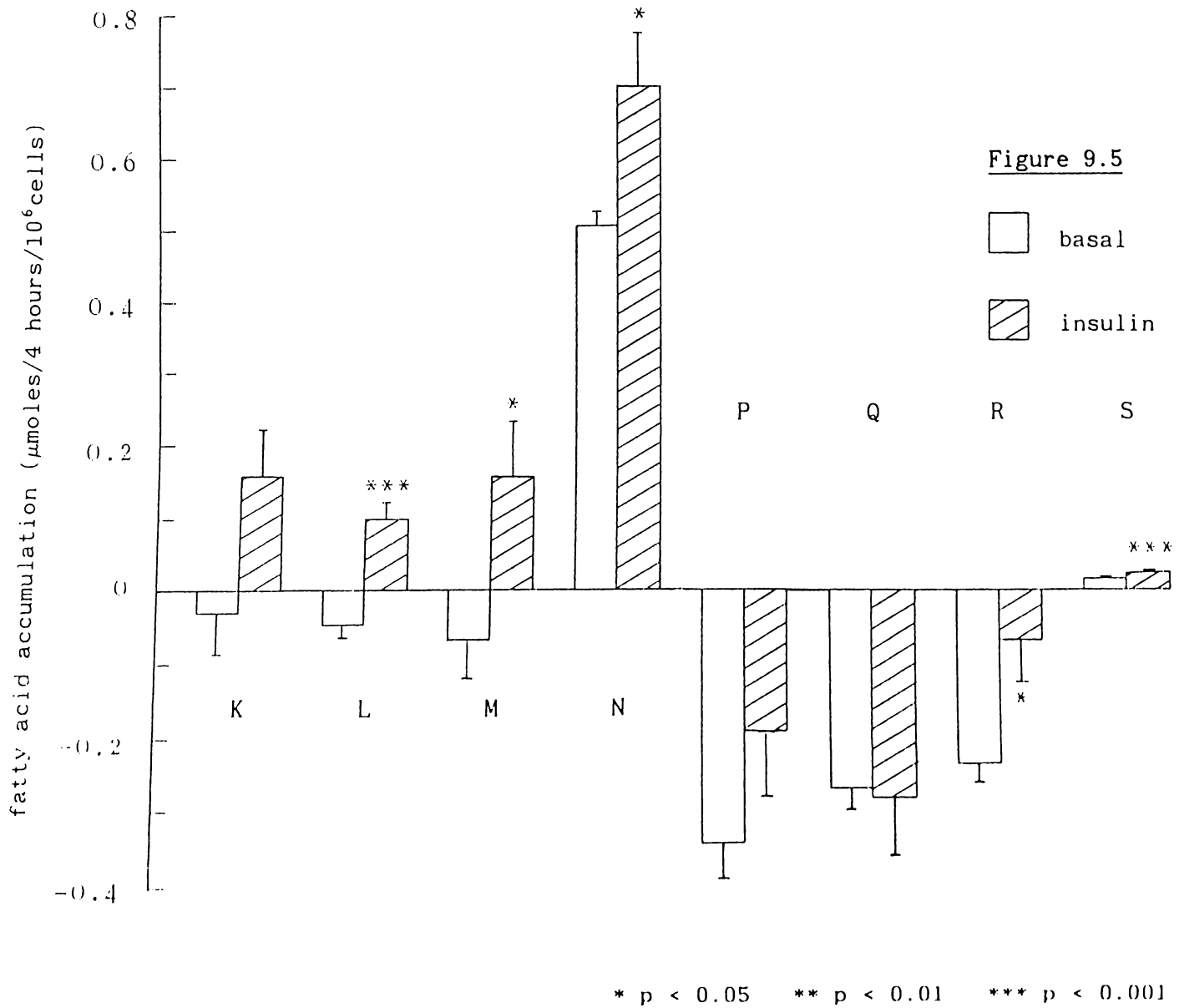


Figure 9.5

* p < 0.05 ** p < 0.01 *** p < 0.001

Figure 9.5 The effect of exogenous bGH *in vivo* on the ability of insulin to alter the rate of fatty acid acumulation *in vitro*

The experimental conditions were as given in Figure 9.1 except that the effects of 2.6 ng/ml insulin in the incubation medium were also determined. The method for calculation of fatty acid accumulation is detailed in Section 3.16.

Each bar represents the mean and S.E.M. of the basal rate of fatty acid accumulation (n = 6) together with that which occurred with insulin (n = 3). The rates are given in units of μmoles/4 hours/10^6cells. The percentage change with insulin and the probability that the change was significant are given in Table 9.5.

Lambs K, L, M and N were control animals while P, Q, R and S received exogenous BGH *in vivo*.

Chapter 10

Discussion

10.1 *In vitro* techniques

Introduction

The technique for the incubation of adipose tissue *in vitro* has several inherent faults which lead to considerable variability in the measurements of metabolic activity in replicate samples. If the variability between replicates is large, then only marked responses to hormones will be demonstrated as being significant. With other responses, the change in the metabolic rate can be so small relative to the error in the measurement that statistical analysis shows the results to be "not significant". This does not mean necessarily that the hormone had no effect on the tissue and the same response *in vivo*, where it may be sustained for months or even years, could result in profound changes in the body composition of the animal.

To be able to demonstrate these small changes in a 6 hour incubation *in vitro*, it was therefore important that the variability between replicates was minimized. To achieve this, the experimental conditions were adjusted to be as near as possible to those *in vivo*. Section 10.1 of this chapter deals with some of the problems encountered when attempts were being made to minimize experimental error.

The preparation of the tissue and the form in which it would be incubated was of prime concern. Also the composition of the medium was thought to be a factor which could contribute to variability in the metabolic rates observed. The use of a chemically defined medium may have overcome this but it was found that the addition of BSA was necessary not only for normal lipid mobilization from the tissue to occur but also to stabilize the hormones being added.

Once the incubation was complete, the chemical analysis of the products of the metabolism also contributed to the overall experimental error, as demonstrated in Chapter 3. Another aspect was the method used to express the metabolic data.

There are obviously many causes of experimental error in a technique which attempts to remove tissue from its normal environment without interupting the metabolic processes that were occurring. But once the errors had been minimized, certain limits were placed on the interpretation of the results obtained. Thus, statistical analysis of the data has been employed and only those results which were clearly significant, despite experimental error, have been discussed. More subtle changes in response to the hormones await the development of methods with smaller experimental errors.

Adipose tissue slices and isolated adipocytes: advantages and disadvantages.

One of the ways in which experimental error can increase is with the use of radioactively labelled precursor molecules to measure lipogenesis *in vitro*. When the labelled precursor molecules are added to the incubation vessel, their specific activity is determined by the amount of unlabelled precursor molecules already present. During the transition phase, as the radioactively labelled molecules pass into the cell and begin to be incorporated into the fatty acid chain (as described in Section 1.1), there are pools of unlabelled precursor molecules and intermediates already present in the cells. The concentrations of each of these unlabelled pools is not known and could vary considerably, even between replicate samples of tissue. Thus, in the initial stage of the incubation, the specific activity of the radioactive component in the cell will change. A steady state will eventually be reached, when all the pools of precursors and intermediates present in the system will be labelled with a single specific activity throughout. The time taken for this steady state to be achieved is not known and will depend on the rate of each of the processes involved. If a steady rate of incorporation of the precursor is observed from the beginning of the incubation (as in Figure 4.1), then this will indicate that a new steady state has probably been reached rapidly. However, if any of the reactions involved are slow, then only a gradual movement towards steady state will be observed and the rate of incorporation will change with time during the incubation, as could be occurring in the first four hours of incubation in Figure 4.2.

One of the principle advantages of the use of isolated adipocytes is that it simplifies the problem of having a different specific activity of labelled precursors in the medium, the interstitial space and the cell cytoplasm. With isolated adipocytes, there is no interstitial space and the cell membrane forms the only barrier between the medium and the cytoplasm. However, this problem in tissue slices can be largely overcome by observation of the variation in precursor uptake with time (see Figure 4.1). Any rise or fall in the specific activity of the precursor in the interstitial space or cell cytoplasm will be followed by an alteration in the slope of the time-course graph.

In isolated adipocytes, the hormones would appear to have more direct access to the cell membrane than in tissue slices. However, in several studies, it has been reported that the sensitivity of the tissue to ovine growth hormone (Duquette *et al.*, 1984) and insulin (Etherton and Chung, 1981) remained the same whether adipose tissue slices or isolated adipocytes were used. This may indicate that the interstitial spaces do not present a barrier to the movement of the hormone towards the cell surface in tissue slices.

A situation similar to that in tissue slices exists *in vivo* where adipose tissue is not well-endowed with capillaries. It has been estimated that rat subcutaneous adipose tissue has a capillary density per unit volume which is one third that in muscle (Gersh and Still, 1945). Thus, the hormones probably diffuse through the interstitial space to reach the cell surface *in vivo* as they do with tissue slices *in vitro*.

There is some evidence that rates of lipogenesis and glucose oxidation are higher in isolated adipocytes than in slices in swine (Etherton and Chung, 1981) and bovine (Vasilatos *et al.*, 1983) adipose tissue. Duquette *et al.* (1984), on the other hand, found higher basal rates of glycerol release in tissue slices than in isolated ovine adipocytes. There is no indication in these studies, however, which of the observed rates best represented the rate *in vivo*. There is a tendency to assume that techniques giving the highest rates are best, an assumption which is not necessarily always correct. It is possible that, in the unphysiological situation of the isolated cell being bathed in copeous amounts of substrate immediately adjacent to the cell surface, an increase in diffusion across the cell membrane would occur. This could lead to an unusually high rate of incorporation of substrates when

compared to the situation *in vivo*. A high basal rate could then mask the effects of hormones which under normal circumstances would be required to boost the rate to that achieved solely by the isolated adipocyte technique. Since resources within the cell will not always allow the cell to boost the metabolic rate still higher, a high basal rate may mean a low hormone responsiveness.

Connective tissue cells are associated with adipose cells and, during the preparation of isolated adipocytes, are removed in the washing solutions since they sink while adipocytes float. This means that only adipocytes will be present in the incubation vessel. During tissue slice preparation, the presence of connective tissue can be minimized by dissection techniques but not eliminated. The connective tissue cells contain little or no stored triacylglycerol, as evidenced by the fact that they sink in solution. They are therefore unlikely to contribute to the lipogenesis and lipolysis that is occurring. In tissue slices, they will not be included in the estimation of the number of cells present, since this is based on the measurement of the weight of lipid.

Indeed, the presence of connective tissue in tissue slices may enhance the ability of the cells *in vitro* to behave in a fashion which is similar to that *in vivo*. Little is known about autocrine and paracrine effects which involve the action of locally produced chemical messengers between cells. However, should they exist in adipose tissue, the tissue slice, with all the cells in their normal juxtaposition, will allow them to function more easily than in isolated adipocytes.

An advantage of the isolated adipocyte technique is that the same number of cells can be dispensed by pipette to each incubation vessel. Therefore similar rates of substrate utilization and waste production will occur in each vessel. This means that, with conditions in the medium being kept constant, there will be less variation between incubation vessels. With slices, on the other hand, the amount of tissue in each vessel is estimated by eye, although an accurate measurement of the number of cells can be made after incubation. However this problem can be largely overcome provided that the amount of tissue in each vessel is not excessive and a check is made on substrate utilization and pH of the medium throughout the period of incubation.

A major disadvantage of the isolated adipocyte method is the damage to the cell that can occur during the collagenase digestion process. In the present study, evidence of the damage was seen in the large quantity of lipid droplets which collected on the edge of the vessel during digestion and subsequent incubation. Also, looking at the cells under a low-power microscope, "florets" were observed on the cell surface of up to 30% of the cells which indicated that the cellular contents were spilling out through ruptured membranes. This would appear to be a problem particularly associated with ovine adipocytes which seem to be more firmly attached to each other and require more digestion to separate them than rat adipocytes do. It has been reported by Etherton and Chung (1981) that, in swine adipocytes, there is little change in the mean cell diameter following collagenase activity, indicating that if rupture does occur, it is in large cells as well as small cells.

However it seems inevitable that, if there is rupture of the cells, there will be damage to cell surface components, even in cells which remain viable. Since collagenase is a crude preparation which could contain other proteases, proteolysis occurring at the cell surface may remove hormone receptor proteins. This means that there is a real possibility that hormone receptors will not function in the same way as they did before collagenase digestion. This was illustrated by Grichting and Goodman (1981) who showed that the cell isolation procedure abolished the ability of insulin to stimulate glucose oxidation or antagonize adrenalin-induced lipolysis in adipocytes from hypophysectomized rats. Thus consideration must be given to the ability of the cells to retain their normal responsiveness following the rigorous, unphysiological procedure of collagenase digestion when interpretations of hormone action are made.

The cells in tissue slices, on the other hand, suffer minimal damage during the slicing process. A rinse with medium after slicing removes the contents of any ruptured cells on the outer surface of the slice, leaving only intact cells for the incubation. The result is that the cell surface receptors, on which the success of any hormone study depends, will be largely intact and as close as possible to the condition they were in before being taken from the animal.

As a result of initial studies, it was found that preparations of isolated adipocytes did not give sufficient viable cells for use on a routine basis. It was felt that the tissue slice technique offered the best opportunity to study adipose tissue under conditions which most closely relate to those *in vivo*.

The use of BSA in the medium

BSA is present in the medium to regulate osmotic pressure and prevent lysis of the cells (Rinaldini, 1959). It has also been shown to offer some protection to the insulin molecule to prevent its degradation in the medium (see Table 4.1). In addition, BSA may prevent the loss of bGH in the incubation medium (see Table 6.1). Another polypeptide molecule, Polypep^{R} from Sigma, did not protect the bGH molecule either in the presence or absence of tissue slices. It is possible that BSA also reduces non-specific binding of the hormone to the walls of the vessel.

One of the most important functions of BSA is the removal of free fatty acid from the medium. Any accumulation of unbound free fatty acid in the medium or cytoplasm will prevent further lipolysis from occurring by driving the equilibrium of the reaction:

$$\text{triacylglycerol} \rightleftharpoons 3 \text{ fatty acid} + \text{glycerol}$$

to the left. This is demonstrated in Figures 3.2 and 3.3, where the presence of BSA enhanced the rate of glycerol release from the medium and the responses of the cells to lipolytic hormones. It was thought that BSA at a concentration of 1% w/v was adequate because there was no increase in the amount of fatty acid leaving the cell when the concentration of BSA was increased to 4% w/v in the present study.

Although it was found that the presence of at least 1% w/v BSA was mandatory for the measurement of the rate of glycerol or fatty acid release, there was little indication that a depression in the rate of lipolysis when no BSA was present affected the rate of lipogenesis and its stimulation by insulin. As seen in Figure 4.1, where all the incubations were carried out in the absence of BSA, both the basal rate of lipogenesis and that in the presence of insulin are linear. This may indicate that, over the six hour incubation period, there was no product inhibition of lipogenesis by excessive accumulation of free fatty acid in

the cell. On the contrary, as will be discussed later in this Chapter, it was shown that, even in the presence of BSA, insulin may stimulate the retention of fatty acid by the cells (Tables 4.8, 4.9 and 4.10).

In fact, the presence of BSA served only to depress the rate of lipogenesis in the presence of insulin (Figure 3.1). BSA could be affecting the cell either by binding to the insulin and reducing its effective concentration or by the presence of an inhibitory factor in the BSA which is acting at all concentrations of insulin to reduce the rate of lipogenesis. This same batch of BSA was used throughout the present study because it appeared to interfere the least of all the batches tested. It has recently been reported that certain batches of BSA from different commercial sources caused a stimulation of lipogenesis in porcine adipose tissue slices and masked the effects of insulin (Walton and Etherton, 1986). Since the BSA used in the present study gave the opposite effect, this illustrates the range of contaminants which may be present in the commercially available product.

It was seen using SDS polyacrylamide gel electrophoresis (Plate 6.2) that there were a large number of different peptide components in the BSA used in this study. It is possible that there may have been some denaturation of the albumin molecules during preparation of the BSA. However, there is also a distinct possibility that other proteins have remained with the albumin molecule throughout the purification process. For example, a 10% solution of the BSA was found to have 0.7 ng/ml IGF I by radioimmunoassay. It can be seen in Plate 6.2 that the BSA contained subunits with estimated molecular weights of less than 10 kDa. Although these could well be lysed fractions of the albumin molecule, they could also be small polypeptide molecules such as insulin or IGF.

Thus one of the major disadvantages in using BSA is that it is a chemically undefined product. The consequences of this are that the measured basal rate of lipid metabolism in the presence of BSA cannot be considered a true control for the observations of hormone responses in the same tissue. Any contaminating hormones which affect the basal rate may or may not have the same effect in the presence of another hormone. Conversely, the contaminating hormone could be having no effect on the basal rate while significantly inhibiting or enhancing the effects of the hormone under study.

An alternative could be to use other proteins such as gelatin, casein (Livesey and Donald, 1982) or Polypep^R. However Polypep^R (a polypeptide) was found to depress adrenalin-stimulated lipolysis (Figure 3.4). It was also shown to offer very little protection to loss of bGH in the incubation medium while BSA was extremely successful in this regard (see Table 6.1). As a result of these experiments, Polypep^R was not used as an alternative to BSA in the present study. Although albumin is normally associated with adipose tissue *in vivo*, none of the alteratives could be considered physiologically normal. Thus if they were present in the medium, their effects on the metabolic processes in the cells would be unknown and therefore they were not used.

It appeared that there was little choice but to continue with the use of BSA. For an experiment where only the rate of lipogenesis was being measured, medium containing no protein was used. However the batch of Sigma BSA shown tested in Figures 3.1, 3.2 and 3.3 as well as Plate 6.2 was used at 1% w/v for all incubations in which lipolysis was being measured or when GH and IGF were being tested.

Cellularity and the measurement of cell dimensions

In the early stages of this work, the metabolic rates were expressed in terms of the wet weight of tissue as described by Vernon *et al.* (1979b). However it became apparent that this method failed to take into account the change in DNA, protein and cell number when the amount of lipid in each cell changed between animals (Hood and Allen, 1973). Since an adipocyte's prime function is to store lipids, its mass is a poor indicator of the ability to metabolize lipids. It is quite possible for an adipocyte to be manufacturing fatty acids at the same rate as a neighbouring cell with twice the volume of stored lipid, since both cells could have similar amounts of DNA, cofactors, enzymes and substrate available to them. As a result, if metabolic activity is expressed in terms of g wet weight or g lipid, it will be considerably less for a fat animal with large, fatty adipocytes than for a lean animal with small adipocytes.

Another problem encountered with the use of wet weight was the change in amount of water associated with the cells during incubation. As illustrated in Table 3.2, the ratio of lipid weight to wet weight, in the presence of insulin, reduced to 75% of that for the tissue with no

hormone in the medium. The indications are that insulin caused an increase in the volume of water associated with the cells which may have caused them to swell. As a result, the wet weight determined after incubation with insulin (*e.g.* Jungas and Ball, 1963; Vernon, 1977b, 1979b; Kasser *et al.*, 1983) may be an over-estimate, making the metabolic rate being examined considerably less than it really was and may therefore under-estimate the response the cells made to the presence of insulin.

To avoid these problems, it was considered preferable to express the metabolic data per cell. There are at least two methods which can be used to estimate cell numbers: the measurement of the DNA content of the tissue or the direct observation and measurement of cell sizes.

Measurement of the amount of DNA in the cells makes the assumption that the DNA content of each cell is constant for a given species. The method, as described by Curtis-Prior *et al.* (1975), involves dividing the total amount of DNA in the tissue by the amount of DNA in one cell. The main disadvantage of this method is that it fails to discriminate between adipocytes and other cell types present in the adipose tissue (Gurr and Kirtland, 1978). As a result, the numbers of adipocytes in the tissue will probably be over-estimated.

The method used to express metabolic data in the present study was that described by Sjostrom *et al.* (1971). The method involves the direct measurements of cell sizes and makes the assumption that the adipocyte consists almost entirely of lipid. The accuracy of the determination of cell numbers depends both on the precision of the extraction of total lipid in the tissue slices and on the extent to which the measurement of the mean cell size is truly representative of the cell size throughout the adipose tissue. For the estimation of total lipid in the slices, it was important that there was no loss due to the extraction and purification processes. One of the main sources of error was the presence of water droplets in the lipid during weighing, caused by condensation during the evaporation of the hexane. It was found that five hours in an oven at 60°C ensured that all traces of water were removed from the lipid extracts. Although a temperature higher than 60°C would drive off the water faster, it could lead to loss of the lipid by oxidation.

It has been suggested (Gurr and Kirtland, 1978) that the direct measurement of the cell diameter must make the assumption that:

(a) the cells have retained their original size.
(b) the cells are perfect spheres.
(c) the cells have been sectioned through their equators, thus revealing their maximum cross-sectional area.

The number of adipose cells were estimated in this study using a fresh, hand-cut tissue slice. The advantage of using fresh slices, apart from the ease of slide preparation, was that there was little opportunity for any change in cell volume to occur as could happen during fixing, freezing or mounting onto a microtome. The microtome method would have taken much longer and probably could not have been begun until after the incubations had finished. This would almost certainly have resulted in cell volume changes.

To overcome the problem of non-spherical cells, two measurements of the diameter of each cell were taken with the second measurement at right angles to the first. The average of these two measurements gave the mean diameter of non-spherical cells. Cells with a honeycomb-like appearance (Plate 3.2), which indicated cell sizes had increased, were not used for diameter measurements. However these changes to cell dimensions were not frequently observed provided that the tissue was kept incubated and the measurements were done within 1 or 2 hours of slaughter. By focusing down through the cell being measured, it was possible to find the maximum possible diameter because the cell membrane came sharply into focus.

An experiment was carried out to measure both wet weight of tissue and cell numbers per sample (Table 3.3). Using the same incorporation data, the variation between measurements of duplicate incubations was reduced from 18% when it was expressed per gm wet weight of tissue to 9% when expressed per 10^6 cells. This indicates that the error for the estimation of the rate of lipogenesis was reduced by half by using cell numbers. Hence all metabolic rates were expressed as per 10^6 cells.

Validation of the *in vitro* model

In a time-course graph, the rate of metabolic activity is plotted against incubation time. When the slope of the time-course graph had a major linear component (*e.g* Figure 4.1), it could be concluded that, throughout the incubation time, the rate of the metabolic activity being measured did not change to any great extent. This type of graph was used to test all the metabolic processes being studied and was repeated frequently when different hormones were being tested. If the rate was constant, then the following assumptions about the incubation of the cells *in vitro* could be made:

(a) Cell structure and the viability of the cells had not been lost at any time during the incubation period. If there had been any cell damage or loss of viability, it could be assumed that lipid metabolism would slow down or stop and the slope of the graph flatten out.

(b) Any reduction of substrate concentration was not significant and was not restricting the rate of lipogenesis. As discussed earlier in this section, any alteration in the specific activity of the substrate or intermediates either in the medium, the interstitial space or the cell cytoplasm will be reflected by a change in the slope of the time-course graph. This also applies to any change in the total concentration of substrate in these three compartments.

(c) Essential enzymes and cofactors required for the metabolic process under study have not been depleted. This would include the oxygen and so a linear graph indicated that any inadequacies in aeration of the tissue during incubation was not gradually restricting cellular activity through the incubation period.

(d) The products of metabolism (*e.g.* free fatty acid, lactic acid, CO_2) are not inhibiting the rate of the metabolic process. Any excessive rise in the concentration of these waste products would lead to a slowing down of the metabolic rate, owing to product inhibition or negative feedback inhibition.

In the presence of insulin, the rate of lipogenesis increased while at the same time remained linear (Figure 4.1). In this case, certain assumptions about the hormone itself can be made:

(a) Any change in the concentration of active hormone in the medium did not have any effect on the rate of lipogenesis during the time of incubation. As discussed in more detail in Section 10.2, a linear graph obtained in an incubation where it was known that the hormone concentration did change will probably indicate that only the initial concentration of hormone in the medium dictates the level of response throughout the six hour incubation time.

(b) The hormone receptors on the cell surface have remained active during the time of hormone interaction with the cells. Any change in their responsiveness will result in a change in the slope of the graph, probably tending towards that of the basal rate.

It became apparent, however, that the time-course graphs were not always linear but could follow a parabolic curve. When the rate was increasing, the graph curved upwards (such as the one shown in Figure 4.2). If this type of curve was observed in cells with no hormone present (the basal rate), it was assumed that changes in cellular conditions such as specific activities of substrates and intermediates had caused the observed increase in the rate. However the same shape of curve observed in the presence of hormone could, in addition, have been caused by a delayed response to the hormone as a result of either delay in receptor-hormone interactions or by cellular processes involved in the response (*e.g.* enzyme induction or synthesis of a "messenger" molecule) requiring time to proceed. In any case, increasing metabolic rates indicated that the cells were viable and the hormone interaction with the cell was proceeding in a way that could be considered normal.

On the other hand, a curve which indicated a decrease in metabolic rate with time (such as that occurring in Figures 4.3 and 4.4 between 4 and 6 hours incubation) could indicate either that the cell was responding to changing conditions by decreasing the rate of metabolism or that the cells were no longer all viable. This type of response was most common in cells after 4 hours incubation when cellular resources could

have been depleted. Thus it is quite possible that the rate of cellular metabolism observed during the first 4 hours incubation was not sustainable in these cells. This situation would not necessarily occur *in vivo* where cellular resources can be replenished and the initial high rate would be more likely to be sustainable.

When the rate of the metabolic process was shown to stop between two observations in this way, the data for the second point was not shown on the graph (*e.g.* Figure 5.2, between 4 and 6 hours incubation). This decision was made based on the assumption that the initial linear rate would have achieved the purpose of the hormone interaction and that, *in vivo*, when some cells shut down in this way, others could be stimulated to respond with the initial linear rate again. Thus cells from different depots could be continually kept at the peak rate observed during the first 4 hours for as long as it was required. To achieve this, the cells could have receptors at differing levels of affinity for the hormone or alternatively use a process such as the internalization of hormone-receptor complexes as is thought to occur with insulin (see section 1.6).

At the outset, it was important to establish that the processes being observed were the result of cellular metabolism and not mere chemical exchange in non-enzymic reactions. For this purpose, blank incubations containing sulphuric acid were carried out for each experiment. These incubations included all the necessary requirements for fatty acid metabolism but, in the extremely acid environment, the proteins present would be denatured. Thus there could be no enzymic activity in the cells and it was found that, under these conditions, fatty acid metabolism did not occur. It was therefore concluded that the processes being observed in the normal incubations were occurring as a result of cellular metabolism which could not proceed unless the cells were viable.

It was necessary to ensure that the characteristics of the cellular metabolism being observed *in vitro* resembled as closely as possible that reported in the literature, both *in vitro* and *in vivo*. It was concluded that the reported rates for lipogenesis in ovine adipose tissue *in vitro*, once converted to the same units, all fell within the range of rates obtained in this study (0.003-3.0 μmoles acetate incorporated into fatty acid/hour/10^6 cells). Lindsay (1970) measured a rate of fatty acid

synthesis in adipose tissue in sheep *in vivo* of 5-7 g/day/animal. If the animals weighed 40 kg and had 10% w/w adipose tissue, this represents 28-38 nmoles acetate converted to fatty acids/minute/g of tissue (as calculated by Vernon (1980)) or 0.32-0.43 μmoles acetate/hour/10^6 cells. These *in vivo* rates are close to the median of lipogenic rates obtained in this study.

However it is apparent that values obtained *in vitro* cover a considerably wider range than the values given for lipogenesis *in vivo*. This could be a reflection of the short-term aspect of measurements *in vitro* whereas the results *in vivo* have been calculated from weight gains of animals over a period of months. It seems likely that the lipogenic rates can vary hourly according to the changing needs of the animal and the extent of the variation is illustrated by the far wider range of rates obtained *in vitro*.

Another process used to validate the *in vitro* model was to test the response of the cells to insulin and adrenalin (see Chapters 4 and 5). Both these hormones have been used extensively in many reported studies and so their effects on the cells were predictable, within certain limits set by the different species being tested. It was assumed that, if the *in vitro* model could show a normal response in the presence of hormones about which a considerable amount is already known, then the model could be used more confidently to study the response to other hormones, the function of which is not so clearly established. The effects of insulin and adrenalin on ovine adipose tissue in the present study and the extent to which they can be considered "normal" are discussed in Sections 10.2 and 10.3.

It was also felt that the cells' response to a hormone such as insulin or adrenalin could be used as a yard-stick by which the responses to other hormones could be measured. Since cellular responses vary considerably between animals, the measurement of a specific response to a hormone is extremely difficult to quantify. It was thought that a comparison of the extent of the response to a hormone under test with that of the same response to a known hormone could be more informative. For this reason, insulin was used as a control for the lipogenic response in adipose tissue and adrenalin for the lipolytic response.

Measurement of lipid metabolism *in vitro*: the assumptions made

As discussed earlier in this section, any changes in pool sizes of unlabelled substrate or intermediates in the cells will lead to changing specific activity of the labelled precursor molecule at the beginning of the incubation period. Thus any changes observed in the rate of acetate incorporation into fatty acid cannot necessarily be attributed to changes in the true rate, at least until a steady state is achieved. Provided that experimental conditions are similar, it is possible to assume that, if the pool sizes do change, the variation will be the same in all incubation vessels. This is because the sizes of the pools *in vitro* are most likely dictated by the physiological condition of the animal at slaughter. Thus any experimentally measured rate of acetate incorporation can only be compared with the control rate of acetate incorporation measured in tissue from the same animal in the same experiment.

As a consequence, non-homogeneous mixing of tissue from several animals in one experiment could lead to changes in the concentrations of intracellular intermediates from one incubation vessel to the next. The result would be that the basal rate of cellular metabolism could not necessarily be used as a control to compare with the rate in the presence of the hormone under test. This is especially the case with tissue slices where only 5 - 6 slices were included in each incubation vessel. For this reason, tissue from several animals was not pooled for one experiment in the present study.

A method to investigate the overall energy balance of a cell *in vitro* has been proposed (Section 3.16). This method takes into account the rates of efflux of fatty acid as well as glycerol from the cell and, together with an estimate of the amount of fatty acid manufactured, can be used to calculate the rate of fatty acid accumulation and re-esterification in the tissue.

Another method to measure the rate of fatty acid re-esterification *in vitro* has recently been proposed (Leibel and Hirsch, 1985). This method uses [^{3}H]-palmitate to measure the rate of esterification and [^{14}C]-glucose to measure the rate of total acylglycerol manufacture. The difference represents the amount of unlabelled fatty acid which is re-esterified. It would be of interest to use this method to check the

accuracy of estimation of fatty acid re-esterification by direct measurements of fatty acid and glycerol efflux.

The advantage of the method proposed in the present study is that the rate of re-esterification can be measured several times during the incubation using samples of medium whereas incorporation of substrates into fatty acid can only be measured once at the end of the incubation. Thus the change in the rate in response to hormones could not be measured in the latter method. However, measurement of the rate of fatty acid accumulation in the method proposed in the present study does require a tissue sample from a terminated incubation. Another advantage of the proposed method is that it does not involve the complications inherent in the use of the double isotope system, as in the method described by Leibel and Hirsch. However, one of the advantages of the method proposed by Leibel and Hirsch is that it does not require assumptions to be made about the alternative destinations for the fatty acid as will be discussed below.

When the calculations of fatty acid accumulation were made in the present study, the amount of fatty acid manufactured during the incubation was used (see Section 3.16). The rate of fatty acid manufacture was calculated from the rate of acetate incorporation into tissue fatty acids assuming that all fatty acids manufactured had a chain length of 16 carbon atoms. Thus it was assumed that the total amount of fatty acid manufactured during the experiment was represented by the amount of acetate incorporated into tissue fatty acids. As demonstrated in Table 4.6 and Table 4.7, the incorporation of glucose into fatty acids may be considerably more than has been previously reported (Hood *et al.*, 1972) and could therefore be used in addition to acetate as a precursor for fatty acid manufactured during the incubation period. It was calculated that 20% of the total fatty acid synthesis from both acetate and glucose came from glucose in one experiment (Table 4.6) and 33% from glucose in another (Table 4.7). If the fatty acid manufacture was increased by 20 - 30% to account for that coming from glucose, the amount of fatty acid accumulated in the cell would be increased as well.

When the rate of lipolysis in the cells was estimated using the rate of glycerol release from the tissue, it was assumed that all the glycerol produced by lipolysis in the cell passes into the incubation medium and is not re-used by the cell. However it is possible that a small fraction

of the glycerol could be either oxidized in the cell or re-incorporated into triacylglycerol. Ovine adipose tissue has very low activities of glycerol kinase, the enzyme necessary for the re-use of glycerol in the cell (Khadachurian *et al.*, 1967). Also it has been observed that the incorporation of [1-^{14}C]-glycerol, in the absence of acetate, was very small, as was the amount of glycerol oxidized to CO_2 (Hood *et al.*, 1972). However, there is a possibility that the action of a hormone under study could cause an increase in the amount of glycerol utilization within the cell to levels where it would be significant.

It was also assumed that all glycerol produced by the cell was as a result of lipolysis. However, it is possible for glycerol to be formed from glycerol-3-phosphate by phosphatase (Steinberg and Vaughan, 1965). But, when rat adipose tissue was incubated with labelled glucose, very little labelled glycerol appeared in the medium, indicating that that the glycerol released was not formed by newly incorporated glucose (Lynn *et al.*, 1960).

The assumption was made that there are three fatty acid molecules produced for each molecule of glycerol during the process of lipolysis. This was based on the premise that all acylglycerol broken down during lipolysis was triacylglycerol and that it is broken down completely to the component fatty acid and glycerol molecules, with no partial hydrolysis occurring. However, there is the possibility that diacylglycerol or monoacylglycerol was utilized, or that triacylglycerol was not completely broken down, in which case the ratio of fatty acid to glycerol would not be 3 : 1.

It has been reported in two studies with bovine adipose tissue *in vitro* that the ratio of fatty acid to glycerol released from the tissue was 6 : 1 (Etherton *et al.*, 1977; Yang and Baldwin, 1973a). However, in ovine adipose tissue *in vitro* the ratio was 0.7 : 1 (Etherton *et al.*, 1977). A ratio of approximately 1 : 1 was obtained in another study with bovine adipose tissue *in vitro* (Metz and van den Bergh, 1977). In the present study, the ratio of fatty acid to glycerol release varied from 0.25 : 1 to 1 : 1 in the incubations with no hormone in the medium. This is in agreement with the latter two studies and probably indicates that there is little partial hydrolysis of triacylglycerol occurring in ovine adipose tissue.

However, the situation is complex and involves simultaneous changes in a number of mechanisms, any one of which could result in the ratio of fatty acid glycerol leaving the cell being different from the theoretical 3 : 1.

(1) Partial hydrolysis of acylglycerol will increase the ratio.
(2) Utilization of mono- or diglycerol for lipolysis will decrease the ratio.
(3) Recycling of fatty acid by re-esterification will decrease the ratio.
(4) Oxidation of fatty acid in the cell will decrease the ratio.
(5) Retention of free fatty acid in the cell will decrease the ratio.

Thus, if the ratio of fatty acid to glycerol leaving the cell is greater than 3 : 1, then this was probably the result of partial hydrolysis of the triacylglycerol molecules. When the ratio is 1 : 1 or less, as was observed in the present study, then this could be the result of a balance between all of the above mechanisms and it is not known which predominates. If it is assumed that the ratio of fatty acid to glycerol originally released within the cell was 3 : 1, then the accuracy of estimates of recycling of fatty acid will depend on the alternative destinations for that fatty acid. However, since the ratio of fatty acid to glycerol actually entering the medium was only 1 : 1, it can be assumed that most of the fatty acid released by lipolysis was retained within the cell and that partial hydrolysis was not occurring to any great extent. On the other hand, the method proposed for the measurement of fatty acid accumulation within the cell does not require that any assumption concerning this ratio is made and is therefore probably the superior method for assessment of the energy balance of the cell.

Since triacylglycerol is predominant in adipose tissue lipids, the contributions of di- and monoacylglycerol to the fatty acids released were assumed to be small. This would also mean that mono- or diacylglycerol was seldom the product of lipolysis either. However, this assumption applies equally to the assessment of lipolysis using glycerol release. When the analysis of glycerol release data alone is used to make judgements about the effects of a hormone on lipid mobilization from the adipose tissue, then such assumptions about the relationship between fatty acid and glycerol flux from the cell must be made. This can be

particularly a problem if, as the result of the action of a hormone, triacylglycerol molecules are broken down only as far as di- or monoacylglycerol. As a result, a large amount of fatty acid could be mobilized from the tissue but no glycerol. The method proposed here, where both fatty acid and glycerol are assessed could, under these conditions, show that the cell is in negative energy balance where observation of glycerol release alone would not. It is suggested that this may be a reason why some studies *in vitro* have failed to demonstrate a lipolytic response in the presence of a hormone known to be normally lipolytic.

When free fatty acid is measured in the medium at the end of the incubation, any fatty acid still remaining inside the cell at this time will be included in the estimations of the amount of fatty acid re-esterified. However, if the glycerol molecule that was associated with this fatty acid has also not passed out of the cell, then it will not be included in the lipolysis figures at all.

It was assumed that the amount of free fatty acid leaving the cell represents the balance between the amount of fatty acid released by lipolysis and the amount re-esterified. There is a possibility that some free fatty acid was oxidized and, as mentioned previously, this fatty acid will be included in the estimate of the amount re-esterified.

At the beginning of the incubation, it is likely that a pool of free fatty acid and glycerol exists within the cell as a result of preceding lipolytic activity. Similarly, at the end of the incubation, some of the free fatty acid and glycerol released by lipolysis would still remain inside the cell (Metz *et al.*, 1973; Metz and Van den Bergh, 1977). The problem would be greatest for short incubations where intracellular free fatty acid could represent a large proportion of the free fatty acid and glycerol produced during the experiment. In the present study, the use of longer incubation times (4 - 6 hours) for mass balance measurements meant that any change in tissue-associated free fatty acid and glycerol concentration was small when compared with the change in free fatty acid and glycerol concentration in the medium during the incubation. However, if the free fatty acid present in the tissue prior to the start of the incubation was excessive, it can be assumed that this would be measured in the basal incubation medium and will therefore be taken into account in any measurements of subsequent hormone action. In this study,

therefore, comparisons were made between free fatty acid movement out of the cell following exposure to the hormone as compared with that without hormone.

One of the disadvantages of using a longer incubation time is that the BSA molecules in the medium could become saturated with fatty acid and further fatty acid mobilization from the cell would be impeded. However, as previously discussed, it was found that, when the concentration of BSA was increased from 1% w/v to 4%, there was no increase in the amount of fatty acid passing out of the cell during a six hour incubation. It was therefore assumed that, even when BSA was present at 1% in the incubation medium, any impedance to fatty acid mobilization was caused by the intracellular processes of re-esterification or oxidation.

There are therefore a number of inherent problems associated with the incubation of adipose tissue *in vitro* which include the choice of methods for tissue preparation and the subsequent incubation conditions. These have been validated in the present study with reference to the situation *in vivo* and by observation of the kinetics of the metabolic activities in the cells *in vitro*. However, the measurements of these activities themselves are subject to a number of limitations owing to assumptions being made about the processes actually occurring in the cells. Interpretation of the metabolic data must therefore be made with reference to these assumptions.

10.2 Insulin

The stability of insulin during incubation

Once the *in vitro* model had been validated, it could then be used to investigate the effects of hormones on the processes of lipid metabolism occurring in the cells. One of the major obstacles to obtaining valid results in these experiments was the instability of the hormone in the medium. As seen in Tables 4.2 and 4.3, there is considerable degradation of insulin by ovine adipose tissue *in vitro*. After 4 hours of incubation, there was only 50% of the insulin molecules in the medium still immunologically active and after six hours, there can be very little active insulin remaining.

It has been postulated that insulin degradation is caused by proteolytic enzymes originating from the cytosol (Gliemann and Sonne, 1978). There seems a real possibility that the contents of any ruptured cells could have spilt cytosolic contents into the medium and caused the observed insulin degradation. Etherton *et al.* (1984) found that a pre-incubation period reduced the amount of insulin degradation in the presence of isolated swine adipocytes. However, a comparison of Tables 4.2 and 4.3 shows that pre-incubation of ovine adipose tissue slices, followed by a change in medium, did not significantly reduce the amount of insulin degradation. It could probably be assumed that a 24 hour pre-incubation of the tissue in excess medium would have removed most of the contents from ruptured cells. Since there was no noticeable reduction in insulin degradation following pre-incubation, it would appear that, with ovine tissue slices, ruptured cells spilling their proteolytic contents into the medium is not causing the insulin degradation.

However, this does not preclude the possibility that cells are able to release insulin-degrading factors even when not ruptured. Rudman *et al.* (1966) have reported that water-insoluble fractions of homogenized rat adipose tissue hydrolysed the internal and terminal peptide bonds of bovine insulin while the disulphide and acidic amide groups remained intact. They suggested that the enzyme which is involved in this hydrolysis is specific for insulin since it had no effect on other peptides. This was confirmed in the present study when the stability of IGF I, a molecule similar in structure to proinsulin, was investigated (see Section 7.2). As predicted by Rudman *et al.*, there was no noticeable degradation of the IGF I molecule, even though tissue slices, and presumably the same proteolytic enzymes, were present.

Carpentier *et al.* (1979) reported that protease inhibitors such as bacitracin can decrease insulin degradation in the medium. However, the decision was made not to use such inhibitors in the present study because of the unknown effects they could have on metabolism within the cell. Juul and Jones (1982) have recently reported that bacitracin, at the concentrations required to inhibit extra-cellular insulin degradation, will interfere with the processing and internalization of insulin in isolated hepatocytes.

It is possible that the BSA could be simply preventing non-specific binding of insulin to the vessel walls (Livesey and Donald, 1982). This would prevent the insulin from coming out of solution and reducing the concentration of insulin measured. However, the results in Table 4.1 show clearly that there was very little insulin loss when there was no tissue present, even without BSA. It is therefore concluded that the observed insulin loss is mostly tissue-associated.

However, when tissue is present in the incubation vessel, it is by no means clear whether BSA offers any significant protection to the insulin molecule. Some samples appear to be better protected than others but this may indicate that there was more proteolytic activity in some tissue than in others. Etherton *et al.* (1984) has suggested that an event associated with pouring the cell suspension of isolated swine adipocytes from the test-tube to the incubation vessel caused some cells to release insulin-degrading activity. It could be postulated that the handling of tissue slices with forceps for transfer to the incubation vessel could also trigger this release. Variation in the degree of handling received by individual cells could then explain why there is so much variation between incubation vessels in the observed amount of insulin degradation.

Although it appears that non-specific binding of insulin to the incubation vessel is probably not a serious problem in these studies, there remains the possibility that at least some of the observed insulin loss (Tables 4.2 and 4.3) was due to either non-specific or specific binding to the adipose tissue slices. The fate of insulin once it is bound to its receptor is still being elucidated. However, it is now known that the insulin-receptor complex is rapidly internalized by endocytosis (Marshall and Olefsky, 1981). The insulin-receptor complexes subsequently accumulate in the endosomal vesicles where they dissociate. It is possible that the receptors then recycle to the cell surface while the insulin is degraded into intermediates and low molecular weight peptides (reviewed by Horuk and Olefsky, 1985). Internalization of insulin could therefore account for at least some of the observed insulin loss.

Another method used to overcome the problem of decreasing concentrations of insulin in the incubation medium has been to use extremely high hormone concentrations at the beginning of the incubation. For instance, Vernon (1979b) has used 10 μg/ml which is about 1000-fold higher than the physiologically normal insulin concentration in sheep

serum. Any insulin degradation, therefore, will not reduce the concentration below physiologically normal levels. The disadvantage of this technique is that there is a distinct possibility that the hormonal effects observed will be far from normal. The phenomenon of insulin-induced down-regulation of insulin receptors (Marshall *et al.*, 1984) could mean that a high concentration of insulin would seriously reduce the normal response of the cells. There is the possibility that high concentrations of a hormone can cause, for example, the opposite effects in the cell to low concentrations of the same hormone. This could happen either by the receptor having different responses at different concentrations or by the hormone, present at the high concentration, interacting with receptors for which it has a low affinity. For these reasons, only physiologically normal concentrations of any hormone were used in the present study.

The fact remains that for a good deal of the six hour incubation period, insulin was not present at the concentration which is specified. However, it has been assumed throughout this study that, provided there is a sustained response to the hormone, the decreasing hormone concentration had not prejudiced the result. A linear trend in the time-course graph would infer that it was the initial concentration of hormone which determined the amount of stimulation throughout the incubation period. If the hormone concentration in the latter stages of the incubation was critical, then the time-course graph could be expected to flatten out. For this reason, time-course graphs were included in the studies of each hormone used.

The effects of insulin on the rate of lipogenesis

The effects of insulin on lipid metabolism in adipose tissue, at least in the non-ruminant, are well established in the literature. Its classical role is that of controlling the rate of glucose transport into the cell. Since glucose is the precursor molecule for lipogenesis in non-ruminants, insulin can affect the rate of lipogenesis either by increasing the rate of glucose entry into the adipocyte or by acting directly on the enzymes of lipogenesis.

However, the role of insulin in ruminant adipose tissue is necessarily different to that of non-ruminants. This is a direct result of the fact that glucose is not the major carbon source in ruminant metabolism. It seems likely that insulin retains its function in glucose transport in ruminants but, since acetate is the main precursor molecule for lipogenesis in these animals, the importance of insulin may be considerably less. Glucose is required by ruminant adipose tissue for production of glycerol and NADPH however, and the supplies of glucose for these processes may well be under the influence of insulin.

Insulin stimulated the rate of lipogenesis in the tissue from most of the animals tested in the present study. Insulin was observed to increase the rate by a maximum of 6-fold (Figures 4.1, 4.6 and 4.12). This is considerably more than has been previously reported for ruminant adipose tissue (see Section 1.6). In a recent review, Vernon (1980) suggested that insulin stimulates acetate incorporation in this tissue by no more than 10 to 50%. Although the effects of insulin on the rate were most commonly 70 to 80% stimulation in the present study, it has been demonstrated that, under some conditions, it can be considerably more than that suggested by Vernon.

It is not known why the tissue in the present study appeared to be more sensitive to insulin than that described in previous reports. It seems unlikely that the use of tissue slices has contributed towards this since the rate of lipogenesis from glucose increased 4-fold with isolated ovine adipocytes (Figure 4.12). Also the explant technique has been used extensively by previous workers to study the effects of insulin in ruminant adipose tissue (Vernon, 1978, 1979a, 1979b, 1982a, 1982b; Baldwin *et al.*, 1973). It has been reported that insulin failed to stimulate lipogenesis in ovine isolated adipocytes (Thornton *et al.*, 1982). However, the choice of an appropriate batch of BSA which does not interfere with lipid metabolism or the response to hormones may be important (see Section 10.1). BSA was shown to inhibit the action of insulin (Figure 3.1). However, it has recently been reported that BSA from other sources stimulated lipogenesis and masked the effects of insulin in swine adipocytes (Walton and Etherton, 1986). Many of the experiments to investigate the effects of insulin in the present study have been carried out in the absence of BSA (Figures 4.1 and 4.6). However, BSA was present in one experiment in which a 4-fold increase in lipogenesis was obtained (Figure 4.12).

On the other hand, in the thirty animals tested in this study, insulin had no significant effect on the rate of lipogenesis in the tissue from approximately one quarter of them (*e.g.* Figure 4.2). Thus the sensitivity of the tissue to insulin varied considerably in apparently similar sheep. For instance, in an experiment where tissue from 4 sheep of similar ages was incubated at the same time under identical experimental conditions, there was considerable variation, not only in the basal rate of lipogenesis but also in the maximum observed stimulation by insulin (Table 4.4).

The accumulation of adenosine in adipose tissue *in vitro* is probably one of the most important causes of the observed variation in response between experiments (Belfrage, 1985). In the same review, it was suggested that adenosine acts by sensitizing the tissue to the effects of insulin and that its presence *in vitro* can be attributed to many factors. Belfrage postulates that these may include cell viability and size, duration of incubation, the presence of adenosine deaminase in the BSA used in the incubation medium, the nutritional state of the donor animal and the concentration of insulin used. BSA was not used in many of the experiments to study the effects of insulin on the rate of lipogenesis but, in those experiments where it was present, only one batch of BSA was used throughout this study. Therefore, if adenosine deaminase was altering the rate of accumulation of adenosine in the incubations, it had the same effect in all experiments where BSA was used. The nutritional status of the donor animals was kept as constant as was possible throughout the study and they were always well fed. It is therefore felt that the accumulation of adenosine, if it is affecting the sensitivity of the tissue to insulin, was not easy to control and that its presence may have been an inherent fault in the *in vitro* method.

There is some indication that the response to insulin may be higher in tissue which has a lower basal rate (Table 4.4). If the basal rate of lipogenesis is already high, it may be close to the maximum possible rate that the cells are able to sustain with present resources. As a result, it is possible that the cells do not respond to insulin by increasing the rate as much as in cells which have a considerably lower basal rate.

There is also evidence that the basal rate decreases with increasing fatness, as measured by GR (Table 4.4). Thus the fatter the animal, the lower the basal rate of lipogenesis and the higher the insulin response.

Although more data would be needed to substantiate these results, it is possible that one of the reasons that the animal is fatter is because the adipose tissue is more responsive to insulin. Although these animals were identical in age, sex, breed and nutritional status, there may be some other physiological factor, perhaps but not necessarily associated with fatness, which has caused the observed differences in the basal rate of lipogenesis and the response to insulin. This factor could be the physiological status of the animal with regard to general health and state of growth or genetically determined susceptibilities to fatness or response to insulin of an individual within a breed. As will be discussed later in this chapter, there is a possibility that a factor associated with lower basal rates of lipogenesis may be the presence of growth hormone *in vivo* (Section 10.7).

The animals used for the experiment described in Table 4.4 were all tested on the same day, with conditions of slaughter and incubation kept identical, and yet showed considerable variation in rate of lipogenesis and insulin response. It is therefore unlikely that the observed differences were due to experimental error but were a reflection of real differences between animals in these characteristics. Thus, in other experiments where tissue from only one animal at a time was tested and considerable variation between animals was found, it is also unlikely that all this difference was due to slight changes in technique between experiments but rather that the observed variation was due to the different physiological condition of each animal.

The animals used in the experiment for Table 4.4 were closely matched for age, and thus for state of growth, as well as being slaughtered in the same season of the year and therefore of identical nutritional status (the animals were pasture-fed and the quality and quantity of pasture varies according to the time of year). Although the animals used in the other experiments in this study were between the ages of 3 and 11 months, they were not so closely matched for age and state of growth and, since they were slaughtered at different times during the year, their nutritional status could have varied slightly. Thus it was not possible to compare results between animals. In contrast to the results seen in Table 4.4, there were no correlations between fatness, basal rate of lipogenesis or insulin response identified when all of the animals tested in the present study were compared because there were so many other factors involved.

The basal rate of acetate incorporation into fatty acid and the response to insulin were similar in tissue from twin animals (Table 4.5). It is possible that the similarity is a conseqence of the animals having similar ages, fatness, nutritional status and genetic background. Conversely, the larger variation observed in tissue from unrelated animals could be associated with the fact that the animals had different genetic characteristics with regard to the rate of lipogenesis and insulin response (Table 4.4). However, more experiments with twin animals would have to be conducted before any conclusions about the relationships between genetic characteristics and lipid metabolism could be made.

The rate of lipogenesis in ovine adipose tissue has been measured using the rate of incorporation of acetate into fatty acids, with the assumption that most lipogenesis in this tissue uses acetate as the principle substrate (Vernon, 1980). However, the overall rate of lipogenesis from all precursors can be measured using the incorporation of ^3H_2O into fatty acid (Windmueller and Spaeth, 1966; Fain and Scow, 1966; Lowenstein, 1971). Since the rate of H atom incorporation into tissue fatty acid seen in Figure 4.5 was measured over a longer time period (96 hours) than that given for acetate incorporation (6 hours), it is possible that there were different processes occurring in response to hormone action in each. The observed increase in response in the long-term experiment with ^3H_2O may have been due to the ability of insulin to increase the amount of enzyme available for the process of lipogenesis. This response could require a considerable length of time and is unlikely to be observed to any great extent in incubations of only 6 hours duration. However, it is possible that during a six hour incubation, insulin could alter the activity of enzymes already manufactured in order to stimulate lipogenesis. This type of response to insulin could occur in either short-term or long-term experiments.

Insulin's prime role in the body is the stimulation of the rate of entry of glucose into the cell, a phenomenon which could play an important part in the ability of insulin to stimulate the rate of lipogenesis in both short and long-term experiments. In the present study, both glucose and acetate were available to the cells and therefore insulin could have exerted its effects either by stimulating glucose uptake or by affecting the rate of lipogenesis more directly by altering the amount or activity of the enzymes.

Insulin stimulated the rate of glucose utilization to manufacture lipid by 12-fold in isolated rat adipocytes (Figure 4.13) and by 4-fold in isolated ovine adipocytes, thus illustrating the diminished importance of insulin in ovine adipose tissue. It has been reported that, in rat adipocytes *in vitro*, there was a 5 to 10-fold stimulation of the rate of glucose incorporation into fatty acids by insulin (Di Girolamo *et al.*, 1974; Holm *et al.*, 1975; Salans and Dougherty, 1971.). Since the stimulation of acetate incorporation into fatty acid by insulin in the 30 lambs tested in the present study was shown to range from no significant effect to a maximum of 6-fold and to average about 70%, it is evident that the sensitivity of ovine adipose tissue to insulin stimulation of lipogenesis is considerably less than that observed in the rat.

The reason for the limited sensitivity of ovine adipose tissue to insulin is unclear but could be associated with several factors. Since glucose is not available from the diet in ruminants and yet is essential for brain, foetal and mammary gland metabolism (Rook and Thomas, 1983), its use by adipose tissue for fatty acid manufacture must necessarily be reduced. Therefore the limited sensitivity to insulin may be a mechanism by which glucose entry into adipose tissue is controlled, and thus blood glucose conserved, in ruminants. This may mean that there is another, perhaps independent, method by which the rate of acetate incorporation into fatty acid is controlled in the ruminant to parallel the way that glucose uptake is controlled by insulin in the non-ruminant.

Another reason for the limited sensitivity of ovine adipose tissue to insulin may be that nutrient availability from the rumen in sheep is continuous, owing to the steady influx of metabolic products from the rumen bacteria (Bauman, 1976). This is unlike the situation in non-ruminants which eat spasmodically in set "meals" which result in changing nutrient availability to the cells. Thus, in the non-ruminant, insulin is required to rapidly stimulate the utilization of nutrients by the cells following each meal and the supplies of fatty acid formed during this period may be essential for the animal to survive until the next "meal". At the same time, it is evident that this function of insulin would not be required in ruminants if there was a steady flow of nutrients to the cells. A constant basal rate of lipogenesis would ensure that surplus nutrients were stored as fatty acids. Changes in the amount of surplus nutrient would be small depending on the changing requirements of the other cells in the body and it could be envisaged that a 70%

stimulation by insulin would be adequate.

In one experiment, it was found that the rate of acetate incorporation into fatty acid was directly proportional to the concentration of insulin, up to a concentration of 4.5 ng/ml (Figure 4.6). Since insulin concentrations in normal sheep are 0.80 ± 0.12 ng/ml (Brockman, 1976), insulin was acting *in vitro* at 4 times the concentration normally observed in serum *in vivo*. If it is assumed that concentrations of insulin in serum are an indication of concentrations in the interstitial fluid immediately adjacent to the adipocytes, then this represents considerable redundant capacity for insulin response *in vivo* which is normally available to the cells. Since a concentration of 2.6 ng/ml insulin was used for most experiments (chosen because this concentration gave about half maximal responses), it is possible that the effects of insulin on adipose tissue lipogenesis in sheep *in vivo* is considerably less than the 70% average observed in the present study.

In another experiment, at concentrations of insulin higher than 4 ng/ml, the rate of acetate incorporation into fatty acid was seen to decrease (Figure 4.7). It is possible that this phenomenon is the result of insulin-induced receptor loss caused by internalization of insulin-receptor complexes as described by Marshall and Oefsky (1981). Thus, this provides a mechanism by which cells already stimulated with excess insulin can cease to respond further until their resources again allow them to. The same mechanism could conceivably be used by cells which, due to overfatness in the animal, have increased to such a size that they can no longer store further fatty acid. Thus the presence of insulin in the adipose tissue could stimulate the cells differentially, according to their individual capacities to store lipid. It has been reported that, in human adipocytes *in vitro*, the larger cells were less sensitive to the stimulating effects of insulin (Smith, 1971). But this was not the case in the present study (Table 4.4) where tissue with the largest cells (*i.e.* from the fattest animals) was the most sensitive to insulin. However, the animals used in this experiment were young and still growing and even the fattest of them had probably not as yet reached the maximum cell capacity. This is unlike the situation studied by Smith where the subjects' ages varied from 37 to 75 years.

The effects of insulin on the rate of acetate incorporation into fatty acid were measured at a constant acetate concentration in the incubation medium (1.1 mmol/l). However, since the acetate used to manufacture fatty acid is thought to pass into the cell by passive transport, the concentration of acetate in the incubation medium would reflect the concentration of acetate in the cell. Thus by varying the concentration of acetate in the medium, it was possible to observe the effects of increasing substrate concentration on the rate of acetate incorporation into fatty acids (Figures 4.8b for the basal rate and 4.9b with insulin). Since the experimental data fitted the curve described by the Michaelis-Menton equation reasonably well, the rate of utilization of acetate for lipogenesis by the cells was probably dictated by the limitations of enzyme kinetics and a maximum rate of lipogenesis attained only when the enzymes were saturated with substrate. In the tissue used for this experiment, insulin did not have a statistically significant effect on the value of V_{max} and K_m in this experiment, although there was a small increase in V_{max}.

The maximum rate of lipogenesis will be limited by the activity of the least active enzyme in the pathway. In the case of acetate incorporation into fatty acid in ruminant adipose tissue, the least active enzyme is thought to be acetyl CoA carboxylase (Vernon, 1980). Thus it is likely that the V_{max} obtained in the present study (Figures 4.8 and 4.9) represents the maximum activity of acetyl CoA carboxylase. The activity of this enzyme in sheep has been shown to correspond to a rate of fatty acid synthesis of 10 - 50 nmoles acetate incorporated/minute/g tissue (Vernon, 1980). Using the conversion factors given in Section 3.14, this converts to 0.455 - 2.277 μmoles/4 hours/10^6cells. Since the V_{max} calculated for the basal rate in the present study was 1.582 ± 0.16 μmoles/4 hours/10^6cells, increasing to 2.024 ± 0.20 in the presence of insulin, the values obtained were within the range of the previously reported maximum activity for acetyl CoA carboxylase.

Since acetate concentrations found in sheep serum vary from 1 - 1.7 mmol/l (Vernon *et al.*, 1981b) and since the K_m is shown here to vary from 0.5 - 0.6 mmol/l, the rate of acetate incorporation observed *in vivo* would be higher than the half maximal rate observed here *in vitro*. If it is assumed that conditions *in vivo* are the same as they are *in vitro*, this may mean that there would not be a great deal of excess capacity to

increase the rate of lipogenesis *in vivo*. Using the Michaelis-Menton curve plotted in Figure 4.8b, the rate of acetate incorporation into fatty acid at 1.1 mmol/l acetate (the concentration of acetate normally used in the present study) is about 1.08 μmoles/4 hours/10^6cells. Since the V_{max} for this curve is 1.58 μmoles/4 hours/10^6cells, the rate can only increase by about 45%. If the excess capacity to increase the rate of lipogenesis is limited, this may go some way towards explaining the apparent insensitivity of the tissue to insulin.

Since the concentration of acetate is unlikely to be limiting, insulin may control the rate of lipogenesis via glucose. If NADPH and glycerol are in short supply, insulin could conceivably stimulate the rate of lipogenesis by increasing the availability of glucose for their manufacture. This means that, when NADPH and glycerol supplies are adequate and therefore not rate-limiting, insulin would influence the rate of lipogenesis only via direct action on the activity or amount of the lipogenic enzymes.

It has been shown that the basal rate of acetate incorporation into fatty acid can be increased markedly simply by increasing the concentration of acetate in the medium (Figure 4.8b). This would imply that NADPH and glycerol must have been available in sufficient quantities to accommodate the increase, despite the fact that glucose transport has not been enhanced by added insulin. Thus, when insulin is introduced into the incubation medium, no stimulation of the rate of acetate incorporation into fatty acid is seen (Figure 4.9b). In other tissue, if insulin does stimulate the rate of acetate incorporation into fatty acid, then it is more likely that the available NADPH or glycerol was limiting. It has been reported that NADPH can be produced from acetate, presumably via NADP-isocitrate dehydrogenase, and this could provide a mechanism whereby the rate of acetate incorporation into fatty acid can increase independently of increased supplies of glucose in the ruminant (Bauman *et al.*, 1972).

To study the effects of insulin on glucose uptake into lipid as compared with its effects on acetate uptake into lipid, several experiments were carried out where the tissue was incubated with ^{14}C-glucose present (Figures 4.10 and 4.11). In one experiment, insulin had no significant effect on the rate of acetate incorporation into fatty acid but stimulated the uptake of glucose into both triacylglycerol and

fatty acid (Table 4.6). Since there was no significant effect of insulin on glucose uptake into glycerol, this may mean that the more important effect of insulin is to channel excess glucose into fatty acid manufacture.

In tissue from Sheep 1, with no insulin present, 20% of total lipogenesis from acetate and glucose used glucose as the substrate and in Sheep 2, 33% came from glucose. However, when Vernon (1976) compared the rates of incorporation from ^{14}C-acetate and from $^{3}H_2O$ (the second to give an estimate of total lipogenesis from all precursors) into tissue fatty acid *in vitro*, with a glucose concentration of 5 mmol/l and an acetate concentration of 2 mmol/l, he concluded that acetate was the precursor for essentially all fatty acid synthesis in sheep. It is postulated that this statement does not apply to all sheep and that, under some circumstances, considerably more glucose is utilized. However, it is unclear why there has been a difference in the results obtained in the two studies.

Vernon (1979b) found that, in the presence of insulin, the fatty acid synthesis from glucose increased several-fold. However, glucose uptake into bovine adipose tissue slices was not stimulated by insulin in another report (Smith, 1984). In the present study, when insulin was included in the incubation medium, the number of C_2 units coming from glucose, that were incorporated into fatty acid, significantly increased in Sheep 1 but decreased in Sheep 2. It is therefore postulated that, in the changing circumstances between animals, insulin can have opposing effects on the contribution made by glucose. The result obtained with Sheep 1 may also indicate that, in studies where insulin is not affecting the rate of acetate incorporation into fatty acid, the rate of lipogenesis from all precursors is increased owing to increasing utilization of glucose for fatty acid synthesis. Since insulin is known to alter the activity of the enzyme ATP-citrate lyase by phosphorylation (Alexander *et al.*, 1982; Pucci *et al.*, 1983), this may be the mechanism by which insulin changes the rate of utilization of glucose for fatty acid manufacture.

In the experiment described in Table 4.7, insulin did not significantly affect the production of glycerol from glucose while, at the same time, it increased the rate of acetate incorporation into fatty acid. These results also imply that the basal rate of NADPH and glycerol production was adequate to sustain the increased lipogenesis measured in the presence of insulin. Glycerol synthesis from glucose in ruminant adipose tissue was stimulated by insulin in some studies (Bartos and Skarda, 1970; Yang and Baldwin, 1973a) but not in others (Skarda and Bartos, 1969; Baldwin *et al.*, 1973, Vernon, 1979b). If glycerol is present in quantities which are not rate-limiting for lipogenesis, this might suggest that the amount produced is in excess of that required for the esterification of newly synthesized fatty acid. If excess is produced, then there must be an alternative destination for glycerol apart from the esterification of newly synthesized fatty acids.

In Sheep 1, the ratio of the number of molecules of fatty acid to the number of molecules of glycerol produced in these cells was 1:1 in both the basal and the insulin-stimulated cells (Table 4.6). In sheep 2, the ratio was 1:6, changing to 1:5 in the presence of insulin (Table 4.7). If it is assumed that three molecules of fatty acid were combining with one molecule of glycerol during the esterification process, then there was an excess of glycerol molecules produced in both sheep. In Sheep 1, the 26 nmoles of glycerol produced each hour by 10^6 cells would have been adequate to esterify 79 nmoles of fatty acid. In fact, there was only 27 nmoles of fatty acid produced during this time, leaving enough glycerol to esterify another 51 nmoles of fatty acid each hour. There was an identical excess of glycerol in the presence of insulin for this sheep.

In Sheep 2, where there were 8 nmoles of fatty acid produced for every 48 nmoles of glycerol, there was sufficient glycerol to esterify 147 nmoles of fatty acid. This means, therefore, that sufficient glycerol was produced to esterify a further 139 nmoles of fatty acid. In the presence of insulin, there was sufficient for a further 137 nmoles of fatty acid. As discussed previously, the presence of this excess of glycerol was also indicated when the rate of lipogenesis could be increased simply by increasing the concentration of acetate in the medium (Figure 4.8b).

It is postulated that the excess glycerol produced is utilized for the process of re-esterification. It has been shown using calculations of the overall balance of fatty acid in the adipose tissue (Figures 4.8, 4.9 and 4.10) that the degree of re-cycling of fatty acids in this tissue could be considerable. This would therefore be the most likely reason for the excess production of glycerol in this tissue. Thus, in Sheep 1, 51 nmoles/h/10^6 cells of fatty acid could have been re-esterified using the excess glycerol, and in Sheep 2, 139 nmoles/h/10^6 cells. Insulin did not affect the capacity for re-esterification in either sheep.

In a recent study, Smith (1984) found that glycerol-3-phosphate was present at high levels in bovine subcutaneous adipose tissue slices *in vitro* and concluded that glycerol-3-phosphate did not have a regulatory role in bovine adipose tissue, a conclusion which would be supported by the results obtained in the present study. However, there have been several reported studies which indicate that the concentration of glycerol-3-phosphate does regulate the rate of lipogenesis in ruminant adipose tissue (Baldwin *et al.*, 1969; Bauman *et al.*, 1972; Hood *et al.*, 1972; Smith and Prior, 1982).

In Sheep 2, in the presence of insulin, the amount of glycerol produced did not change and the amount of glucose being incorporated into fatty acid significantly decreased. It is therefore possible that insulin had the effect of channelling the glucose elsewhere. The most logical destination for this glucose would be to the pentose phosphate pathway for production of the NADPH required for the maintenance of the observed 60% increase in acetate incorporation into fatty acids. In the case of Sheep 1, where there was no increase in the incorporation of acetate into fatty acids in the presence of insulin, there was also no channelling of glucose away from fatty acid production and in fact the excess glucose appears to have been incorporated into the fatty acid molecule itself. This means that there is unlikely to be any increase in the production of NADPH from this source. Since fatty acid production from glucose did increase, it is possible that there was a deficit of NADPH and this may account for the observation that there was no change in the rate of acetate incorporation in the presence of insulin in the tissue from this animal.

The effects of different insulin concentrations on glucose incorporation into triacylglycerol in the absence of acetate is shown in Figure 4.12 in isolated ovine adipocytes and in Figure 4.13 in isolated rat adipocytes. The basal rate of incorporation in rat adipocytes is similar to that in ovine adipocytes. This may indicate that, when there is glucose present but no insulin or acetate, ovine adipocytes have the capacity to utilize glucose to the same degree as rat adipocytes. Since lambs obtain glucose and no acetate from milk, cellular mechanisms for glucose utilization could therefore be expected to be present. However the animals used in the present study were all fully ruminant and therefore no longer obtaining glucose from the diet. Thus, the capacity to utilize glucose exclusively would normally be redundant *in vivo*. Abomasal or intravenous infusion of glucose to ruminants *in vivo* increased fatty acid synthesis from glucose (Ballard, 1972; Lindsay, 1970). Therefore, it would appear that the mechanisms for the utilization of glucose in adult ruminants remain essentially in place, both *in vitro* and *in vivo*.

The effects of insulin on the rates of lipolysis and mass balance of fatty acid

In addition to influencing the rate of lipogenesis, insulin was seen to significantly decrease the rate of glycerol release from the tissue in some experiments (Figure 4.14) but not in others (*e.g.* Table 4.8). In tissue where the antilipolytic response was observed, insulin was effective over a wide range of concentrations (Figure 4.15). As discussed in Section 1.8, it is thought that insulin causes a dephosphorylation of hormone-sensitive lipase which in turn could reduce the amount of triacylglycerol being degraded in the adipose tissue (Nilsson *et al.*, 1980; Belfrage, 1985).

Insulin administration to ruminants *in vivo* results in a rapid decrease in plasma NEFA and glycerol (Bergman, 1968; Bauman, 1976; Hertelendy, 1973; Trenkle and Kuhlmeier, 1966; West and Passey, 1967) and also a decrease in output of NEFA and glycerol from adipose tissue in sheep (Brockman *et al.*, 1975; Khadachurian *et al.*, 1966). However, studies *in vitro* have previously failed to demonstrate this phenomenon (Metz and Van den Bergh, 1972, 1977; Yang and Baldwin, 1973b). Hence the results obtained in the present study would support those observations *in vivo but not those in vitro*.

The effects of insulin on the mass balance of fatty acid in the adipose tissue was also investigated. Insulin stimulated lipogenesis by 92% in tissue from this animal (Sheep 3; Table 4.8), while stimulating the total accumulation of fatty acid within the cell by 132%. Thus insulin's effect on fatty acid accumulation was more than its effect on fatty acid manufacture. This was achieved, not by altering the rate of lipolysis as measured by glycerol release, but by reducing the rate at which fatty acid passed out of the cell. Insulin's effect on the rate of re-esterification was not statistically significant but, since the percentage recycling was already 91%, only a small increase in the rate was needed to take up the extra molecules of fatty acid retained. As discussed previously, insulin also did not appear to affect the capacity of the tissue for re-esterification when the amounts of excess glycerol produced were calculated (Table 4.6 and 4.7). This therefore supports the contention that the rate of re-esterification was largely unaffected by the action of insulin.

In another experiment (Table 4.9, Sheep 4), insulin increased the rate of fatty acid manufacture within the cells by 34% while increasing the rate of fatty acid accumulation within the cells by 50%. Thus, as with Sheep 3, insulin's effect on fatty acid accumulation is more than the effect on fatty acid manufacture. This was achieved by small and not always statistically significant changes to the total fatty acid recycling within the tissue. The rate of lipolysis decreased by 12% while the amount of fatty acid leaving the tissue decreased by 52%. Thus, as with Sheep 3, there was a high rate of recycling of fatty acids occurring in the tissue (89%) and a small change in the rate of re-esterification in the presence of insulin.

As discussed earlier in this section, ovine adipose tissue is relatively insensitive to the actions of insulin, when compared with that of the rat. In approximately one quarter of the experiments with tissue from sheep in the present study, insulin failed to significantly effect the rate of acetate incorporation into fatty acids. One of these experiments is illustrated in Figure 4.10 (Sheep 5). Although having little effect on the rate of lipogenesis or lipolysis in this tissue, insulin significantly decreased the amount of FFA leaving the tissue and thus increased the number of moles of fatty acid remaining in the tissue by 0.64 μmoles/6 hours/10^6cells. This represents about 75% of the extra fatty acid accumulated as a result of the action of insulin. Thus, as

with Sheep 3 and 4, insulin significantly increased the amount of fatty acid accumulated within the tissue.

In the incubations with no hormone in the medium, fatty acid was leaving the tissue from Sheep 5 at a rate which was 4 to 5-fold higher than in the tissue from Sheep 3 and Sheep 4. It is possible that this may be linked with the failure of insulin to influence the rate of lipogenesis in the tissue from Sheep 5. The large amount of fatty acid leaving the tissue may be as a result of prior stimulation by a lipolytic hormone *in vivo* which could also be affecting the ability of insulin to increase the rate of lipogenesis *in vitro*. Nevertheless, the tissue responded to insulin by decreasing that high rate at which the fatty acid passed out of the cells.

When the results from Sheep 3, 4 and 5 are compared, insulin stimulation of fatty acid manufacture has varied from no significant effect to +90% and yet in the same experiments the effect of insulin on the rate of fatty acid accumulation in the tissue varied from +50% to +132%. Thus, in all three experiments, insulin appears to have a greater effect on the amount of fatty acid accumulated in the tissue than it has on the rate of fatty acid manufacture. In all cases, it significantly decreased the rate at which FFA passed out of the tissue.

Thus the overall effect of insulin on the adipose tissue is to significantly increase the amount of fatty acid stored within it. Insulin appears able to elicit this response whether or not it stimulates the rate of acetate incorporation into fatty acids. Insulin also appears to increase the rate of fatty acid recycling in all three experiments. Although the percentage increase was not great (5 - 15%), this illustrates a consistent trend towards energy conservation within the adipose tissue in the presence of insulin. It is therefore postulated that the measurement of the mass balance of fatty acid within the cell could give less variation between animals because, as seen in Table 4.10, the cells may adjust their overall energy balance in the presence of insulin even when there is little change in the rate of lipogenesis. For this reason, it is likely that, in all three experiments, the measured rate of fatty acid manufacture would have provided an under-estimate of the energy-storing effects of insulin.

It is known that insulin's role in the adipose tissue is to control both the utilization of nutrients and energy conservation during times of plentiful food intake. Thus its function during these times of positive energy balance would logically be to encourage the storage of fatty acids in the adipose tissue. The observation, in this study, that its overall effect on fatty acid storage is greater even than its effects on fatty acid manufacture would therefore conform with the established theory. Thus it is considered that the method used to measure the total accumulation of fatty acid in the tissue gives a better estimate of the functions of insulin than a simple measurement of fatty acid manufacture.

Many studies of adipose tissue metabolism have examined only the rate of fatty acid manufacture (*e.g.* Vernon 1978, 1979a, 1979b, 1981b, 1982b; Yang and Baldwin 1973a; Etherton and Evock, 1986; Walton and Etherton, 1986). Since most investigations of adipose tissue metabolism are prompted by concerns about overfatness or obesity, it is the rate of fatty acid accumulation which is of prime importance. It is therefore suggested that if the rate of fatty acid accumulation is measured, in addition to the rate of lipogenesis, this could lead to a better understanding of the effects that outside factors, such as hormones, are having on the actual amount of fatty acid finally stored in the adipose tissue.

For the process of re-esterification to occur, supplies of glycerol would obviously be required. In previous experiments (Table 4.6 and 4.7), the amount of glycerol manufactured in the adipose tissue was estimated using ^{14}C-glucose and ^{14}C-acetate incorporation and it was of interest to compare the results with the rate of re-esterification measured in the tissue in the subsequent experiment. Re-esterification of fatty acid occurred in the tissue without hormone present in the medium at the rate of 1.37 μmoles/4 hours/10^6 cells in Sheep 3, 2.34 μmoles/4 hours/10^6cells in Sheep 4 and 1.88 μmoles/4 hours/10^6 cells in Sheep 5 (Table 4.8, 4.9 and 4.10). In the other experiments, it was calculated that there was an excess of glycerol produced sufficient to re-esterify 0.204 and 0.554 μmoles/4 hours/10^6 cells in Sheep 1 (Table 4.6) and Sheep 2 (Table 4.7) respectively (data converted from nmoles/h/10^6cells). Thus considerably less glycerol was available in tissue from Sheep 1 and 2 than would be needed for the re-esterification calculated to be occurring in tissue from Sheep 3, 4 and 5.

Although this difference between the amount of glycerol required and amount manufactured could be caused by variations in the rates of lipid metabolism occurring in tissue from different animals, it could also be a reflection of the assumptions made in the calculations used for both methods. The factor most likely to be contributing to this difference is the method used to calculate the rate of re-esterification using the rate of glycerol and fatty acid release from the tissue. As previously discussed (Section 10.1), the fatty acid calculated as "re-esterified" in this method included any fatty acid which was oxidized or remained as free fatty acid within the cell. There could also have been partial hydrolysis of triacylglycerol or utilization of mono- or diacylglycerol for the process of lipolysis. All these factors could contribute to an incorrect assessment of the rate of re-esterification actually occurring in this tissue. If it is assumed that 3 fatty acid molecules were released for every glycerol within the cell, then the difference in the two figures indicates that there may have been only enough glycerol to re-esterify about half the fatty acid remaining in the tissue after lipolysis. It would be of interest to combine these two experiments and measure the rate of fatty acid "re-esterification" using glycerol and fatty acid efflux from the tissue while at the same time estimating the amount of glycerol manufactured using ^{14}C-acetate and ^{14}C-glucose in tissue from the same animal.

The percentage of fatty acid released by lipolysis and subsequently recycled was measured in ten animals in the present study and the basal rate was found to vary from 61% to 100%, with the average being about 80%. Wilson (1983) estimated the apparent re-esterification of fatty acids *in vivo* in pregnant ewes on a normal diet by measuring the rates of entry into the blood of both NEFA and glycerol. Using the glycerol entry rate and assuming that 3 fatty acids were released for each glycerol molecule, it was calculated that the amount of fatty acid released by lipolysis within the tissue was 493 g/day and that the actual fatty acid entry rate was 162 g/day. This represents an apparent re-esterification rate of 331 g/day. These figures can be converted to a percentage recycling, as used in the present study, by dividing the fatty acid re-esterified by the fatty acid released within the tissue. Thus 67% of the fatty acid was recycled in this experiment *in vivo*. It could be expected that the energy demands of the foetus in these pregnant ewes could have required that fatty acid was mobilized and the rate of fatty acid recycling reduced. However, the percentage of fatty acid recycling

measured in these animals *in vivo* compares favourably with those rates of re-esterification obtained *in vitro* in tissue from the young growing lambs used in the present study.

In a study with human adipose tissue slices *in vitro*, it was found that 54% of FFA newly formed by lipolysis was re-esterified (Leibel *et al.*, 1985). In this experiment, a different method was used to assess the rates of re-esterification in which it was not necessary to make the assumption that 3 fatty acids were released for each glycerol (see Section 10.1 for a description of the method used by Leibel *et al.*). Since the values for fatty acid recycling in tissue from humans obtained by Leibel *et al.* are not too different from those obtained in the present study with ovine adipose tissue, this could indicate that the assumption that 3 fatty acids are released for each glycerol during lipolysis may be valid. Vernon and Clegg (1985) state in their review, "the proportion of fatty acids that is re-esterified varies and in unstimulated adipose tissue it can approach 100% of the FFA produced by lipolysis both *in vitro* and *in vivo*."

If it is accepted that recycling of fatty acids does occur, then glycerol must necessarily be produced in quantities in excess of that required to esterify only newly synthesized fatty acids. This excess of glycerol has been measured in a previously discussed experiment (Table 4.6 and 4.7). Thus it seems unlikely that glycerol can be rate-limiting for lipogenesis as has been suggested in several previous studies (Baldwin *et al.*, 1969; Bauman *et al.*, 1972; Hood *et al.*, 1972; Smith and Prior, 1982). If they are correct and glycerol is rate-limiting, this would mean that re-esterification must in some way have priority over lipogenesis for the supplies of glycerol.

The high rate of recycling observed in adipose tissue is an example of what is known as a "futile cycle", where a molecule is broken down and resynthesized at the expense of considerable energy consumption. It is a phenomenon that was observed in all adipose tissue examined in this study. There are several reasons why the use of a "futile cycle" could be advantageous to the animal. As has already been discussed, it can mean that a slight alteration in the rate of re-esterification has far greater consequences than the same percentage alteration would have if the rate of recycling had been low. The opposite could also be the case, so that a slight decrease in the rate of recycling will result in a large increase

in the rate of fatty acid passing out of the cell. This could be a feature of adipose tissue metabolism which allows for rapid mobilization of fatty acid when required.

Another advantage of the futile cycle could be in heat production. It is possible that the operation of the "futile cycle" generates the heat required to maintain body temperature in the animal. It is thought that the main function of brown adipose tissue is in heat production but, since this tissue is not present in adult sheep, the same functions could conceivably be carried out by white adipose by fatty acid recycling. This would therefore provide a mechanism whereby hormones, such as insulin, could increase or maintain non-shivering thermogenesis by influencing the rate of fatty acid recycling in the adipose tissue.

It was noticed in the present study that the two youngest animals tested (3 months old) both had re-esterification rates of 100%. This could be explained by the fact that it was winter and that the younger animals had a higher ratio of surface area to volume and thus a higher heat loss. In this case, the increased rates of recycling observed may have had the purpose of generating more heat to compensate for this loss.

Conclusions

The results of all the experiments carried out with insulin confirmed that this hormone exerts a lipogenic, anti-lipolytic effect on ovine adipose tissue slices. Insulin has the overall effect of increasing the retention of fatty acid by the tissue as well as increasing the amount of fatty acid actually stored there. This conforms with insulin's accepted role in nutrient utilization and energy conservation in mammalian species. It was therefore concluded that the *in vitro* model being used gave a valid estimation of lipid metabolism in these animals and that the responses of the tissue to insulin could be used as a standard against which the lipogenic, anti-lipolytic and energy-storing effects of other hormones could be compared and contrasted.

10.3 Adrenalin and nor-adrenalin

Although insulin was used to convert the tissue into a state of positive energy balance, it was felt that the use of a hormone known to alter the tissue to a negative energy balance would allow the various measurements of negative energy balance to be tested and validated. For this purpose, the effects of catecholamines, principally adrenalin, on the ovine adipose tissue slices *in vitro* were investigted.

The effects of adrenalin on the rate of lipolysis

As confirmed by the results seen in Figures 5.1 and 5.2, one of the most important functions of adrenalin in ovine adipose tissue is to increase the rate of lipolysis in the cells. This could be expected to lead to increased fatty acid release from the cells which would thereby provide the additional energy demanded by the emergency situation that prompted the release of adrenalin. In the present study, adrenalin was shown to stimulate the release of glycerol from ovine adipose tissue by up to 4-fold. This is similar to that reported in other studies with ruminant adipose tissue *in vitro* where adrenalin and nor-adrenalin were observed to stimulate the rate of lipolysis by 2 to 10-fold (Khadachurian *et al.*, 1967; Metz and Van den Berg, 1972, 1977; Metz *et al.*, 1973; Pothoven *et al.*, 1975; Bartos *et al.*, 1975a, 1975b; Etherton *et al.*, 1977; Yang *et al.*, 1978).

In one experiment in the present study, adrenalin stimulated the rate of glycerol release by 120% while insulin decreased the rate by 38% (Figure 5.3). When adrenalin interacts with adipose tissue, it is thought to trigger a sequence of events which leads to the reversible phosphorylation of hormone-sensitive lipase. In this activated form, the enzyme then catalyses the degradation of triacylglycerol into its component fatty acid and glycerol molecules (Steinberg and Huttunen, 1972). On the other hand, insulin may be involved in the dephosphorylation of hormone-sensitive lipase (Nilsson *et al.*, 1980). Thus the two hormones may have opposite effects on the rate of lipolysis because of their different effects on the activity of this lipolytic enzyme. Since insulin is a hormone normally associated with times of plentiful food intake, it is unlikely that it would be required to mobilize energy from the adipose tissue. Thus its observed effect, in

this case, of decreasing the rate of glycerol release illustrates its opposing role to that of adrenalin in ovine adipose tissue.

The effect adrenalin had on the rate of lipolysis was significant when measured after 2 hours incubation (Figures 5.1 and 5.2). This was observed when adrenalin was infused into young lambs and there was an increase in the levels of NEFA in the blood after 2 - 4 hours (Alexander *et al.*, 1968).

The effects of adrenalin and nor-adrenalin on the rate of lipogenesis

Since adrenalin is a hormone associated with times of stress when energy is required by the rest of the body, it would not be expected to increase the rate of lipogenesis in the adipose tissue and in fact it was seen to decreased the rate by 17 - 40% (Figure 5.4 and 5.5). Adrenalin usually either did not significantly affect the rate of lipogenesis during the time of the experiment or there was a small depression of the rate, not exceeding 40%. Yang and Baldwin (1973a) reported a similar small depression of the rate of acetate incorporation into fatty acid when incubating bovine adipose tissue in the presence of adrenalin. Thus adrenalin appears to increase energy mobilization from ruminant adipose tissue without altering the basal rate of lipogenesis to any great extent.

Adrenalin is seen to affect the rate of lipogenesis only after four hours incubation (Figure 5.4 and 5.5). Thus it is likely that only during prolonged exposure to adrenalin, lasting more than 2 hours, will any decrease in the rate of lipogenesis greatly influence the amount of energy available to the rest of the body. It is possible that adrenalin affects the rate of lipogenesis only indirectly by product inhibition. Thus increased levels of FFA in the cell, as a result of increased lipolysis, may result in a decreased rate of fatty acid synthesis. Any effect on the rate of fatty acid synthesis via this mechanism would only become apparent following a time delay while the levels of FFA in the cell increased to a level which would inhibit the rate. On the other hand, the effect of adrenalin on the rate of lipolysis was seen to be more rapid.

Nor-adrenalin depressed the rate of lipogenesis from 17-19% and was seen to have an effect on lipogenesis similar to that of adrenalin in the adipose tissue of this animal (Figures 5.5 and 5.6). Both hormones would therefore have similar effects in conserving energy by decreasing its utilization by adipose tissue for long-term storage.

A comparison of the effects of adrenalin and insulin on the rate of lipogenesis showed that, after 2 hours incubation, both insulin and adrenalin stimulated the rate of lipogenesis, although after 4 hours incubation of the same tissue, insulin no longer affected the rate of lipogenesis but adrenalin decreased the rate (Figure 5.7). The results of this experiment illustrate the importance of observing the actions of hormones over a period of time. Some hormones act faster than others and a comparison of their actions at only one incubation time may not necessarily convey the correct impression of the effects of hormones on the tissue. As seen with insulin in Figure 5.7, an initial stimulation of a metabolic process by a hormone can be followed by a period of comparatively slow metabolic activity, possibly while the cells recover their resources or because the hormone is no longer effectively interacting with the tissue.

The initial increase in the rate of lipogenesis in the presence of adrenalin is a surprising result, since it would appear to be counter-productive, but was observed on several occasions during this study. It has been shown that addition of adrenalin to rat epidydimal adipose tissue *in vitro* results in an immediate increase in the consumption of oxygen by adipose tissue (Ball and Jungas, 1965). This increase was sometimes transitory and the levels of oxygen consumption were similar to basal after two hours incubation. As concluded by Ball and Jungas, it is possible that this increase in oxygen demand reflects the increased utilization of ATP for re-esterification of the excess FFA produced in response to adrenalin. However, since fatty acid synthesis has been shown to result in increased oxygen consumption (Flatt and Ball, 1965), the observation that adrenalin increases initial oxygen consumption in adipose tissue may also be as a result of an immediate but transitory increase in the rate of fatty acid manufacture.

The effects of adrenalin on the mass balance of fatty acid

The effects of adrenalin on the mass balance of fatty acid in ovine adipose tissue *in vitro* were investigated. In the tissue from Sheep A, adrenalin increased the rate of lipolysis within the tissue by 2-fold while increasing the rate at which fatty acid left the cells by 6-fold (Table 5.1). The tissue was therefore seen to mobilize the fatty acid which would be required for the event that stimulated the release of adrenalin. However, the actual amount of fatty acid which left the cells represented only a fraction of the amount of fatty acid which was released within the tissue by the process of lipolysis, as measured by glycerol release into the medium. By far the major part of this fatty acid remained in the cells and was presumably re-esterified.

A similar result was obtained in tissue from Sheep B, where although adrenalin increased both the rate of lipolysis within the tissue and the release of free fatty acid, it also increased the rate of re-esterification (Table 5.2). Thus, although there was an extra 0.34 μmoles of fatty acid/6 hours/10^6cells released within the tissue following adrenalin action, 0.29 μmoles/6 hours/10^6cells of this was retained by the tissue.

It is known that during shock, when adrenalin would almost certainly be present, the level of lactate rises in both adipose tissue and blood (Fredholm and Hjemdahl, 1979). Plasma lactate levels in sheep have also been shown to rise following an intravenous injection of 1.5 mg of adrenalin, as did levels of glucose and NEFA (Bassett, 1970). Since lactate is known to inhibit fatty acid release by causing an increased rate of re-esterification (Fredholm, 1971), it is possible that the observed increase in the rate of fatty acid re-esterification in both Sheep A and Sheep B was a direct result of the increased presence of lactate in the cells.

The apparent increase in re-esterification in the presence of adrenalin is in agreement with the observation by Ball and Jungas (1965) that there is an increase in oxygen consumption by adipose tissue during the initial stages of stimulation by adrenalin. This could reflect the increased utilization of ATP for re-esterification of excess FFA. However, the increased oxygen demand may be due to the unexplained phenomenon of increased lipogenesis in the initial stages of adrenalin

stimulation, as described earlier in this section. Thus it is possible that both the transitory increase in lipogenesis and the more continuous increase in re-esterification may both be responsible for the observed increase in oxygen demand in the cells following adrenalin stimulation.

Injection of adrenalin into Syrian fat-tailed sheep *in vivo* stimulated the uptake of glucose by the adipose tissue of the tail pad (Khadachurian *et al.*,1966), an effect which could have been due to the increased requirements of glucose for glycerol manufacture. Thus this observation *in vivo* could also indicate that fatty acid re-esterification was increased as a result of the action of adrenalin.

Therefore the observation that adrenalin has caused an increase of nearly 2-fold in the rate of re-esterification of fatty acid is supported by evidence from other studies. On the other hand, there is a possibility that it was the *in vitro* conditions which were causing the increased retention of fatty acid by the tissue. It has been reported that the release of glycerol and fatty acid from bovine adipose tissue *in vitro* was gradually inhibited when the fatty acid : albumin ratio increased (Metz *et al.*, 1973). However, as discussed previously, an increase in the concentration of BSA in the incubation medium to 4% did not result in any increase in the rate at which fatty acid left the cells in the present study. It therefore seems most likely that the rate of fatty acid efflux from the cells *in vitro* reflects that which normally occurs *in vivo*.

Since most *in vitro* experiments reported in the literature are carried out with BSA at a concentration of 1 to 4% w/v, the effects of accumulating FFA in the tissue ought to be examined. If the fatty acid released by lipolysis is not being released from the cell and, at the same time, is not being re-esterified, then the accumulating FFA would almost certainly begin to depress the rate of lipolysis, as measured by glycerol release.

An alternative explanation for the observed increase in the rate of re-esterification in both Sheep A and Sheep B may be that the estimation of fatty acid re-esterified includes the amount of fatty acid which remained inside the cells in the form of free fatty acid or which was oxidized. (For a discussion of the method for estimation of re-esterification, refer to Section 10.1). Adrenalin may well have had the effect of increasing the amount of fatty acid oxidized within the

adipocytes. Since nutrients normally supplied to the adipose tissue by the blood will be required elsewhere in the body (*e.g.* brain and muscle) following adrenalin stimulation, it would seem logical if the adipose tissue began to oxidise fatty acid for its own maintenance. Under more normal circumstances, when there is no adrenalin present, there would be no need for adipose tissue to oxidise fatty acids because substrates such as acetate and glucose would be present in sufficient quantities. This could explain why there was an increase in the estimated rate of "re-esterification" in the adrenalin-stimulated cell as compared with the normal situation in the basal incubations.

In the tissue from both Sheep A and B, the rate of glycerol release increased considerably more than the rate of accumulation of fatty acid in the tissue. This means that although the estimation of the amount of fatty acid release accomplished by measuring the rate of glycerol release into the incubation medium is probably providing the best indication of the amount of lipolysis occurring in the tissue, it gives a very poor indication of the overall effect of the hormone on the cell.

It can be seen that, because of the simultaneous effects of adrenalin on fatty acid retention within the cell, the effects of adrenalin on the adipose tissue in this study would have been over-estimated if only the rate of glycerol release had been observed. This means that any study that reports only glycerol release (*e.g.* Duquette *et al., 1984; Vernon and Finley, 1984; Fain et al.*, 1984; Larsen *et al.*, 1985) should not necessarily be used to draw conclusions about the overall effects of the lipolytic hormone on fatty acid accumulation within the adipose tissue.

The rate of glycerol release, as opposed to the rate of fatty acid release, has been used to estimate the rate of lipolysis *in vitro* because there is less chance that glycerol would re-enter the cell and be re-used, as fatty acid can. Ovine adipose tissue appears to lack the means (glycerol kinase) to re-utilize glycerol for esterification (Khadachurian *et al.*, 1967) and uses glucose to manufacture new glycerol molecules (Vernon, 1980). However if, following a large increase in the amount of lipolysis occurring in the cell, there is a corresponding increase in the rate of re-esterification, then glycerol release does not give a good indication of the energy balance in the cell. Although free fatty acid may pass in and out of the cell, its net efflux may be a

better estimate of the effects of lipolytic hormones than glycerol release. However, the superior method may be a measurement of fatty acid accumulation, which uses the difference between the amount of fatty acid manufactured and the amount of fatty acid released from the tissue. As has already been discussed, this measurement does not require an assumption to be made about the ratio of fatty acid to glycerol released following lipolysis.

If triacylglycerol was broken down only as far as mon- or diacylglycerol, fatty acid but no glycerol would be released. In this case, observation of glycerol release alone would indicate that the tissue was not mobilizing fatty acid. An observation of fatty acid release or fatty acid accumulation however would indicate that the tissue was in negative energy balance.

There is another disadvantage of the use of glycerol release to estimate the overall effect of a hormone on the cell. If there was a large increase in glycerol release, accompanied by a large increase in re-esterification, the cell would require a correspondingly large increase in the supply of glucose to manufacture the glycerol needed for re-esterification. The glucose would probably be drawn from outside the cell and would thus drain immediately available glucose away from the rest of the body. Although the glycerol will ultimately be converted to glucose by the process of gluconeogenesis, it will not be available in the short term because most of the glycerol kinase required for its conversion is present in the liver (Vernon and Peaker, 1983). This is in an animal where, if only the glycerol release data had been observed, it could be concluded that energy supplies were passing from the adipose tissue into the rest of the body. In fact the adipose tissue may have become a net user of glucose.

However, the fact that the rate of glycerol release is high compared with the rate of fatty acid release from the tissue following stimulation by adrenalin may imply that this is a mechanism by which energy is mobilized from the adipose tissue. Considering that the cell must manufacture new supplies of glycerol for re-esterification while that released by lipolysis passes out of the cell, this may suggest that the adipose tissue is manufacturing glycerol for utilization elsewhere. This could imply that an alternative destination for the glycerol, apart from the liver for gluconeogenesis, may in fact exist, particularly during

times of stress. As has already been suggested, the more likely explanation is that the tissue is producing large quantities of glycerol, only to immediately release it, as a by-product of the "futile cycle" which may occur for the maintenance of non-shivering thermogenesis.

In the tissue from Sheep A, the percentage of fatty acid recycling within the cells following lipolysis decreased from 90% to 77% and in tissue from Sheep B remained largely unchanged. When the results are compared with those in Tables 4.8, 4.9 and 4.10, it can be seen that once more adrenalin had the opposite effect to that of insulin, with insulin increasing the rate of recycling and adrenalin having either no effect or decreasing it. When Leibel *et al.* (1985) measured the rate of re-esterification in human adipose tissue *in vitro*, they found that the basal rate of recycling of 54% was reduced to 20% in the presence of a mixture of adrenalin and phenolamine.

The effects of adrenalin on the actions of insulin

Even though the handling of the sheep during preparation for slaughter was minimized, this was undoubtedly a stressful situation for these pasture-fed animals. Thus serum levels of adrenalin were almost certainly high at the time of slaughter (Pearson, 1979). Although adrenalin is known to have a very short half-life in the body, there is still the possibility that some of this adrenalin was present in the adipose tissue when it was prepared for incubation and would not have been washed out during the slicing process. Also the effects of the adrenalin on the cells could have been still evident during the subsequent incubation, even though the hormone itself was no longer present.

An experiment was therefore carried out to measure the influence of adrenalin on the actions of insulin. Adrenalin, although acting alone to depress the rate of lipogenesis by a maximum of 35%, was seen to have no significant effect on the action of insulin in these cells (Figure 5.8). Insulin stimulation of the rate of lipogenesis was slightly depressed by adrenalin but the difference was not significant. It was therefore assumed that if any adrenalin was present in the tissue after slaughter it was unlikely to affect the observations of the actions of insulin to any great extent. Since insulin and adrenalin are acting via different receptors, this result is not surprising.

However, it is not known to what extent the presence of adrenalin in the tissue at slaughter could have effected the basal rate of both lipogenesis and lipolysis in the subsequent incubations. As discussed earlier in this chapter, the activity of a hormone present in the tissue at slaughter can affect the resources of the cells which will in turn affect the ability of the cell to respond to a hormone introduced in the subsequent incubation. Thus the presence of adrenalin in the tissue immediately after slaughter could conceivably alter the basal rate of lipid metabolism within the cell in the first few hours of the incubation. The basal rate of lipolysis could be higher than it should be while the rates of lipogenesis and fatty acid accumulation could be lower. thus giving a false impression of the any changes occurring as the result of the subsequent actions of hormones. For instance, if the cells were already mobilizing energy, they would be less likely to respond to any further stimulation. Thus, the effects of other lipolytic agents on the cells could be considerably under-estimated.

Degradation of the adrenalin *in vitro* will not necessarily negate the effects of this animal-derived adrenalin since this did not occur in the experiments where adrenalin was added to the incubation medium. Although only one dose of adrenalin at a physiological concentration was added to the medium at the beginning of the incubation, its effects on lipolysis (*e.g.* Figure 5.1) and fat accumulation (*e.g.* Table 5.1) were observed 4 - 6 hours later. These observations illustrate the importance of minimizing the stress of the animal at the time of slaughter.

Conclusion

The results of all the experiments carried out with adrenalin in this study confirmed that the hormone exerts a lipolytic, anti-lipogenic effect on ovine adipose tissue slices *in vitro* which has the overall effect of decreasing the amount of fatty acid stored in the tissue. This conforms with the accepted role of adrenalin in energy mobilization during emergency situations in mammalian species. It was therefore concluded that the *in vitro* model of adipose tissue metabolism being used gave a valid estimation of the energy balance of the tissue and that the responses to adrenalin could be used as a standard with which the lipid-mobilizing effects of other hormones could be compared.

10.4 Growth hormone

One of the hormones thought to have an influence on lipid metabolism in adipose tissue is GH. It is a hormone known to be associated not only with increased growth but also with a reduction in the deposition of fat in the adipose tissue *in vivo* (Chung *et al.*, 1982, Johnsson *et al.*, 1985). However, the direct effects of GH on the processes of lipid metabolism in ruminant adipose tissue *in vitro* are not so clearly documented.

The purity and stability of growth hormone preparations

Before any experiments were carried out to determine the biological function of GH, preliminary investigations of the structure and immuno-reactivity of the several GH preparations to be used were undertaken.

The bGH, in the presence of 1% w/v BSA, was not degraded during a 6 hour incubation period with ovine adipose tissue slices (Table 6.1). This is in contrast to the situation with insulin where losses of immuno-reactive hormone in the presence of tissue were high (Table 4.2). Since there was loss of immuno-reactive hormone when BSA was not present and even when another polypeptide in the form of Polypep^{R} was used, it was concluded that BSA prevents hormone degradation. Even storage of the hormone preparation at 4°C for several hours until the RIA was carried out resulted in considerable loss unless BSA was present. Thus all incubations with GH in the medium were carried out in the presence of BSA. It was also seen that the immuno-reactive concentration of bGH was considerably higher than expected when measured with BSA (Table 6.1). This may be due to the heterogeneous nature of both the hormone and also the antibody used for the assay. Thus the apparent increase may be due to cross-reaction of degradation products of GH with the antibody during the radioimmunoassay. However, since the concentration has not changed during the incubation, it would appear that any degradation was not tissue-associated.

The advisability of initial investigations of GH components using electrophoresis is illustrated in Plate 6.1. A recombinant DNA-derived bGH preparation obtained from Genentec for use in these studies was found to contain a continuous array of different protein molecules, very little

of which resembled GH. As a result of this, the use of the preparation was discontinued.

Using SDS polyacrylamide gel electrophoresis, the purified oGH was seen to consist of five major components, only two of which had the same molecular weight as the bGH (Plate 6.2). There were also five major bands in the oGH preparation seen by isoelectric focusing on polyacrylamide gel electrophoresis (Plate 6.1). In view of the heterogeneous nature of the oGH preparation, its use in the present study was also discontinued.

The recombinant DNA-derived bGH from Cyanamid and the purified bGH from Dr I. Hart were used for most of the bioassays carried out in this study. It was shown by SDS polyacrylamide gel electrophoresis that these two hormones both contained a components of 20 kDa (Plate 6.2). The recombinant DNA-derived product was more homogeneous than the purified product. This may indicate that there had been some cleavage of the pituitary-derived GH (Singh *et al.*, 1974) which increased the effective radius of the molecule and resulted in retardation of one component (Lewis *et al.*, 1980).

The heterogeneity of GH has been attributed to the presence of modified forms such as aggregates, dimers and cleaved fragments. It is also possible that multiple genes for the hormone exist. It is thought that the variants of GH may serve as prohormones for other peptide hormones, each with a specific restricted metabolic action. Proteolytic modification of all the various forms of GH, each acting as a specific substrate, may result in a variety of molecules each of which interacts with a specific receptor and produces a different response (reviewed by Lewis *et al.*, 1984). It has been reported that the variants of bGH may have individual biological functions (Hart *et al.*, 1984a). Since the purified bGH used in the present study has a heterogeneous nature (Plate 6.2), it is likely that any biological responses observed could be a result of interaction of any one component with the tissue.

The effects of growth hormone on the rate of lipogenesis

GH was seen to increase the rate of lipogenesis in the adipose tissue in several experiments (Figures 6.1 and 6.2). However, it was apparent that bGH does not consistently increase the rate of acetate incorporation in tissue from these animals (*e.g.* Table 6.2). Although incubation conditions were as similar as possible between experiments, there may be underlying physiological conditions in the cells which were determined before slaughter and subsequently caused the differences observed *in vitro*. One of the most important of these could be the occurrence of episodic GH release *in vivo* and the amount of time that had elapsed since this had occurred when the tissue was being incubated. It is quite likely that recent exposure of the cells to GH could render them insensitive to further stimulation by GH for some considerable time (Eden *et al.*, 1982). In some studies, this problem has been overcome to a certain extent by using hypophysectomized rats. A transient increase in the rate of lipogenesis in tissue from these animals has been reported (Goodman and Schwartz, 1974). However, as discussed in Section 1.9, the use of hypophysectomy causes the loss of many other hormones and could result in unknown changes occurring in the physiology of the cells. In the present study, the use of a pre-incubation time of several hours, as suggested by Eden *et al.* (1982), was intended to overcome the problem of previous exposure of the tissue to GH but it is not known to what extent this was successful. It is possible that, in tissue from some animals, this length of time was not enough to allow the cells to again interact with GH.

The observed response to bGH was identical to the response to insulin in increasing the rate of acetate incorporation into fatty acid (Figure 6.3). In rat epidydimal fat cells, hGH was shown to cause several insulin-like effects, such as increased glucose oxidation as well as increased incorporation of glucose into lipid. Unlabelled GH, but not insulin, reduced the binding of ^{125}I-hGH to these cells and it was concluded that GH was not interacting with the insulin receptor binding sites to achieve the responses (Grichting *et al.*, 1983). It is possible that, when acetate incorporation into fatty acid is stimulated by GH in ovine adipose tissue, the tissue is responding in a similar way to rat adipocytes when glucose incorporation into fatty acid is stimulated.

It has been observed in several studies with rat adipose tissue *in vitro* that there is an initial, usually transitory, enhancement of glucose transport following exposure to GH (Goodman, 1965; Grichting *et al.*, 1983). This is a possible mechanism for the observed response to bGH in ovine adipose tissue. Addition of glucose to the incubation medium increased acetate incorporation into lipid in ruminant adipose tissue (Hanson and Ballard, 1967; Ballard *et al.*, 1969; Ballard *et al.*, 1972; Vernon, 1977b). It was shown that glucose, rather than insulin, was the major stimulant of this response (Vernon, 1977b; Yang and Baldwin, 1973a). If additional glucose entered the cells in the present study as a result of the action of bGH then it is possible that the same stimulating effect of glucose caused the increase in acetate incorporation into fatty acid. But, as discussed in Section 10.2, increased glucose in the cells will only result in increased acetate incorporation into fatty acid if supplies of NADPH and glycerol-3-phosphate are rate-limiting. When NADPH and glycerol-3-phosphate supplies are adequate and therefore not rate-limiting, then GH, like insulin, may not be observed to enhance acetate incorporation into fatty acid.

The effects of growth hormone on the rate of lipolysis

A lipolytic response to purified GH *in vitro* has been demonstrated in rat adipose tissue in some studies (Katosc *et al.*, 1973; Wieser *et al.*, 1974; Sengupta *et al.*, 1981; Goodman and Grichting *et al.*, 1983; Goodman, 1984; Hart *et al.*, 1984a, 1984b) but not in others (Frigeri, 1980; Frigeri *et al.*, 1983). It was thought in the latter studies that the lipolytic activity was associated with an acidic fraction removed during the purification process. There has also been no demonstrable lipolytic activity in adipose tissue from rabbit (Bowden *et al.*, 1985) or sheep (Duquette *et al.*, 1984). However, in the present study, GH stimulated the rate of lipolysis, as measured by glycerol release from the ovine adipose tissue slices (Figures 6.4 and 6.6). The effect appeared to be transient in both experiments, lasting only 2 - 4 hours. However, as with the lipogenic effects of this hormone, the lipolytic effects were not consistently observed.

The lipolytic activity of GH seen in Figures 6.4 and 6.6 was obtained using adipose tissue from normal animals which had not been hypophysectomized. As discussed previously, the tissue could have been exposed to recently released GH *in vivo* and this could account for the

lack of response seen in other experiments. Also the responses were obtained in the absence of glucocorticoid. The lipolytic response to GH has been shown to be synergistic with glucocorticoid in rat adipose tissue (Fain *et al.*, 1965). It is possible that the presence of glucocorticoid could enhance the lipolytic response to ovine adipose tissue, although this was not observed by Duquette *et al.* (1984).

In the present study, both adrenalin and bGH stimulated the rate of glycerol release by similar amounts after 2 and 4 hours of incubation, while insulin had the opposite effect (Figure 6.5). When these hormones were injected into goats, there was a significant increase in plasma NEFA levels in animals injected with either adrenalin or bGH, while the levels fell significantly in animals injected with insulin (Radloff and Schultz, 1966). Considerably more NEFA was mobilized in those animals injected with bGH than in those injected with adrenalin and the authors concluded that bGH was the more potent lipolytic hormone. The results shown in Figure 6.5 would indicate that bGH can be at least as potent as adrenalin in enhancing lipolysis in ovine adipose tissue *in vitro*. The differences in the relative potencies seen in these experiments *in vitro* compared with those observed *in vivo* may be due to the synergistic effects of other hormones, such as glucocorticoids, which could have influenced the responses *in vivo* but not those *in vitro*.

Both recombinant DNA-derived bGH and purified bGH were seen to be lipolytic in ovine adipose tissue (Figures 6.4 and 6.6). The two hormone preparations probably have similar protein components although the recombinant DNA-derived product appears to be more homogeneous (Plate 6.2). It is thought that the different molecular forms commonly found in pituitary-extracted GH may account for the multiple metabolic activities of the hormone (Lewis *et al.*, 1980, Hart *et al.*, 1984a). It is therefore apparent that a lipolytic component is present in both the bGH preparations used in the present study. It would be of interest to separate the two main components of the purified bGH, as seen in Plate 6.2, and determine whether the lipolytic activity was associated with the component which most closely resembled the recombinant DNA-derived bGH and not with the other.

Hart *et al.* (1984b) have reported that, although purified bGH significantly increased the rate of glycerol release from rat adipose tissue *in vitro*, recombinant DNA-derived bGH did not. Although the

recombinant DNA-derived bGH used by Hart *et al.* was not from the same source as that used in the present study, and may therefore contain other GH components, it appears that the hormone can be lipolytic in ovine adipose tissue where it is not in the rat.

In addition, both preparations of bGH used in the present study must have contained a component which, under some circumstances, could elicit a lipogenic response in the tissue. Thus bGH appears to have multiple effects on lipid metabolism in ovine adipose tissue, as has been observed in rat adipose tissue.

The time at which the maximum lipolytic response was observed in the present study varied, with the response being observed only when measured at 2 hours in one experiment and at 2 and 4 hours in another. Duquette *et al.* (1984) used an incubation time of 4 hours only and this may be one of the reasons why they failed to observe a lipolytic response to GH. The transitory nature of the lipolytic response in this tissue could be associated with the affinity or availability of the receptors involved or alternatively with the activity of the lipolytic enzymes (*e.g.* hormone-sensitive lipase and monoacylglycerol lipase). It is possible that the receptors or alternatively the enzymes remain active for only a few hours following stimulation by bGH in ovine adipose tissue. It is possible that the effects of glucocorticoids, which have been shown to enhance RNA synthesis, could be involved in the lipolytic actions of GH (Fain *et al.*, 1965). In the absence of glucocorticoids, the activity of the enzymes could be affected if the protein synthesis does not proceed and this may be the reason why the response was not sustained in the present study.

There is a possibility that the difference in observed effects of GH between different laboratories is due to a difference in incubation times. Hart *et al.* (1984b), Frigeri (1980) and Frigeri *et al.* (1982) all used incubation times of 4 hours. But Frigeri *et al.* (1983) found no response to purified hGH when they measured the rate of glycerol release after 1 hour. The lipolytic response to the acidic fraction of the hGH was still sustained after 4 hours incubation. On the other hand, Goodman *et al.* (1983) found a significant increase in the rate of release of glycerol in response to both the highly purified hGH and the acidic fraction following 2 hours of incubation. The effect was still observed after 4 hours. It is possible that, even in the presence of

glucocorticoid, the time taken for the hormone to interact with the cells and cause a lipolytic response is varying with different tissue. This could indicate that time-course investigations are necessary in experiments with GH *in vitro*.

When Hart *et al.* (1984b) administered both purified and recombinant DNA-derived bGH to sheep *in vivo*, there was an immediate significant increase in plasma NEFA levels 2 hours after the injection of either hormone and the effects were sustained for approximately 7 hours. The plasma NEFA levels had fallen to pre-injection levels after 9 hours. There were no plasma NEFA levels reported for times earlier than 2 hours and it would be of interest, in the light of results in the present study, to know how long the hormone takes to have this effect *in vivo*. Since there was a significant lipolytic response to bGH *in vivo* by 2 hours and since the response may be transitory *in vitro*, it is indicated that measurements of biological activity, at least in ovine adipose tissue, should not be done solely at incubation times longer than 2 hours.

It is also possible that the range of concentrations at which the hormone is active is very narrow and can change in tissue from different animals and depending on the hormone preparation being used (Figures 6.7 and 6.8). An immuno-reactive concentration of 70 - 115 ng/ml was found to be the most suitable for demonstration of the lipolytic activity *in vitro* in the present study. Duquette *et al.* (1984) measured glycerol release from ovine adipose tissue following exposure to 1 - 25 μg/ml oGH and failed to observe a lipolytic response. It is apparent that the concentrations of GH they used were well outside the narrow operating range for GH in this tissue.

In sheep *in vivo*, the baseline level of GH is 3 - 4 ng/ml with the episodic release increasing the concentration to approximately 30 - 50 ng/ml (Davis *et al.*, 1977). Thus, if these plasma concentrations reflect those in the adipose tissue *in vivo*, it would appear that GH may not normally be active in mobilizing fatty acids in the adipose tissue during the baseline levels of 3 - 4 ng/ml and only becomes lipolytic following an episodic release of the hormone.

The effects of bGH on the mass balance of fatty acid

As discussed previously, the effects of hormones on fatty acid accumulation in the adipose tissue could give a better indication of the changes occurring in the overall energy balance of the cells. However, because of the variety of effects that GH had on the tissue and because the nature of these effects was sometimes contradictory, a generalization about the influence of GH on fatty acid accumulation *in vitro* is not possible.

In one experiment, although GH had little effect on the rate of either fatty acid manufacture, release or re-esterification, the release of FFA from the tissue was increased (Table 6.2). This may be an indication of previous lipolytic activity of bGH in this tissue. Since the measurements of mass balance were not done until after six hours of incubation, the increase in the rate of release of glycerol may have disappeared by the time the samples were taken. If the effects of bGH on glycerol release were significant but transitory, then it appears that the FFA so released was slower to come out of the cell than was the glycerol. The overall effect was that the rate of fatty acid accumulation in the cells during the incubation decreased by 8-fold. This means that, even though no actual increase in glycerol release was observed, the cells appear to be in negative energy balance. The recycling of fatty acid in this tissue decreased from 61% to 42%, thus also indicating that the cells were in negative energy balance.

An alternative explanation for the the observation that bGH did affect glycerol release from this tissue could be that bGH caused tissue-associated FFA to pass out of the cell without the need for any further lipolysis to release the FFA from the triacylglycerol molecules. The same effect would be observed if bGH had caused the triacylglycerol molecules to be broken down only as far as mono- or diacylglycerol. This mechanism would be feasible if GH was acting only to increase the activity of hormone-sensitive lipase and not that of monoacylglycerol lipase (see Section 1.1). As a result, there would be release of FFA from the cell but no glycerol. This further illustrates one of the disadvantages of placing too much reliance on observations of glycerol release as an indication of the lipid-mobilizing actions of hormones *in vitro*.

GH acted only at a concentration of 100 ng/ml to decrease the rate of fatty acid accumulation in the tissue (Figure 6.9). As was the case with the effects of GH on lipolysis, this implies that the adipose tissue only enters a period of negative energy balance during the peaks of plasma GH concentration following episodic release *in vivo*. A decrease in fatty acid accumulation in the tissue when GH was present *in vitro* is similar to the observation *in vivo*, where administration of exogenous GH to sheep caused a decrease in the deposition of fat in the carcass (Chapter 9; Muir *et al.*, 1983; Johnsson and Hart, 1985).

The bGH had no significant effect on the mass balance of fatty acid in tissue from five other animals, one after 6 hours and four after 4 hours of incubation. Because of the difficulties involved in assessment of tissue-associated fatty acid, the effects of the hormones on mass balance of fatty acids was not measured at 2 hours. However, as shown in Table 6.2, it may still be possible to measure a negative energy balance in the tissue even when no stimulation of glycerol release is observed.

Summary

There were both lipogenic and lipolytic responses in adipose tissue *in vitro* from some sheep as a result of bGH activity while in tissue from others bGH appeared to have no effect. The lipolytic response was observed to be transitory and had disappeared by 4 hours in tissue from one animal and by 2 hours in tissue from another. It is suggested that the lack of observed lipolytic activity of GH in some experiments was due to the transitory nature of the response which had become non-significant by the time the sample for glycerol analysis was taken.

The transitory nature of the response may also have been associated with the observation that bGH seldom affected the rate of fatty acid accumulation in the cells. The hormone caused the adipose tissue from one animal to go into negative energy balance following 6 hours incubation, even though there was no significant effect of the hormone on glycerol release. This was achieved by the cells releasing a significant amount of FFA into the incubation medium following exposure to the GH. This may be an indication of previous lipolytic activity in the tissue.

The action of bGH on both the rate of lipolysis and fatty acid accumulation was effective only at a narrow range of hormone concentration. This suggests that, if GH causes the adipose tissue to mobilize fatty acid, the phenomenon will only occur at the peaks of episodic release *in vivo* and not during times when GH concentration in the blood is at the low background level.

10.5 Insulin-like growth factors

IGFs have only recently become available in sufficient quantities to allow investigations of their biological activity. Little is known about the actions of IGFs in ruminant adipose tissue although their activity is thought to be linked with that of GH (Wien *et al.*, 1983: Clemmons and Van Wyk, 1984). It has been reported that the functions of IGFs in adipose tissue from the rat *in vitro* appear to have insulin-like characteristics (Zapf *et al.*, 1978; Poggi *et al.*, 1979; Froesch *et al.*, 1985).

The stability of IGF

Although there was no method available to measure the immuno-reactive concentration of IGF II, a preliminary experiment was carried out to measure the stability of the IGF I in the incubation medium. There was no detectable loss of immuno-reactive concentration of IGF I when it was incubated in the presence of tissue slices and BSA. As discussed in Section 10.2, this was a surprising result when compared with that obtained when insulin was incubated in the presence of tissue slices and loss of hormone was considerable (Table 4.2). It is thought that the action of proteolytic enzymes from the tissue causes insulin degradation (Gliemann and Sonne, 1978) and it would seem likely, since the IGF I molecule has many similarities in structure to that of the pro-insulin molecule, that the same enzymes degraded IGF I. However, it would appear that either the enzymes are specific for insulin or that the losses of insulin were caused by some other factor.

The effects of IGF on the rate of lipogenesis

Both IGF I and IGF II, at concentrations of 40 ng/ml, significantly stimulated the rate of acetate incorporation into tissue lipids throughout the 6 hour incubation period (Figures 7.1, 7.2 and 7.3). Etherton and Evock (1986) also demonstrated a significant increase in the rate of acetate incorporation into lipid in bovine adipose tissue slices *in vitro* using IGF I concentrations of 30 - 500 ng/ml. In another study with rat adipocytes, IGF I at a concentration of 300 ng/ml and IGF II at a concentration of 100 ng/ml stimulated glucose transport and at concentrations of 10 - 100 ng/ml stimulated glucose oxidation and uptake into lipid (Zapf *et al.*, 1978). Thus it would seem that the IGFs cause insulin-like responses in ovine adipose tissue in the same way as they do in rat and bovine adipose tissue

Insulin stimulated the rate of lipogenesis by almost the same amount when present at a concentration of approximately 1/20th of that of IGF I (Figure 4.1 compared with Figure 7.1). A similar effect has been observed *in vitro* in muscle cells from the mouse where IGFs stimulated glucose transport, glycolysis and protein synthesis and were 5 - 10% as potent as insulin when mediating these insulin-like effects (Poggi *et al.*, 1979). It has also been reported that the IGFs are 1/5th to 1/100th as potent as insulin in exerting the insulin-like effects in rat adipose tissue. Thus there is some indication that the relative potencies in ovine adipose tissue may be similar to those rat and mouse adipose tissue.

Both IGF I and MSA (rat IGF II) behave very similarly to insulin in their effects on lipogenesis when the IGFs are present at a concentration of 40 ng/ml (Figures 7.2 and 7.3). It has been reported that IGF I and IGF II have equal potencies when stimulating the rate of glucose uptake and net gas exchange but that IGF II is 2 to 3-fold more potent in stimulating the rate of glucose incorporation into fatty acids (Zapf *et al.*, 1978). However, the insulin-like effect being observed in the present study was acetate incorporation into fatty acid as opposed to glucose incorporation into fatty acid observed in the rat adipose tissue. Thus it is likely that there could be different hormone potencies as a result.

It has been suggested that IGF interacts with the insulin receptor to exert the insulin-like responses in rat adipose tissue (King *et al.*, 1980; Zapf *et al.*, 1981a). There is a possibility that the same is occurring in these experiments with ovine adipose tissue although the insulin-like effect being observed here (incorporation of acetate into fatty acids) is different from that investigated in the rat (incorporation of glucose into fatty acids). As discussed in Section 10.2, ovine adipose tissue appears to be relatively insensitive to the actions of insulin on lipogenesis compared with adipose tissue from the rat. If IGFs are interacting with the insulin receptor to increase the rate of lipogenesis, this would imply that ovine adipose tissue would also be relatively insensitive to the actions of IGF I compared with the rat. However, IGF I at 40 ng/ml stimulated the rate of acetate incorporation into lipid by 100 to 500% and IGF II at the same concentration stimulated the rate by only 53% in the present study while it has been reported that both IGFs at 100 ng/ml stimulate glucose incorporation into lipid in rat adipose tissue by 100% (Zapf *et al.*, 1984). It is unclear why ovine adipose tissue appears to be more sensitive, at least to IGF I, than rat adipose tissue. If insulin stimulation of lipogenesis in rat adipose tissue is at least 2-fold greater than in ovine adipose tissue (Section 10.2) and yet IGF I stimulation of lipogenesis in rat adipose tissue is about the same or considerably less than in ovine adipose tissue, it would appear that there is some possibility that IGF I is using a different mechanism to insulin when exerting this insulin-like effect.

The concentrations of IGF used in these experiments was 40 ng/ml to simulate that normally seen in sheep serum. However, IGF I and IGF II are present in blood bound to specific binding proteins which may restrict their biological activity (Zapf *et al.*, 1979). It is not known what the relationship is between bound and unbound IGF nor whether IGF at the cell surface is active in the bound or free state. It is thought that the presence of binding proteins restricts the movement of IGF through the capillaries and inhibits access of the hormone to the membrane receptors and binding sites (Meuli *et al.*, 1978; Zapf *et al.*, 1979; Daughaday *et al.*, 1980). This could be interpreted to mean that the concentration of IGFs normally seen in the serum is effectively higher than the concentration of active hormone at the cell surface.

Thus the use of concentrations of IGF in excess of 20 to 50 ng/ml, which are similar to the concentrations of both bound and free IGF in the serum (see Section 9.3), could mean that the responses observed *in vitro* are being mediated by an IGF concentration which may well be higher than that normally found at the cell surface. These high concentrations of IGF have been shown to exert insulin-like effects in adipose tissue which may be mediated via the insulin receptor. Since this would mean that the IGFs are interacting with a receptor for which they have little affinity (Kasuga *et al.*, 1981; Massague and Czech, 1982; Rechler and Nissley, 1985), it is possible that the concentrations of IGF being used in the experiments to demonstrate the insulin-like effects *in vitro* are higher than those which would be seen at the cell surface *in vivo*. If this were not the case then it is likely that the animal would be in a state of permanent hypoglycaemia (Zapf *et al.*, 1984). When IGFs were tested in the present study at concentrations less than 40 ng/ml, they did not stimulate the rate of acetate incorporation into fatty acids. Thus it seems likely that, at these low concentrations, IGFs do not interact with the insulin receptor.

The effects of IGF on the rate of lipolysis

It is not known what the concentration of IGF at the cell surface actually is, neither is it known whether the presence of binding proteins are necessary for IGF to interact with the cell membrane (Hintz, 1984). However, since BSA does not contain IGF binding proteins, there were no binding proteins in the incubation medium in the present study. Therefore if, as has been suggested by Hintz (1984), the interaction between IGF and the cell receptor requires the presence of binding protein, then the responses observed in the experiments conducted in the present study would not be biologically relevant. On the other hand, if only the free IGF is active (Meuli *et al.*, 1978; Zapf *et al.*, 1979; Hintz, 1984), then the experiments described in the following section will simulate the presence of low concentrations of active, unbound hormone at the cell surface.

The results of these experiments indicate that both IGF I and IGF II, when present at concentrations lower than 4 ng/ml, stimulate the release of glycerol from ovine adipose tissue *in vitro*. There is some evidence that IGF II was more effective than IGF I when mediating this response (Figures 7.6 and 7.8). There was little difference between the response with adrenalin and that with IGF II. However, there was a significantly lower response to IGF I than to adrenalin. In some of these experiments, hIGF II was used and in others MSA but for the purposes of the discussion, both are referred to as IGF II. Since neither molecule is specific for ovine adipose tissue, there is a possibility that the interactions with the cells by either could have been impeded by species-specific structural differences.

The concentration at which the IGFs were effective varied considerably between experiments (Figures 7.9, 7.10, 7.11, 7.12). Also the hormones were found to be effective over a wide range of concentrations in some experiments. This is unusual when compared with hormones such as GH and insulin where the mode of action probably depends on rapid changes in plasma concentrations to regulate acute metabolic responses. Thus, with GH and insulin, the cells must be able to recognize and respond to these changing concentrations. It has been suggested by Hintz (1984) that the presence of binding proteins for IGF necessarily effects the mode of action of the hormone and that it is possible that these hormones will be present at a more constant concentration at the cellular level. If the regulation of cellular metabolism by the hormone is less dependent on rapid fluctuations in concentration, this may explain why the IGFs, unlike insulin and GH, were active over such a wide range of concentrations.

The effectiveness of IGFs at low concentrations has also been observed in cultured muscle cells (Saltman and Gavin, 1985). In these experiments, amino acid uptake was stimulated by IGF II when present at concentrations of approximately 0.05 - 0.5 ng/ml and by IGF I at 0.5 - 5 ng/ml. This also represents a wide range of effective concentrations as seen with adipose tissue in the present study.

It is postulated that the insulin-like responses, which have been observed when IGFs are present at high concentrations, do not normally occur *in vivo* due to the presence of binding proteins. They are only observed *in vitro* because the high concentrations of IGF allow the

hormone to interact with the insulin receptor. Instead, at concentrations of IGF that are 100 to 1000-fold lower than this, the unbound IGF molecules interact with their own receptors and mediate the observed lipolytic response, at least in ovine adipose tissue. Binding affinity studies of IGFs with ovine adipose tissue will be needed to confirm that the hormone is interacting with the IGF receptors at these low concentrations.

It has been reported that both IGF I and IGF II, when present at concentrations of 30 to 125 ng/ml, are antilipolytic in adipose tissue from rats (Zapf *et al.*, 1978). Insulin has also been observed to be antilipolytic (Figure 4.15) and so, like the other insulin-like responses observed by Zapf *et al.*, the antilipolytic activity of IGF at high concentrations was probably mediated via the insulin receptor. Since the stimulation of glycerol release by IGFs at low concentrations observed in the present study had the completely opposite effect on the cell, it is proposed that the two responses are mediated by different receptors (Figure 1.2). At high concentrations IGF interacts with the insulin receptor and inhibits lipolysis while at low concentrations, IGF interacts with its own receptor and stimulates lipolysis.

There is another better documented example of opposing effects being stimulated by one hormone. As has been discussed in Section 10.4, when GH interacts with adipose tissue, it has been shown to be lipogenic and lipolytic in different experiments. It is not known which receptors are involved in mediating each of these responses although it is thought that GH is not interacting with the insulin receptor when mediating the insulin-like response (Grichting *et al.*, 1983). There is some indication that there may be different forms of GH which mediate different biological responses (Hart *et al.*, 1984a).

Thus it would seem that IGF is not unique in this regard and, although there is no evidence that IGF exists in different forms comparable with those seen with GH, it is possible that two receptors are involved. If this were true, then it could be expected that insulin, when present at high concentrations, would interact with the IGF receptor for which it is thought to have a low affinity (Kasuga *et al.*, 1981; Massague and Czech, 1982; Rechler and Nissley, 1985). If such an interaction did occur, then it might be expected that insulin would stimulate lipolysis when present at high concentrations. This phenomenon has in fact been

observed in experiments with rat adipocytes *in vitro* where the antilipolytic action of insulin when present at low and moderate concentrations altered to lipolytic action at high concentrations (Kono and Barham, 1973; Solomon *et al.*, 1970). Also, the action of insulin to inhibit the production of hormone-stimulated adenylate cyclase at lower concentrations becomes stimulatory at higher concentrations (Hepp and Renner, 1972; Illiano and Cuatrecasas, 1971). These reports may indicate that, at high concentrations, insulin is interacting with the IGF receptor to induce a response in the tissue which is similar to that observed at low concentrations of IGF in the present study.

In rat adipose tissue, it is thought that the type I IGF receptor has a low affinity for insulin and that the type II receptor has no affinity for insulin (Kasuga *et al.*, 1981; Massague and Czech, 1982; Rechler and Nissley, 1985). This would imply that insulin is interacting with the type I IGF receptor to mediate the lipolytic response. However, it has also been reported that rat adipocytes, although possessing a type II receptor, have no type I receptor (Zapf *et al.*, 1978; Massague *et al.*, 1982). It is not known whether ovine adipose tissue is similar to rat adipose tissue in this regard.

Insulin, when present at physiological concentrations, has been shown in several studies to have no significant effect on the rate of lipolysis in ruminant adipose tissue (Metz and Van den Bergh, 1972, 1977; Yang and Baldwin, 1973b). In the present study, insulin either had no significant effect on the rate of lipolysis (Figures 7.6 and 7.8) or depressed the rate (Figures 4.14 and 4.15). Thus, at physiological concentrations, insulin does not appear to stimulate lipolysis. This implies that, when IGFs are seen to stimulate lipolysis, they are not interacting with the insulin receptor to mediate the response.

In two experiments, IGF II caused a greater response in the tissue than IGF I (Figures 7.6 and 7.8) which may indicate that, if a single receptor is involved in mediating the response, then the receptor must have a higher affinity for IGF II than for IGF I. This suggests that the receptor involved in mediating the lipolytic response to IGFs is the type II IGF receptor. However, since high concentrations of insulin are lipolytic in rat adipose tissue which does not possess IGF II receptors, it is still unclear which of the two IGF receptors is mediating the lipolytic response. It is likely that there are species differences in

the presence or absence of receptor types and more information about the receptors present in ovine adipose tissue will be needed before a final conclusion can be made.

The effects of IGF on the mass balance of fatty acid

The effects of the IGFs on the overall balance of fatty acid in the adipose tissue was examined to determine the influence that the IGFs have on the ability of the tissue to mobilize or store lipid. IGF I, when present at a concentration of 0.4 ng/ml, had no effect on fatty acid manufacture but increased the rate of lipolysis in the cells by 2-fold (Table 7.1). There was a concomitant increase in the rate of FFA leaving the cells which was another indication that the tissue was in a state of negative energy balance. The lipolytic activity of IGF I can lead to a 4-fold increase in fatty acid mobilization from the adipose tissue.

There was an increase in the amount of fatty acid which remained in the tissue following IGF I-induced lipolysis. As was observed to occur with adrenalin (Table 5.1 and 5.2), only a small fraction of the fatty acid originally released in the tissue by lipolysis actually passed out of the cell. The result is that there was only a small non-significant decrease in the rate of fatty acid accumulation in the tissue during the incubation.

The effect of IGF II at a low concentration on the mass balance of fatty acid was also investigated in the same experiment (Table 7.2) and was found to be very similar to IGF I and adrenalin. There was a 4-fold increase in the amount of fatty acid leaving the tissue. However, only a fraction of the fatty acid released by lipolysis was observed to actually pass into the incubation medium and there was a large increase in the amount of fatty acid remaining within the tissue following release by lipolysis.

In response to the presence of IGF I, the percentage of recycling of fatty acid in the tissue decreased from 90% to 84% during the 6 hour incubation (Figure 7.1). In the same experiment, IGF II decreased the percentage to 82% (Figure 7.2) and adrenalin decreased the percentage to 77% (Figure 5.1). Thus all three hormones have had very similar effects on the tissue in this regard. With all three hormones, there was a dramatic increase in the rate of FFA leaving the cell and this accounted

for most of the decrease in the amount of fatty acid accumulated in the cell during the experiment.

Adrenalin and the IGFs increased by about 2-fold the rate at which the fatty acid released by lipolysis was retained by the tissue and was presumably re-esterified. As discussed in Section 10.3 with regard to the same phenomenon in the presence of adrenalin, it is possible that the discrepancy between the amount of fatty acid released by lipolysis and that subsequently released from the tissue could be caused by the method used to calculate the results. If the ratio of fatty acid to glycerol released within the tissue as a result of lipolysis was not 3 : 1 or alternatively if some of the fatty acid was oxidized or remained as FFA in the tissue, then the estimation of "fatty acid re-esterified" would be inaccurate.

Thus, although the changes in the rate of "re-esterification" observed in the presence of either IGF I, IGF II or adrenalin all appear to be similar, they may in fact involve different mechanisms by which the change has occurred. For instance, the method employed to measure the rate would not discriminate between the effect of one of the hormones in increasing the amount of di- or monoacylglycerol being utilized for lipolysis and another in increasing the amount of fatty acid being oxidized. Both phenomenon would increase the amount of "fatty acid re-esterified" as calculated by this method. Further work will be required before such distinctions could be made. This will include measurements of tissue-associated fatty acid and CO_2 output from fatty acid oxidation as well as accurate estimations of the ratio of fatty acid to glycerol released by lipolysis within the cell in ovine adipose tissue.

Nevertheless, when either IGF I or IGF II were present in the medium, it would appear that the tissue has been altered to a state of negative energy balance which is similar in many ways to that in the presence of adrenalin. This would imply that, when active IGFs are present in the adipose tissue at a low concentration, the tissue begins to mobilize fatty acids in the same way that they do in the presence of adrenalin.

Summary

The IGFs were seen to elicit an insulin-like lipogenic response in ovine adipose tissue when present in the incubation medium at a concentration of 40 ng/ml. However, when present at concentrations 100 to 1000-fold lower than this, IGFs mediated a lipolytic response in the tissue. The IGFs were effective over a wide range of concentrations when stimulating the lipolytic response.

When comparing either the amount of FFA leaving the cell, the amount of fatty acid accumulated in the cell or the amount of lipolysis, IGF I or IGF II at low concentrations have similar effects in the tissue to adrenalin. Both adrenalin and IGFs have the opposite effects on the overall energy balance of the tissue to that of insulin. Insulin usually increased lipogenesis whereas adrenalin and low concentrations of IGFs did not. Insulin either had no effect on or decreased the rate of lipolysis whereas IGFs and adrenalin significantly increased it. Insulin decreased the rate of fatty acid leaving the cell whereas IGFs and adrenalin significantly increased the rate. Insulin had a small, often negative effect on the rate of re-esterification in the cell whereas IGFs and adrenalin significantly increased it. Insulin increased the rate of fatty acid accumulation in the tissue; IGF and adrenalin decreased it. Insulin increased the rate of recycling of fatty acid in the cell, IGF and adrenalin decreased it.

10.6 A comparison of the effects of growth hormone and IGF

In experiments where both IGF and GH were tested simultaneously on tissue from the same animal, it was possible to compare their actions on the processes of lipid metabolism. When IGF I was present at 40 ng/ml and bGH at 100 ng/ml, both hormones were as effective as insulin in stimulating the rate of lipogenesis although low concentrations of GH did not elicit this response (Figure 8.1). It would appear therefore that the lipogenic effects of insulin, GH and IGF I can be very similar. This might be interpreted to mean that the responses are mediated via the same mechanism. However, although it is thought that the insulin-like effects of IGF are mediated through the insulin receptor (King *et al.*, 1980; Zapf *et al.*, 1981a), it appears that GH's are not (Grichting *et al.*, 1983).

But, if binding proteins restrict the activity of IGF I (as discussed in Section 10.5), it is more likely that IGF I will be present in the adipose tissue at much lower concentrations than that used to demonstrate insulin-like effects. Therefore the action of IGF I, when present at a low concentration, was compared with the action of GH at physiological concentrations. It was found that only GH and not IGF stimulated lipogenesis at these concentrations (Figure 8.2). As discussed in Section 10.5, it is likely that this comparison of the actions of the two hormones may have more relevance to the situation *in vivo* than that shown in Figure 8.1.

When a comparison of the actions of bGH and IGF I on the rate of lipolysis in adipose tissue was made, IGF I was found to stimulate the rate by as much as adrenalin although bGH, like insulin, had no effect (Figure 8.3). GH can have a transient effect on lipolysis and may therefore have stimulated an early release which was not detected when the sample was taken at 4 hours. However, in this experiment, IGF I was obviously a more effective lipolytic hormone than GH.

The effects of glucocorticoids on the actions of growth hormone in ovine adipose tissue have not been investigated in the present study. However, glucocorticoids and GH have been shown to enhance each other's effects on the release of fatty acid and glycerol from isolated rat adipocytes (Fain *et al.*, 1965). Duquette *et al.* (1984) showed that the presence of glucocorticoids made no difference to the insensitivity of ovine adipose tissue slices to oGH. However, there is a possibility that

the lipolytic effects of bGH on ovine adipose tissue demonstrated in the present study could be further enhanced by the presence of glucocorticoid.

The results shown in Figure 8.3 illustrate the problem discussed previously (Section 10.1), that a high basal rate will mask the effects of a hormone. Thus neither adrenalin nor IGF I stimulated the rate by as much as was observed in other experiments (*e.g.* Figure 8.4 where the basal rate of glycerol release was approximately 5-fold lower after 2 hours incubation). It is possible that the high basal rate of glycerol release from this tissue was caused by adrenalin released during the slaughter process.

However, in the second experiment, IGF I, IGF II, recombinant DNA-derived bGH and purified GH were equally lipolytic in this tissue (Figure 8.4). Insulin had no effect on the rate of glycerol release. These results indicate that, on those occasions when GH is observed to be lipolytic in this tissue, its effects are indistinguishable from those of IGF.

There is considerable evidence that GH mediates the release of IGFs *in vivo* (see Section 1.10 and 10.7). It has been suggested that IGFs are produced in many, if not all, tissues where they may be able to act on cells close to their site of synthesis (D'Ercole *et al.*, 1984). Recent evidence suggests that the "direct" actions of GH on chondrocytes may in fact be modulated by local IGF I release (Watenabe *et al.*, 1985).

It is not known if GH is involved in the release of IGF from ovine adipose tissue but it would be at least a possibility. The Medium 199 used in the present study contained many of the amino acids that may be necessary for the manufacture of IGFs *in vitro*. These amino acids would not be available in the Krebs-Ringer buffer used in most investigations *in vitro* reported in the literature. During the process of IGF production, it is possible that paracrine effects between adipocytes could occur in the tissue slice where the cells are still packed in their normal juxtapositions. On the other hand, these processes would be unlikely to function with isolated adipocytes across the wide gaps of medium between cells. Also there is a possibility that the connective tissue associated with the adipocytes is either responsible for releasing the IGF or mediates the release from the adipocytes by other paracrine

factors. As discussed in Section 10.1, the method used to prepare isolated adipocytes will exclude connective tissue whereas it is probably present in tissue slices in much the same quantity as *in vivo*. This may mean that, if GH was mediating the release of IGF from the adipose tissue *in vitro*, that it is more likely to occur in tissue slices incubated in Medium 199 than it is in isolated adipocytes incubated in Krebs-Ringer buffer.

Thus any observed lipolytic activity of GH in the present study may have been due not to the direct actions of GH but to the effects of IGFs released by the adipose tissue under the influence of GH. The action of locally released IGF *in vitro* would seem more feasible when it is recognized that only an extremely small amount of IGF would be required to elicit the observed lipolytic response.

The phenomenon of locally produced IGF could account for the transient nature of the lipolytic response to GH; if the cells were able to produce only very limited amounts of IGF, then the response would not necessarily be sustained. As seen in Figure 8.4, under some circumstances, the effects of GH and IGF can be very similar, evidence which may indicate that a similar mechanism is involved. On the other hand, IGF was lipolytic in tissue where GH was not (Figure 8.3). This may indicate that, in this case, no local release of IGF occurred because if it had, the tissue would have shown a lipolytic response to GH in the same way it did when IGF was added to the medium. It is postulated that the presence of glucocorticoids, which are known to enhance protein synthesis (Fain *et al.*, 1965), may have the effect of stimulating the production of IGF in these cells. The presence of glucocorticoids would thereby enhance the lipolytic response to GH.

However, since the lipolytic response to GH was not consistently observed in the present study, it seems likely that the paracrine effects of GH are variable and do not occur in tissue from some animals. Thus there may be other physiological pre-conditions (*e.g* the presence of glucocorticoids) which have occurred with some animals and not others and which are necessary before any local release of IGFs will proceed.

An experiment could be conducted to determine the validity of the postulation that the mode of action of GH is via the action of IGF *in vitro*. If adipose tissue which had been incubated in GH was prepared so that IGF could be extracted and measured, then any increases compared with adipose tissue incubated without hormone could be determined. The concentrations of IGF found are likely to be very small and present methods may not be able to detect any changes unless large quantities of tissue (*e.g.* 50 g) were incubated and extracted. It could also be determined whether glucocorticoids enhanced any IGF production.

It has been shown that GH (Chung *et al.*, 1985; Johnsson *et al.*, 1985) and IGFs (Schoenle *et al.*, 1982; Schoenle *et al.*, 1985) are associated with growth *in vivo*. The energy required for such growth could not always be provided unless there is a mechanism to simultaneously stimulate the adipose tissue to release fatty acids. Although not all the fatty acid released by IGF-stimulated lipolysis actually left the cell, there was subsequently a significant increase in the amount of fatty acid mobilized from the tissue (Table 7.1 and 7.2). GH may be capable of stimulating that release directly but these results indicate that IGFs could also be involved. It seems likely that at least some of the observed increase in plasma NEFA on administration of GH *in vivo* (Hart *et al.*, 1984b) may be due to the actions of IGF. IGF would thereby provide an alternative mechanism for the mobilization of energy for GH or IGF-induced growth.

10.7 Events *in vivo*: their subsequent effects *in vitro*

One of the major disadvantages of the method for studying the mechanisms of cellular metabolism in adipose tissue *in vitro* is that the tissue from each animal has a different previous history, depending on the physiological state of the animal. As a consequence, at the start of each *in vitro* experiment, the ability of the tissue to respond to a hormone in the incubation medium is likely to vary considerably from animal to animal. It could be easier to explain the variation in cellular activity between animals if more was known about the preceding physiological events which determine the variation in individual responses *in vitro*.

It was found that the circulating levels of GH, insulin and IGF I were raised in the animals which received exogenous GH (Section 9.3). As a result of this, it is likely that the levels of other hormones not measured have also changed. These endocrinological changes *in vivo* may then alter the responsiveness of the adipose tissue to any subsequent stimulation of lipid metabolism by hormones *in vitro*.

Exogenous GH *in vivo*: effects on the rate of lipid metabolism *in vitro*

Administration of bGH to lambs for two months resulted in a marked growth-promoting effect, with significant increases in live-weight and carcass weight in the bGH-dosed animals. Deposition of fat in the carcass was significantly reduced in these animals and since the percentage protein in the carcass increased, it is possible that there was a re-partitioning of energy from the adipose tissue towards protein synthesis as a result of the presence of exogenous GH. Chung *et al.* (1985) found that there was an increase in intramuscular lipid content of the longissimus muscle following administration of porcine GH to pigs and they suggest that this may be due to increased delivery of NEFA from the adipose tissue.

The *in vitro* rate of lipogenesis in the tissue from the bGH-dosed animals showed a marked decrease as compared with that in the tissue from the control animals (Figure 9.1). At the same time, there was little difference between the two groups in the rate of lipolysis in the tissue *in vitro* (Table 9.3). A similar study with pigs has been reported and the

authors found no significant effect of porcine GH administration on rates of lipogenesis *in vitro* (Chung *et al.*, 1985). However, the samples for incubation *in vitro* were taken after only 30 days of GH administration when there was no significant effect on adipose tissue mass. In the present study, the live-weight gain in the bGH-dosed animals was not apparent until the second month of the trial and this could indicate that the effects of exogenous bGH on the rate of lipogenesis *in vitro* may also not be apparent until the second month of bGH administration. On the other hand, Fain *et al.* (1962) showed that a single dose of GH to rats depressed the rate of ^3H_2O uptake into adipose tissue fatty acids *in vivo* 6 - 14 hours later, although there is a possibility that this effect could have been due to pituitary-derived contaminants in the GH.

The presence of GH *in vitro* was not observed to be anti-lipogenic in the present study and, on the contrary, was observed on occasions to be lipogenic (Section 10.4). Thus it seems unlikely that it is the presence of excess GH in the tissue slices *in vitro* which caused the observed depression in the rate of lipogenesis in tissue from the bGH-dosed group. However it is possible that the observed depression was caused by some other factor associated with the presence of exogenous GH in these animals *in vivo*.

Since the presence of exogenous bGH caused a marked growth-promoting effect, it could be assumed that the reduction in the rate of fat deposition in the carcass was caused by a reduction in the amount of energy being available to the adipose tissue. Such molecules as glucose and acetate could be expected to be used to supply the energy required to synthesize protein and bone for growth. However, if this lack of glucose and acetate were the only factor responsible for the reduction of fat deposition in the carcasses of the GH-dosed animals, then it could be expected that in the incubation *in vitro*, where the supplies of glucose and acetate were optimal, the rates of fatty acid manufacture and accumulation would immediately return to normal. Since in fact they have remained severely depressed during the incubation *in vitro*, it must be assumed that some other factor, which is still present *in vitro* as it was *in vivo*, is causing the low rates in tissue from the bGH-dosed animals.

There was a significant decrease in the amounts of active enzymes for NADPH-generation in the tissue from the bGH-dosed animals (Table 9.2). Since NADPH would be required for lipogenesis to proceed, these results support the evidence that there was a significant decrease in the rate of lipogenesis in the adipose tissue of these animals (Figure 9.1). Thus it is postulated that the physiological changes in the animals, which occurred as a result of the presence of exogenous GH *in vivo*, has resulted in a decrease in the amounts of NADPH-generating enzymes in the adipose tissue which in turn dictated the basal rate of lipogenesis *in vitro*.

On the other hand, O'Hea and Leveille (1969) reported that the overall changes in the activities of these enzymes in porcine adipose tissue were less pronounced than the corresponding lipogenic changes as measured by ^{14}C-glucose incorporation into fatty acid and they suggest that the loss of enzymatic activities was a result rather than a cause of reduced lipogenesis following dietary manipulation. When the activities of the same enzymes were measured in adipose tissue from two groups of pigs, one group growing at a fast rate and the other growing at a slow rate, it was concluded that the fast-growing group had a higher enzyme activity than the slow-growing group but that this was associated with a similar deposition of fat (Hood and Allen, 1973). Thus it would appear that the relationship between the level of enzyme activity for the generation of NADPH and the rates of lipid deposition in the carcass may not be as clear as the data in the present study would indicate.

Administration of exogenous bGH *in vivo* led to a marked decrease in the rate of fatty acid accumulation in the adipose tissue *in vitro* (Figure 9.2). This result is supported by the fact that these animals showed a significant decrease in fat deposition in the carcass compared to the control animals (Section 9.3). The administration of bGH also slightly decreased the percentage of fatty acid recycled in the tissue *in vitro* (Figure 9.3). This suggests that the adipose tissue was in negative energy balance *in vitro* and may indicate that at least some mobilization of fatty acid was occurring. The direct measurements of fatty acid leaving the cell *in vitro* showed a slight, non-significant increase in the tissue from the bGH-dosed animals and it is this which is contributing to the observed decrease the rate of fatty acid accumulation in the tissue.

It is therefore evident that the physiological characteristics of the adipose tissue *in vivo* were retained in the tissue during the incubation *in vitro* even when hormones were not present in the medium. If it is assumed that a factor associated with the presence of exogenous GH caused the depression of the rate of fat deposition *in vivo*, then the factor appears to maintain its influence *in vitro*. This may mean that either it can influence the rate of lipid metabolism even when not present or that it was still present in the tissue during the incubation. If its influence was solely to decrease the activity or amount of the enzymes involved in lipogenesis, then it may not necessarily need to be present, at least in the short term, to exert its influence.

It would be of interest to know how long the influence could be maintained in long-term incubations without hormones in the medium and whether the presence of such hormones as GH or insulin are necessary to sustain the reduced rates of lipogenesis and fatty acid accumulation *in vitro* in this tissue or alternatively can act to return the rate to that seen in tissue from the control animals. However, there is always the possibility that the factor was present in the tissue slices, either intra or extra-cellularly, and could therefore maintain its influence on the rates of lipid metabolism even in long-term incubations.

On the other hand, the basal rate of lipolysis in the adipose tissue *in vitro* was largely unaffected by the presence of exogenous GH *in vivo*. Although there was little evidence of any change in NEFA in the bGH-dosed animals in the present study, others have observed that administration of bGH resulted in an increase in plasma NEFA in sheep (Hart *et al.*, 1984b) and pigs (Chung *et al.*, 1985). However, in the latter study the means of the two groups were not statistically different. The presence of bGH *in vitro* resulted in a transient stimulation of the rate of lipolysis in tissue from some of the normal sheep tested (Figures 6.4 and 6.6) and in tissue from another was seen to stimulate the release of fatty acid (Table 6.1). However, it is postulated that at least some of the lipolytic activity observed on administration of GH *in vivo* is due to the increased activity of IGF in the adipose tissue (Section 10.5 and 10.6). It would appear that the influence of the lipolytic hormones on the tissue is not maintained *in vitro* as it was with the anti-lipogenic effects.

Exogenous GH *in vivo*: effects on hormone action *in vitro*

When exogenous GH is administered to sheep, there is a rise in the plasma concentrations of a number of hormones. However, whatever their concentration in the blood, these hormones will only influence the rate of lipid metabolism in the adipose tissue if the adipocytes continue to interact with the hormones. It was therefore of interest to determine whether there had been any change in the responsiveness of the adipose tissue to some of the hormones known to rise in the blood of bGH-dosed animals.

It is thought that insulin and GH affect each other's biological actions. Administration of exogenous bGH to sheep was found to impair the ability of insulin to reduce the concentration of plasma glucose (Hart *et al.*, 1984b). The effect was similar for either pituitary or recombinant DNA-derived bGH, indicating that this is an intrinsic characteristic of the GH molecule and not the result of the action of pituitary-derived contaminants. Exogenous porcine GH in pigs increased plasma levels of insulin and glucose and the authors suggest that an insulin-resistant state had developed (Chung *et al.*, 1985). Thus the observation in the present study that the presence of exogenous bGH in lambs resulted in a marked decrease in the magnitude of the responses to insulin in the adipose tissue *in vitro* (Figures 9.4 and 9.5) may be another indication that GH is interfering with the ability of insulin to influence cellular metabolism.

Because the basal rate of acetate incorporation into fatty acid in the tissue from the bGH-dosed animals was significantly lower than in the tissue from the control animals, the response to insulin, even though occurring with a similar percentage stimulation (Table 9.4), is significantly reduced. Thus it is the response of the tissue to insulin which has been affected by the presence of exogenous GH while the responsiveness was largely unchanged. As a result, the importance of insulin in stimulating the rate of lipogenesis and fatty acid accumulation is diminished. Since the percentage stimulation by insulin was similar in both groups, this might indicate that insulin is responsible for the appearance in the cell of some factor, possibly glucose or an activated lipogenic enzyme, the percentage increase of which cannot exceed a certain level. Thus when the basal rate of lipogenesis is low, the response will be small.

Although there was an increase in the plasma concentration of insulin in the bGH-dosed animals *in vivo*, this did not result in any increase in the rate of lipogenesis in these animals. On the contrary, the rate, as measured *in vitro*, was significantly depressed (Figure 9.1). Thus this could be interpreted to mean that there was an insensitivity of the adipose tissue to these high concentrations of insulin *in vivo*. If the tissue had been sensitive to insulin, then it would be expected that the rate of lipogenesis in this tissue would have been similar to that in the control animals. If insulin can only stimulate the rate of lipogenesis by a small amount, even when active, then this will not greatly enhance the rate of fat deposition in these animals.

If the influence of insulin in increasing the amount of acetate incorporated into fatty acid in the adipose tissue is decreased *in vivo*, as has been observed *in vitro*, then it is also likely that it would not be greatly influencing the amount of glucose incorporation into lipid either. Thus reduced uptake of glucose by the adipose tissue may be one of the reasons why it has been observed *in vivo* that the ability of insulin to reduced the levels of plasma glucose is impaired in the presence of exogenous GH (Hart *et al.*, 1984b).

GH has been shown to antagonize the lipogenic action of insulin *in vitro* in adipose tissue from sheep (Vernon, 1978, 1979, 1982b) and pigs (Walton and Etherton, 1986). This would indicate that GH may interfere with the ability of insulin to interact with the cell and mediate the response. Thus it is possible that the high levels of circulating GH *in vivo* in the bGH-dosed animals were responsible for the apparent insensitivity of the tissue to the lipogenic activity of insulin and led to the marked reduction in the basal rate of lipogenesis.

High concentrations of insulin can lead to a reduced response to insulin (Figure 4.7), a phenomenon which could also be responsible for the apparent insensitivity to insulin following administration of GH *in vivo*. Since plasma levels of insulin in these animals were elevated, there is a possibility that this could have caused down-regulation of insulin receptors. However, this was not observed in a similar study with humans where insulin resistance following GH administration could not be attributed to a change in insulin receptor concentrations (Rosenfeld *et al.*, 1982). The authors concluded that decreased insulin sensitivity was probably due to a post-receptor phenomenon.

In the present study, it was found that insulin could interact with the tissue from the bGH-dosed animals to elicit a percentage increase in lipogenesis which was similar to that in tissue from the control animals. This would also indicate that there was no decrease in the binding of insulin to the adipocytes from the bGH-dosed animals compared with tissue from the control animals. On the other hand, the reduction in the effectiveness of the hormone in this tissue would also indicate that a post-receptor mechanism, such as a reduction in the activities of NADPH-generating enzymes, were involved.

However, there is also the possibility that the sensitivity of the tissue to insulin has been affected by a third, possibly GH-dependent, factor which was present in the adipose tissue of the bGH-dosed animals and not in the control animals. In addition to insulin, the hormones which may be associated with the activity of GH are thyroxine and triiodothyronine (Trenkle, 1981), hydrocortisone (Etherton and Walton, 1984) and prolactin (Bauman *et al.*, 1982). However, the actual role these hormones could play in the actions of GH have yet to be elucidated.

It is also possible that lipid metabolism in the adipose tissue is regulated by intracellular mechanisms. There has been a suggestion that glucose transport and insulin sensitivity may be regulated by the presence of free fatty acids and intracellular long chain fatty acyl-CoA. In rats fed a high fat diet, insulin binding, glucose transport, glucose oxidation and the rate of lipogenesis were all decreased (Olefsky and Saekow, 1978). Thus any increase in the levels of NEFA in the adipose tissue in the presence of exogenous GH could be one of the factors influencing the rate of lipogenesis in the adipocytes.

There is however another possible mechanisms by which GH could have induced the apparent insensitivity to insulin *in vivo*. Exogenous GH has been shown to affect the enzymes and cofactors that mediate the intracellular response to insulin *in vitro*. As has already been discussed, the presence of exogenous GH reduced the activity of NADPH-generating enzymes in the adipose tissue (Table 9.2). It has also been reported (Bornstein *et al.*, 1983) that hGH can inhibit acetyl CoA carboxylase activity in rat adipocytes *in vitro* by mediating the release of a "second messenger" which in turn inhibits acetyl CoA carboxylase activity by enhancing its phosphorylation. It is thought that acetyl CoA carboxylase is a crucial, possibly rate-limiting, enzyme for lipogenesis

in ovine adipose tissue (Vernon, 1980). Thus its inactivation could be expected to result in a decrease in the rate of lipogenesis as was in fact observed in tissue from the bGH-dosed animals. Inactivation of acetyl CoA carboxylase could also interfere with ability of insulin to stimulate lipogenesis, as could inactivation of the NADPH-generating enzymes. The presence of reduced amounts of active enzyme would account for the decrease in the magnitude of the response to insulin *in vitro* in tissue from the bGH-dosed animals.

However, this view of GH action is inconsistent with the results of other experiments with GH *in vitro* (Figures 6.1 and 6.2) where GH did not depress the rate of lipogenesis during the 4 - 6 hour incubations. Although Bornstein *et al.* (1983) observed the inactivation of acetyl CoA carboxylase following only 10 minutes exposure to GH, it is possible that the response may take longer in ovine adipose tissue. Goodman *et al.* (1984) also found that the initial response to GH in isolated adipocytes from hypophysectomized rats was insulin-like. Bornstein *et al.* suggest that there may be marked differences in the time taken to alter the activity of acetyl CoA carboxylase between animals of different ages.

On the other hand, the exogenous GH was present in the blood of the animals in the present study for two months prior to the experiment *in vitro*. During this two month period, it is possible that changes not seen in short-term *in vitro* or *in vivo* experiments could have occurred. These changes are most likely to involve alterations in the amounts and activities of crucial lipogenic enzymes. As suggested by O'Hea and Leveille (1969), this could be a result rather than a cause of reduced rates of lipogenesis in the tissue. One of the mechanisms by which the rate of lipogenesis was reduced in the bGH-dosed animals could be that GH or IGF was stimulating the uptake of nutrients by muscle for protein synthesis and less was available to the adipose tissue.

The lipogenic effects of high concentrations of IGF I were not investigated in this experiment and so it is not known whether the insulin-like response to this hormone in the tissue was similarly reduced by the presence of exogenous GH. However, the actions of IGF I at 0.7 ng/ml and bGH at 100 ng/ml *in vitro* were not affected by the presence of the exogenous GH *in vivo*. This means that the presence of exogenous GH in the animals either did not affect the action of these hormones, or alternatively any interference with their action *in vivo* was a

short-lasting effect which was not sustained through the slaughter and tissue preparation procedures. Since it is most likely that the IGF I and GH present *in vivo* were interacting normally with the cells in the adipose tissue, their lipolytic activity could then account for any raised levels of NEFA in bGH-dosed animals.

Conclusions

The observations that the rate of lipogenesis and fatty acid accumulation *in vitro* were depressed by the presence of exogenous GH *in vivo* and that the action of insulin is no longer so effective suggests that the passage of acetate and glucose into the adipose tissue of these animals would be considerably reduced, as would the rate of fatty acid accumulation when compared with that in the adipose tissue from the control animals. If the nutrient molecules are not being used by the adipose tissue, even in the presence of the higher plasma concentrations of insulin in the bGH-dosed animals, then they will be available in increased amounts for uptake by the muscle tissue for protein synthesis.

This would suggest that the "growth-promoting" and "lipolytic" effects of exogenous GH are dependent on the ability of GH to mediate a reduction in the rate of lipogenesis and a reduced ability of insulin to stimulate that rate. In fact reduced utilization of nutrient by the adipose tissue and increased utilization of that nutrient by the muscles for growth may have caused post-receptor changes in the adipose tissue which resulted in the *in vitro* observations of reduced lipogenesis and fatty acid accumulation.

At the same time, it is postulated that the "lipolytic" effects of exogenous GH may also be mediated by the increased levels of IGF in the adipose tissue. This effect is more likely to be a receptor-dependent phenomenon, as opposed to a post-receptor one, because increased lipolytic activity was not observed in the subsequent *in vitro* incubation and the sensitivity of the tissue to IGF was unchanged.

This would therefore provide a mechanism for the growth-promoting effects as well as the resulting reduction in fat deposition in the adipose tissue following administration of GH to these animals.

It appears that the decrease in fat deposition in the adipocyte, as observed both *in vitro* and *in vivo* in this experiment, is basically a long-term phenomenon. The rates of lipogenesis and fatty acid accumulation in the adipose tissue *in vitro* appear to have been determined by physiological events associated with the presence of exogenous GH *in vivo*.

10.8 The relevance of *in vitro* experiments with adipose tissue to lipid deposition *in vivo*

One of the main problems associated with investigations of the controlling factors influencing the rate of lipid deposition *in vivo* is the complexity of the synergistic and antagonistic effects of hormones and feedback mechanisms, all of which operate simultaneously. Experiments *in vivo* with exogenous hormone, such as that described in Chapter 9, is one method by which the effects of a hormone on lipid deposition *in vivo* have been studied. However, the disadvantage of this approach is that the complexity of the resulting physiological changes in the animal means that, although there may have been a measurable change in the metabolic rates in the animal, it is extremely difficult to detect which factor has been responsible for the change.

When exogenous GH was administered to lambs in the present study, there was an increase in growth accompanied by a decrease in lipid deposition in the carcass. There were marked changes in the circulating concentrations of several hormones that were measured but there could have been equally significant changes in the concentrations of hormones which were not measured. The concentration of insulin in the presence of exogenous GH was shown to rise but at the same time it appeared that the adipocytes were not responding to that hormone by increasing the rate of lipogenesis. Thus a change in the circulating concentration of a hormone may not necessarily mean that the expected increase in responses to that hormone will occur.

The endocrinological changes occurring in an animal as a result of the presence of exogenous GH will also cause changes in levels of substrates, enzymes and cofactors present in the adipose tissue. Although it is possible to measure the concentrations of substrates (glucose and acetate) or products (NEFA) in the blood, these are only the final concentrations found at a particular instant following both input and output from many tissues around the body. These plasma concentrations may not necessarily reflect the quantities of substrate being utilized by the adipose tissue or the amount of fatty acid being mobilized from the adipose tissue. The changed hormone balance will alter the rate at which these substances leave or enter the adipocyte such that the concentrations of substrates in the cytoplasm may be completely different from the concentration circulating in the blood.

One of the ways in which this complexity can perhaps be better understood is by the use of experiments with adipose tissue *in vitro*. However, as has been demonstrated in preceding chapters, even when the tissue has been isolated from all the other influences *in vivo* which could normally affect hormonal interaction with the cell, the effects of a single hormone present in the medium are still difficult to interpret.

On the other hand, when enough *in vitro* experiments have been carried out, it may eventually be possible to piece together the individual effects of each hormone on both the physiology of the cell and the action of other hormones. Firstly, the responses to each hormone alone must be determined and hence changes when a second hormone is added to the medium will be easier to interpret. Alterations in the order of introduction of the two hormones into the medium could then be investigated. In addition, the effects of changing substrate concentration on all the hormonal influences should be examined. Then sufficient information may be available to allow interpretation of the results of experiments such as the introduction of exogenous GH *in vivo*.

In vitro experiments can also be used to determine which hormones are likely to be influencing the cellular metabolism so that their concentrations can be measured in experiments *in vivo*. The changes in circulating concentrations of all hormones likely to be influencing the observed changes *in vivo* can then be examined and this may lead to a better understanding of the mechanisms that operate.

However, one of the problems associated with the analysis of biological activity in adipose tissue *in vitro* is that different physiological conditions *in vivo* can cause profound alterations in both the basal rates of lipid metabolism and the sensitivity of the tissue to different hormones *in vitro*. Although the effects *in vitro* were probably exaggerated by the pharmacological doses of GH administered to the lambs in the experiment described in Chapter 9, it has nevertheless illustrated that any differences in the physiological state of the animal can cause changes which are sustained throughout the subsequent 4 hour incubation. Thus it is likely that variation in responses between animals obtained in incubations of tissue from normal animals in the present study could have been the result of physiological changes *in vivo* as much as being the result of changing conditions between experiments *in vitro*. Therefore, interpretation of results obtained *in vitro* must be made with limited knowledge of preceding events which could be relevant to that interpretation.

This problem was encountered when observing the responses of the adipose tissue to GH in the medium *in vitro* (Chapter 6). In some experiments, the hormone was lipolytic while in others it was lipogenic. Presumably these opposing responses would be counterproductive if occurring at the same time and may normally be controlled *in vivo* by synergistic or antagonistic effects of other hormones. Thus, when the tissue is incubated *in vitro*, the responses observed will depend on the presence or absence of any synergistic or antagonistic effects of other hormones. These effects can be caused by either the hormone itself being in the incubation vessel or previous activity of the hormone before slaughter. In the case of GH, where cleaved products of the molecule may have individual biological functions, the presence or absence of specific proteolytic enzymes in the tissue may also determine the type of response obtained *in vitro*.

One of the major differences between *in vivo* and *in vitro* experiments to assess the rates of cellular metabolism is the time scale. Experiments *in vitro* are necessarily short, when compared with the life span of an animal. Thus, during a 4 or 6 hour experiment *in vitro*, the hormone being tested may cause a small, barely detectable, change in the rate of lipid metabolism. However if the same effect was sustained over a period of months or years *in vivo*, it could have significant effects on the body composition of the animal.

It is not known to what extent the effects of a hormone are cumulative and this also could be assessed *in vitro*. Thus the small effects of hormones might be very effective if each was added to the last, rather than, as is often assumed, the rate returns immediately to the original rate once the hormone is no longer present. There is evidence from the experiment with exogenous GH that the effects of hormones may be cumulative. The basal rate of lipogenesis in the tissue from the bGH-dosed animals was depressed *in vitro* and did not return to normal during the 4 hour incubation. If the factor causing the depression in the rate of lipogenesis in this experiment had acted by altering the concentration of the lipogenic enzymes involved, then the effects could well be cumulative. Any subsequent changes may take many hours and only occur in the presence of the appropriate hormone.

The assessment of cellular metabolism *in vitro* is complicated by the variation in responses obtained in tissue from different animals. The magnitude of the basal rate of lipid metabolism *in vitro*, which was probably determined in the tissue before slaughter, could be linked with the variation in responses to hormones observed. For instance, a high basal rate may be associated with a low stimulatory effect of a hormone because the available resources of the cell do not allow further stimulation. At the same time, a low basal rate could result in only a small effect of the hormone if that hormone influences the activity or amount of enzymes available by a fixed percentage. A fixed percentage of a small amount of enzyme will be less than for a large amount of enzyme present when the rate is high.

Another factor which may influence the responses to hormones in tissue from different animals concerns the characteristics of the individual interaction of a hormone with the cell. It is possible that each hormone takes a different length of time to cause its maximum response, that it acts at its own particular concentration and requires its own particular pre-conditions before it can interact with the cell or cause a response. These criteria for the observation of a maximum response may vary in tissue from different animals. The length of time taken to observe a maximum response will depend on the availability of all the membrane receptors, secondary messengers, intermediates, cofactors and enzymes present in the cell throughout the incubation period. The availability of these parameters will depend not only on their initial concentrations. which will be determined by the

physiological state of the animal, but also on the rate at which the hormone can cause them to change during the incubation.

The optimal concentration of the hormone will depend on the affinity of the cell receptors for the hormone being tested. This also may be influenced by previous exposure of the receptors either to the hormone actually being tested or to another inhibitory or synergistic hormone prior to slaughter. It is possible that, unless pre-conditions such as these have occurred, there will be no response to the hormone *in vitro*. Thus the interaction of the hormone with the cell *in vitro* may be almost as complex as it is *in vivo*, with the main exception being that the interaction of only one major hormonal component is being observed.

Another factor affecting the interpretation of experiments *in vitro* is the variation which occurs between duplicate incubation of tissue from the same animal. Although this variation may be the result of experimental error, there could be a number of other factors involved. Since tissue slices, as opposed to isolated adipocytes, were used in the present study it is possible that there may have been characteristics of each of the sections of tissue used which were retained *in vitro*. Although tissue was taken from one depot, sliced and then 5 - 6 randomly selected slices dispensed into each incubation vessel, some vessels could have contained tissue which was metabolically more active than others.

Different metabolic activities in different slices could be the result of contours which occur in the subcutaneous fat depot *in vivo*. Since subcutaneous fat is not deposited in a layer of equal thickness over the entire dorsal region, it would seem that there must be some variation in the rate at which lipid is being deposited across the area. If a layer of the tissue were thin, then it is likely that there could be a predominance of small, relatively inactive cells, while in thick layers, the cells could be larger and more active. This could cause an alteration in not only the metabolic rate being measured but also the sensitivity to the action of the hormones. As a result there may be errors associated with different sections of the tissue giving different responses.

The situation could possibly be avoided by using internal fat depots, such as perirenal adipose tissue, for the incubations *in vitro*. However, the relevance of the rates of lipid metabolism and hormonal responses in tissue from these depots to the problems of overfatness and obesity are uncertain. For example, when exogenous bGH was observed to significantly decrease the rate of fat deposition in the subcutaneous region (as measured by GR), there was little change in the weights of either the perirenal or mesenteric adipose tissue depots. Thus it was alterations in the subcutaneous fat depot as they pertain to overfatness which was of interest in the present study

If the animal was in positive energy balance, tissue with a predominance of large cells could have a higher affinity for lipolytic hormones than tissue with a predominance of small cells. Conversely, small cells may be so because they have a higher affinity for lipolytic hormones than large cells. On the other hand the reverse could be true if the animal were in negative energy balance, when it is conceivable that fatty acid would be mobilized from tissue containing the largest stores of energy. This whole situation could vary between animals, as it is well known that certain, possibly genetically determined, factors lead to particular adipose tissue depots, such as the abdominal subcutaneous fat in the human, being retained at the expense of other smaller depots. The factors which control the size of a fat depot *in vivo* are poorly understood but may be associated with different affinities of the cells in each depot for the hormones controlling their rates of lipid metabolism. Thus the source of each tissue slice, even on a microscopic scale, could determine the variation obtained in both the basal rate of lipid metabolism and the response to hormones being tested.

Within each depot of subcutaneous adipose tissue, it was observed that there were a variety of differently sized cells. Thus it would appear that each cell is accumulating lipid at a different rate. This could mean that, eventually, some cells must reach a maximum capacity of stored lipid and will no longer interact with lipogenic hormones. It is also not known what mechanism, if any, determines the maximum size of an adipocyte and causes a cessation of further storage in that cell. It could possibly be a biochemical feedback mechanism operating intracellularly so that the process of lipogenesis will no longer occur. On the other hand, there could be a hormonal control mechanism either from the central nervous system or paracrine effects from adjacent cells

which are able to detect the confluence of the tissue via mechanisms in the adjacent cell membranes.

By whatever mechanism the cell size is controlled, the changes occurring when a cell reaches its maximum size could involve the affinity of the hormone receptors. Thus a tissue slice with a predominance of cells which have reached their maximum size will probably not have the same basal rate of lipid metabolism nor will it respond to the action of hormones in the same way as tissue with no cells which have reached maximum size. It was noticed, in the present study, that there was a tendency for the responsiveness to the lipogenic activity of insulin to be less in tissue from older animals. This could possibly be caused by the tissue having a predominance of larger cells in the depot being studied.

From observations *in vivo*, it is thought that some animals, including humans, may have different propensities to fatten. This is a complex situation which is associated with many factors such as quality and quantity of food intake, level of exercise, basal rates of cellular metabolism, the presence of brown adipose tissue and the amounts of energy utilized in heat production. However, it has been found in the present study that adipose tissue from some animals responds significantly more to lipogenic and lipolytic hormones than others. The rate of fatty acid recycling, which is one of the main factors leading to the increased retention of fatty acid by the adipose tissue, is under the control of hormones and can be affected to different degrees by the same hormones in tissue from different animals. Increased recycling of fatty acid in the adipose tissue could lead to increased heat production.

Any increase in heat production in the adipose tissue will have the overall effect of causing less lipid to be deposited there. This means that, for two animals with similar levels of food intake and exercise levels, there will then be less fat deposition in the animal with the higher rates of fatty acid recycling. This could be one of the reasons for the observation that some individuals have a decreased tendency to fatten. If this hypothesis is correct, then it is possible that hormones which increase fatty acid recycling but do not affect lipogenesis may have a future role in the control of obesity.

Although the responses to hormones *in vitro* are almost as complex and difficult to interpret as those *in vivo*, they may be the best method presently available to study the effects of individual hormones on the rates of lipid metabolism. It will only be as a result of many such experiments with individual hormones *in vitro* that enough information will eventually become available to allow conclusions to be drawn concerning the overall control of overfatness in animals and obesity in humans.

Appendix A
Gibco Culture Medium 199

Gibco Catalogue Number: DM01

Dry powder reconstituted to 1000 ml.

NaCl	8.0	g	Guanine	0.3	mg
KCl	0.4	g	Xanthine	0.3	mg
$MgSO_4.7H_2O$	0.2	g	Hypoxanthine	0.3	mg
Na_2PO_4	0.048	g	Thymine	0.3	mg
KH_2PO_4	0.06	g	Uracil	0.3	mg
$CaCl_2$ (fused)	0.14	g	Pyridoxal.HCl	0.025	mg
$Fe(NO_3)_3.9H_2O$	0.72	mg	Pyridoxine.HCl	0.025	mg
Glucose	1.0	g	Niacin	0.025	mg
Adenine Sulphate	0.01	g	Niacinamide	0.025	mg
L-Arginine.HCl	0.07	g	p-Aminobenzoic acid	0.05	mg
L-Histidine.HCl	0.02	g	i-Inositol (meso)	0.05	mg
L-Lycine.HCl	0.07	g	Riboflavin	0.01	mg
DL-Tryptophan	0.02	g	Thiamine.HCl	0.01	mg
L-Tyrosine	0.04	g	Calcium Pantothenate	0.01	mg
L-Cystine	0.02	g	Choline dihydrogen Citrate		mg
DL-Penylalanine	0.05	g		1.1	mg
DL-Methionine	0.03	g	D-biotin	0.01	mg
DL-Serine	0.05	g	Folic Acid	0.01	mg
DL-Threonine	0.06	g	Calciferol	0.1	mg
DL-Leucine	0.12	g	Cholesterol	0.2	mg
DL-Isoleucine	0.04	g	α-Tocopherol Phosphate		
DL-Valine	0.05	g	disodium salt (vit.E)	0.01	mg
DL-Glutamic Acid.H_2O	0.15	g	Menadione	0.01	mg
DL-Aspartic Acid	0.06	g	D-Ribose	0.5	mg
DL-α-Alanine	0.05	g	D-2-deoxyribose	0.5	mg
L-Proline	0.04	g	Adenylic acid	0.2	mg
L-Hydroxyproline	0.01	g	Phenol Red	0.02	g
Glycine	0.05	g	L-Cysteine.HCl	0.1	mg
L-Glutamine	0.10	g	Glutathione	0.05	mg
$CH_3COONa.3H_2O$	83.00	mg	Ascorbic acid (vit.C)	0.05	mg
Adenosine Triphosphate	1.00	mg	Vitamin A acetate	0.1	mg

Appendix B
Difco Culture Medium 199

Difco catalogue number: TC Medium 199

Dry powder reconstituted to 1000 ml.

L-Arginine	70	mg
L-Histidine	20	mg
L-Lysine	70	mg
L-Tyrosine	40	mg
DL-Tryptophane	20	mg
DL-Phenylalanine	50	mg
L-Cystine	20	mg
DL-Methionine	30	mg
DL-Serine	50	mg
DL-Threonine	60	mg
DL-Leucine	120	mg
DL-Isoleusine	40	mg
DL-Valine	50	mg
DL-Glutamic acid	150	mg
DL-Aspartic acid	60	mg
DL-Alanine	50	mg
L-Proline	40	mg
L-Hydroxyproline	10	mg
Glycine	50	mg
L-Cysteine	0.1	mg
Adenine	10	mg
Guanine	0.3	mg
Xanthine	0.3	mg
Hypoxanthine	0.3	mg
Thymine	0.3	mg
Uracil	0.3	mg
Thiamine Hydrochloride	0.01	mg
Riboflavin	0.01	mg
Pyridoxine Hydrochloride	0.025	mg
Pyridoxal Hydrochloride	0.025	mg
Niacin	0.025	mg
Niacinamide	0.025	mg
Calcium Pantothenate	0.01	mg
Biotin	0.01	mg
Folic acid	0.01	mg
Choline	0.5	mg
Inositol	0.05	mg
p-Aminobenzoic acid	0.05	mg
Vitamin A	0.1	mg
Calciferol	0.1	mg
Menadione	0.01	mg
α-tocopherol phosphate	0.01	mg
Ascorbic acid	0.05	mg
Glutathione	0.05	mg
Cholesterol	0.2	mg
L-Glutamine	100	mg
Adenosinetriphosphate	1	mg
Adenylic acid	0.2	mg
Ribose	0.5	mg
Deoxyribose	0.5	mg
Bacto-dextrose	1000	mg
Tween 80	5	mg
Sodium Acetate	50	mg
Iron(Ferric Nitrate)	0.1	mg
Sodium Chloride	8000	mg
Potassium Chloride	400	mg
Calcium Chloride	140	mg
Magnesium Sulphate	100	mg
Disodium Phosphate	60	mg
Monopotassium Phosphate	60	mg

Appendix C
Modified Krebs-Ringer Buffer

118.0	mmol/l NaCl
4.8	mmol/l KCl
1.3	mmol/l $CaCl_2$
1.2	mmol/l KH_2PO_4
1.3	mmol/l $MgSO_4$
25.0	mmol/l Hepes buffer
0.25	mmol/l glucose
1.1	mmol/l sodium acetate
1%	w/v BSA

the pH was adjusted to 7.4 with NaOH.

Appendix D
The Galettii Method for analysis of glycerol

Glycerol concentrations in the medium samples were measured using the method described by Galletti (1967).

Solutions required:

1. Phosphoric acid 2 mol/l
2. Potassium metaperiodate 0.015 mol/l
3. Phenyl hydrazine hydrochloride 1% w/v in distilled water. This solution was prepared freshly just prior to commencing the assay.
4. Potassium ferricyanide 1% w/v in distilled water.
5. Sulphuric-acetic acid reagent:
   80 ml water + 120 ml concentrated sulphuric acid + 200 ml concentrated acetic acid. Solution was cooled before use.
6. Isopropanol

The procedure involves the oxidation of the glycerol with periodic acid, followed by determination of the formaldehyde thus formed. The colour development is caused by the reaction of formaldehyde with phenylhydrazine and potassium ferricyanide giving a red dye.

It was found that the accuracy of the assay depended on strict adherence to a timed schedule of a series of additions. Each addition was made in turn to the tubes which were arranged in the same order for each addition. The samples were vortexed after each addition. A blank, 4 standards and about 15 samples could be handled in each run. The additions and the time schedule are given below:

Time(in minutes)	Addition
0	50 μl phosphoric acid + 100 μl KIO_4
15	200 μl phenylhydrazine
25	200 μl KFeCN samples placed on ice after this addition.
30	samples removed from ice add 1 ml H_2SO_4-acetic acid reagent and 1 ml isopropanol
50	read the absorbance of the samples at 520 μm

A standard curve was constructed for each set of samples with glycerol concentration plotted against absorbance (Figure 3.7). A computer was used to fit a line to this plot using linear regression analysis and to calculate the glycerol concentration for each sample's given absorbance value. The intra-assay variation was 8% and the inter-assay variation 12%.

Appendix E
Measurement of fatty acid in medium

Free fatty acid was measured in 3.0 ml aliquots of medium or tissue extract using the semi-automated method described by Crane and Lane (1977).

Five ml of Dole's extraction medium (see Section 3.14), 3 ml heptane and 2 ml of boiled distilled water were added to the 3 ml of medium sample. The tubes were capped and shaken thoroughly to extract the FFA into the heptane layer. Nitrogen was bubbled through the samples to remove any volatile acids, such as carbonic acid, which cause high readings. When the layers had settled, 4 ml of heptane layer was transferred to another tube. In the case of tissue extracts, the heptane layer from total extraction of lipid was used. For samples of tissue in which FFA was to be measured, no isotope had been added to the medium to prevent contamination of the auto-analyser. Contamination from the medium samples was not a problem because the isotope present in the medium was still in the form ^{14}C-acetate and therefore did not appear in the heptane layer. The 4 ml of heptane containing the FFA to be measured was washed with 0.05% v/v sulphuric acid and shaken thoroughly. This removed any lactic acid and phospholipid which may interfere with the assay.

The upper layer was used for FFA analysis in the auto-analyser. Standard solutions of palmitic acid were prepared at a range of concentrations from 160 - 1000 μmol/l. One ml of the standard was extracted in the same manner as the samples. The auto-analyser used 2.5% w/v sodium barbitone and 1% w/v phenol red in sodium barbitone. The change in red colour caused by the presence of FFA was measured at 550 nm. A standard curve of μmoles/l FFA against absorbance was constructed and the samples read off.

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