BIOCHEMICAL ASPECTS of ENERGY UTILISATION in RUMINANTS

by

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DECLARATION

This thesis was composed by myself and none of the work therein has been presented in any previous applications for a degree.

All sources of information are shown in the text and listed in the references. All help given by other people is indicated in the acknowledgements.

N.D. SCOLLAN

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ABSTRACT

The activities of acetyl-CoA hydrolase and acetyl-CoA synthetase and the influence of diet and feeding level on them were investigated in various ovine tissues and used to determine both the potential rate of substrate cycling between acetate and acetyl-CoA and the contribution of this cycle to energy expenditure in the ruminant. Two experiments, using lambs, were conducted in an attempt to further understand biochemical pathways which may influence the efficiency of utilisation of ME and explain why this is lower for fibre (sugarbeet pulp) when compared to starch (barley) based diets. The influence of carbohydrate source, feeding level and protein level on plasma concentrations of acetate, glucose and insulin and the rates of acetate incorporation into CO_2 and lipid were studied.

ATP-stimulated acetyl-CoA hydrolase is present in rumen epithelium, muscle and the cytoplasm of ovine liver but not in perirenal adipose tissue, and it is not inactivated by cold. "Mitochondrial" acetyl-CoA hydrolase was detected in all tissues investigated. The activities of acetyl-CoA hydrolase and acetyl-CoA synthetase tended to be higher in perirenal adipose tissue of lambs fed on sugarbeet diets and their activities decreased with increasing level of feeding ($P<0.05$).

Variation in plasma characteristics were more easily observed in lambs fed semi-continuously compared to lambs which received their ration once daily. Higher levels of crude protein in the diet resulted in lower plasma acetate concentrations ($P<0.05$). The rates of acetate incorporation into CO_2 and lipid were influenced by acetate ($P<0.01$), glucose ($P<0.0001$) and insulin ($P<0.01$). Higher levels of crude protein in the diet resulted in greater rates of acetate incorporation into lipid ($P<0.05$) whereas feeding sugarbeet pulp resulted in increased rates of acetate incorporation into CO_2 ($P<0.05$).

A technique involving open column ion exchange chromatography, freeze drying and HPLC was developed for the concentration and separation of plasma organic acids. Organic acid recoveries were 43-69%.

An experiment was conducted to investigate the activity of the substrate cycle between acetate and acetyl-CoA in calf liver *in vivo*. Several problems were encountered, notably huge variation in blood flows (including negative rates). This introduced large variation into the calculated fluxes and no meaningful conclusions were made.

It was calculated from enzyme measurements made *in vitro*, that the substrate cycle in ovine liver may potentially account for 2.5% of basal heat production. It is suggested that the efficiency of utilisation of ME is related to glucose homeostasis, involving VFA and protein metabolism.

CONTENTS

ABBREVIATIONS

ADP	- Adenosine Diphosphate
AMP	- Adenosine Monophosphate
AHG	- Autumn Harvested Grass
Ap_5A	- P^1,P^5 di(adenosine-5') pentaphosphate
ATP	- Adenosine Triphosphate
B	- Barley (Diet)
BH	- Barley/high level of Feeding
BL	- Barley/low level of Feeding
BPH	- Barley/high Protein
BPL	- Barley/low protein
BSA	- Bovine Serum Albumin
C_2/C_4	- 2 or 4 Carbon compound
CF	- Crude Fibre
CoA	- Coenzyme A
CP	- Crude Protein
^{14}C	- Radioactive Carbon
D/F	- Diet/Feeding Level
DB	- Digestion Buffer
DE	- Digested Energy
DPM	- Disintegrations per minute
DTT	- Dithiothreitol
DTNB	- 5,5 Dithiobis (2-Nitrobenzoic Acid)
E	- Enzyme
EDTA	- Ethylene Diamine Tetracetic Acid
EGTA	- Ethylene Glycol-bis(ß-aminoethyl Ether)N,N,N',N'-Tetraacetic Acid
G	- Grass/Rice husks (Diet)
GH	- Grass/Husks High Level of Feeding
GL	- Grass/Husks Low Level of Feeding
HEPES	- N-[2-Hydroxyethl]piperazine-N'-[2-ethanesulfonic acid]
HIF	- Heat Increment of Feeding
HPLC	- High Performance Liquid Chromatography
H_f	- Heat of Fermentation
H_t	- Metabolic Heat Production
^3H	- Tritium
I	- Incubation
IMP	- Ion Moderated Partition

INT	- Iodonitrotetrazolium Chloride (INT)
K_f	- Efficiency of Energy Utilisation for Growth
K_m	- Efficiency of Energy Utilisation for Maintenance
ME	- Metabolisable Energy
M	- Molassed Sugarbeet Pulp
MADF	- Modified Acid Detergent Fibre
MH	- Molassed Sugarbeet/high level of Feeding
ML	- Molassed Sugarbeet/low level of Feeding
MOPS	- Morpholinopropan-Sulphonic Acid
NAD^+	- Nicotinamide Adenine Dinucleotide (oxidized)
NADP	- Nicotinamide Adenine Dinucleotide Phosphate
NADPH	- Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NDF	- Neutral Detergent Fibre
NEFA	- Non-Esterified Fatty Acids
PAH	- p-aminohippuric Acid
P_i	- Inorganic Phosphate
PP_i	- Pyrophosphate
PPP	- Pentose Phosphate Pathway
q	- Metabolisability
RDP	- Rumen Degradable Protein
S.E.M.	- Standard Error of the Mean
SHG	- Spring Harvested Grass
SH	- Sugarbeet Pulp High Level of Feeding
SL	- Sugarbeet Pulp Low Level of Feeding
S/SBP	- Sugarbeet Pulp (Diet)
SPH	- Sugarbeet/High Protein
SPL	- Sugarbeet/Low Protein
T	- Transport
TCA	- Tri-carboxylic Acid (cycle)
TEA	- Triethanolamine
TRT	- Treatment
Tris	- Tris(Hydroxymethyl) Aminomethane
U	- Plain Sugarbeet Pulp
UDP	- Rumen Undegradable Protein
UH	- Plain Sugarbeet/high level of Feeding
UL	- Plain Sugarbeet/low level of Feeding
VFA	- Volatile Fatty Acids
W	- Washing

CHAPTER ONE

INTRODUCTION and LITERATURE REVIEW

1.1.1 INTRODUCTION

The efficiency with which substrates are converted into animal products is of immense importance to animal production. This efficiency depends upon many factors including the animal's genotype, sex, age, physiological state such as pregnancy or lactation and also upon external factors such as climate, quality and quantity of food available. The purpose of this thesis is to achieve a greater understanding of selected biochemical pathways and processes which underlie energy utilisation. From a knowledge of these processes and their control, it may be possible to alter energy expenditure in specific ways, thus improving the efficiency of productive processes by farm animals.

1.1.2 ELEMENTS of ANIMAL ENERGY EXPENDITURE

The basic terminology used in energy metabolism has changed relatively little since Armsby (1917). Metabolisable energy (ME) is the portion of dietary energy which is available to the animal for metabolism. It may be calculated as the gross energy of the food minus the energy contained in the faeces, urine and gaseous products of digestion.

An animal when fasted, rested and held in a thermoneutral environment exhibits a basal level of energy expenditure which is supported by the oxidation of substrates, principally lipids and amino acids, mobilised from body tissues (Milligan & Summers, 1986). Baldwin *et al.*, (1980) have categorised basal energy expenditure into the costs for service functions and functions associated with cell maintenance (Table 1.1). Service costs are energy expenditures by tissues which are of benefit to the animal as a whole, whereas, cell maintenance functions are essential for the sustenance of individual tissues or cells. However, it is recognised that at the cellular level a considerable degree of overlap will exist between the two categories. For example, the majority of the energy cost of neuronal activity and kidney work actually entails energy expenditure on ion transport. These processes are frequently referred to as basal energy expenditures,

Table 1.1 *Energy expenditures in several major maintenance functions.*

Function	% basal energy expenditure
Service functions:	
Kidney work	6-7
Heart work	9-11
Respiration	6-7
Nervous functions	10-15
Liver functions	5-10
Total	36-50
Cell maintenance:	
Protein resynthesis	9-12
Lipid resynthesis	2-4
Ion transport	30-40
Total	40-56

(Baldwin *et al.*, 1980)

ancillary energy expenditures or since they utilise ATP (the form in which cellular energy is made available), ATP expending processes.

Studies have shown that nutritional history and physiological state influence the relative proportions of the metabolically intense organs (liver, kidneys pancreas, heart and intestines) and basal energy expenditure varies linearly with these changes (Baldwin *et al.*, 1980; Armstrong & Blaxter, 1984).

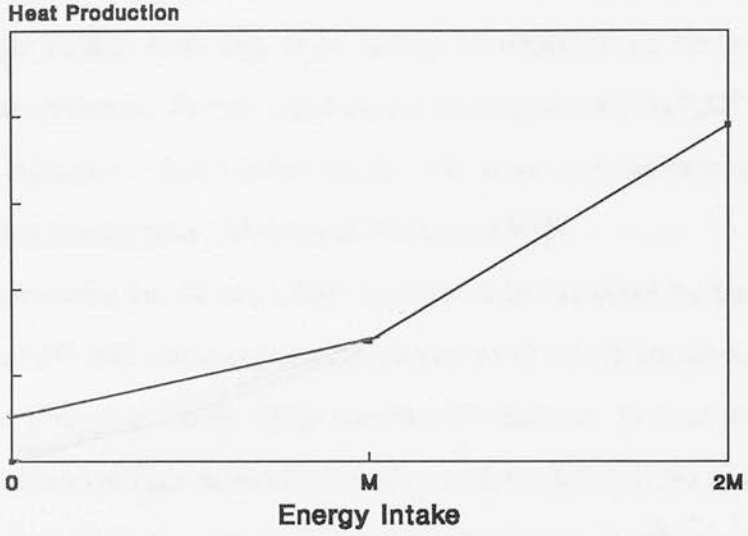
Baldwin *et al.* (1980) did not include the energy costs of muscle tone, posture or vascular circulation, but omission of these may not be very significant (Milligan & Summers, 1986). As stressed by Milligan & Summers (1986) there are many other energy expending processes which require investigation. These will be discussed presently.

Evolution of energy as heat from the body is the inevitable consequence of the inefficiency of the many complex processes underlying the maintenance of vitality. Even in a state of basal energy expenditure an animal still evolves considerable quantities of heat, the average for mammals is approximately 308kJ per metabolic kg ($kg^{0.75}$) (Robinson *et al.*, 1983).

The level of energy intake resulting in a state of energy balance, where energy is neither retained nor lost by the body, is referred to as the maintenance energy requirement. This requirement is assumed to be the dietary ME required to meet basal energy expenditure plus the energy cost of minimal activity and urinary energy excretion (Agricultural Research Council, 1980). Energy provided which is excess to maintenance requirements is available for productive purposes. On feeding the efficiency of ME utilisation declines exponentially with increasing ME intake (or alternatively heat production increases) (MacRae & Lobley, 1982). For simplicity the efficiency of ME utilisation by ruminants is illustrated graphically as two straight lines with an inflection point at maintenance (Fig.1.1). Efficiency of ME utilisation for maintenance and production are termed k_m and k_f respectively.

The Agricultural Research Council (1980) indicates that ruminants require 40% more ME to achieve energy balance than basal energy expenditure. A proportion of

Fig. 1.1 *The Relationship between Energy Intake and Heat Production.*



this discrepancy may be accounted for in terms of the energy costs of food consumption (ingestion, digestion, absorption), the lower efficiency of volatile fatty acid use compared to oxidation of fatty acids derived from the body, the utilisation of body fatty acids and the energy costs of their subsequent resynthesis (Milligan & Summers, 1986). A large proportion remains unaccounted for and evidence exists which indicates that major energy expending processes underlying energy expenditure are quantitatively different at maintenance than when compared to basal state. For example, Reeds & Fuller (1983) reviewed evidence which suggests that protein synthesis varies with energy intake. Protein synthesis at zero energy retention was reported to increase two fold over that occurring at fasting. Milligan & Summers (1986) calculate that this would require a 10-15% increase in ME intake to achieve energy balance. Oxygen uptake in support of Na^+,K^+-ATPase, a major ion transport process, increased 1.82 fold for duodenal epithelium and 2.65 fold for hepatocytes, at intake levels which provided for energy balance over expenditures during fasting (McBride & Milligan, 1985a,b).

This suggests basal energy expenditures are not constant and in addition these processes may be further potentiated above the maintenance state. Reeds & Fuller (1983) have derived positive linear relationships for whole body protein synthesis and breakdown with intakes extending from fasting to intake levels three times that required for maintenance. Energy expenditures associated with Na^+,K^+-ATPase in the duodenal mucosa of sheep increased by 37% from maintenance to an intake allowing for twice maintenance (McBride & Milligan, 1985b).

The non-fermentative (or aerobic) heat production in the digestive tract of sheep increased from 3.2% ME consumed at maintenance to 15.6% of ME consumed above energy balance (Webster, 1980). This considerable increase in heat production is thought to be related to high rates of cell and protein turnover by the gut epithelium and as mentioned above the activity of Na^+,K^+-ATPase by duodenal epithelium is markedly increased with increasing energy intake.

Hence, it is realised that energy expending processes are influenced by the level of ME intake. Energy is expended to support processes which are not directly associated with productive pathways and furthermore it appears that control of these processes lies not only with ME intake but they are also influenced by dietary constituents. For example, protein turnover is specifically more responsive to increased intake of dietary protein rather than non-protein dietary energy (Reeds *et al* 1985). Adeola *et al.*, (1989) investigated oxygen consumption, Na^+,K^+-ATPase dependent and independent respiration and protein synthesis in vitro, in several muscles taken from pigs which had received either 130, 170 or 210 g protein/kg diet. Oxygen consumption and Na^+,K^+-ATPase dependent respiration increased linearly with increasing proportion of protein in the diet. Protein synthesis rate was influenced by dietary protein which is in accordance with Reeds *et al.* (1985). A close association between Na^+,K^+-ATPase dependent respiration and protein synthesis was demonstrated. This suggests interactions between dietary constituents, ancillary energy expenditures and productive processes.

Milligan (1971) concluded that animals do not differ in the pathways utilised for the

conversion of precursors to products and therefore variation in the efficiency of product formation will depend upon ancillary energy expending processes linked to product synthesis. Hence if modification of basal energy expenditure by an animal is to be achieved, a greater understanding of those ancillary processes is a priority. As discussed above an insight into cellular productive processes and ATP-utilising processes is developing. Activation of protein synthesis is a controlled process and a concomitant alteration in the cellular environment occurs, which involves changes in Na^+,K^+-ATPase activity, phosphatidyl inositol metabolism, release of specific mediator peptides and adenyl cyclase activity. All these processes are integrated and activation of one results in co-activation of the others, resulting in increased energy expenditure. Hence as discussed by Reeds *et al.* (1985) part of the increase in basal metabolic rate that accompanies the storage of nutrients is energy expended by processes which control the metabolic response. Therefore it is important to qualitatively identify the processes underlying energy utilisation and to then quantify their role.

One such process that can be considered as a component of basal energy expenditure is substrate cycling, which hydrolyses ATP. Such cycles exist at certain key points in intermediary metabolism. The substrate cycle which operates between acetate and acetyl-CoA may be particularly pertinent in ruminant metabolism, since acetate is a major source of energy to the ruminant animal and acetate has also a high endogenous entry rate (Milligan & Summers, 1986).

This cycle has been shown to operate in rat hepatocytes *in vitro* (Jessop *et al.*, 1986; Crabtree *et al.*, 1989) and the rate of cycling increased linearly with increasing acetate concentrations. This project will investigate the potential for cycling between acetate and acetyl-CoA in various tissues obtained from ovines and bovines, by determining the activity of the enzymes involved in the cycle namely acetyl-CoA hydrolase and acetyl-CoA synthetase, *in vitro.* If sufficient potential exists in bovine liver, an attempt will be made to detect and assess the activity of this cycle in the liver of calves *in vivo*.

1.1.3 THE HEAT INCREMENT of FEEDING

The conundrum of the inefficient utilisation of some fibrous diets has been approached on many occasions, but a satisfactory solution remains tentative. Blaxter (1980) concluded that *"the major question in energy metabolism of ruminants is probably still that discovered and formulated by Zuntz nearly a century ago, namely what is the origin of the heat increment and why is it greater for roughages than for concentrate feeds?"*

Table 1.2 *The efficiency of ME utilisation from fibre and starch based sources.*

	k_m	k_f
fibre	0.59	0.25
starch	0.74	0.55

(Blaxter, 1980)

The heat increment of feeding (HIF) is the heat associated with food consumption (the inverse of k_m or k_f). Table 1.2 indicates it is greater for a fibre diet than a starch diet and this is particularly so above maintenance.

Webster (1980) has drawn up a list of factors which, from first principles, contribute to the HIF.

1. The increase in heat production directly associated with
   (a) eating and (b) rumination.
2. Heat production by microbial fermentation in the gut.
3. Heat arising from metabolic activites in the gut
   (portal-drained viscera).
4. Heat associated with metabolic activity in tissues other than those
   included in activities described above.

Many heated debates have taken place as to the exact cause of the increased heat

increment observed with feeding fibre when compared to that observed with feeding starch based diets. Various theories have emerged but new information arises which repeatedly challenges the original hypothesis, and the issue continues to remain unresolved.

These theories will be discussed subsequently, but one theory which has received a considerable amount of attention has suggested that the increased inefficiency of ME utilisation from fibrous diets may be related to the efficiency of acetate utilisation for fat synthesis. This research project proposes to investigate this theory by studying metabolite levels in lambs which will be fed on either a fibrous or starch based diet and after slaughter to investigate acetate metabolism in adipocytes isolated from perirenal adipose tissue, *in vitro*.

1.2 ACETATE METABOLISM

1.2.1 *Acetate in Relation to other Energy Yielding Nutrients*

Acetate is a major energy source for the ruminant and together with propionate and butyrate form the major volatile fatty acids (VFA), that are produced in the rumen as a result of microbial fermentation of the diet. The volatile fatty acids are absorbed across the rumen wall into the portal blood and supply 70-80% of the fed animal's energy requirement (Bergman & Wolff, 1971).

The proportions of VFA's produced vary with the nature of the diet (degree of fibre/starch, grinding and pelleting of the diet (Ørskov *et al.*, 1974), frequency of feeding and addition of feed additives such as monensin (Armstrong & Spears, 1988).

Approximately 5-7% of rumen propionate is metabolised to lactate by the rumen epithelium, and the majority of the residue is metabolised to glucose by the liver (Weekes, 1974; Bergman & Wolff, 1971). Butyrate is extensively metabolised by rumen epithelium producing the ketone bodies acetoacetate and 3-hydroxybutyrate (Annision & Armstrong, 1970). Acetate is metabolised relatively little by rumen epithelium (Bergman & Wolff, 1971) and is the only short chain fatty acid common in peripheral blood.

Ruminants absorb little or no glucose from the digestive tract and hence the glucose requirement must be met from gluconeogenesis. The major precursors for glucose synthesis are propionate, lactate and certain amino acids such as alanine and glutamate. Consequently the ruminant's metabolism has adapted to using VFA (principally C_2 & C_4) to generate energy whilst "sparing" glucose for essential body functions.

1.2.2 *Sources of Acetate*

In the fed ruminant, circulating concentrations of acetate are usually 1-2mM, but in sheep starved for 48h, plasma acetate levels drop to 0.3-0.4mM, similar to values found in non-herbivores (Annison & White, 1962). It is clear that there are two sources of

plasma acetate, the proportion absorbed from the alimentary tract (exogenous acetate) and that arising from tissue metabolism (endogenous acetate).

Studies by Annision (see Annison & Armstrong, 1970) suggested that approximately 40% of the total acetate supply could not be accounted for by ruminal acetate production. Further experiments (Bergman & Wolff, 1971; Pethick *et al.*, 1981) using isotope dilution techniques found endogenous acetate to be relatively constant in the normal fed animal at approximately 30% of plasma acetate. Pethick *et al.* (1981) suggested that since the endogenous contribution is quite stable in the fed animal, it may be possible to use blood acetate as a indicator of nutritional status.

Many tissues have been shown to contribute to endogenous acetate but in general the liver is of major importance and Palmquist (1972) has reported the liver to be responsible for 80% of this fraction. Studies suggest that endogenous acetate production is influenced by food intake and by physiological status. Knowles *et al.* (1974) found the rates of acetate output by the heart and liver to increase on starvation. In lactating ewes hepatic acetate production would appear to be correlated to the stage of lactation, high initial values at peak lactation declining as milk output falls (Costa *et al.*, 1976).

In the fed ruminant the main sources of endogenous acetate are butyrate, ethanol, non-esterified fatty acids (NEFA) and possibly amino acids (Annision *et al.*, 1963; Buckley & Williamson, 1977), whereas in the fasted state NEFA are the primary source (Palmquist, 1972). It is not entirely clear what controls acetate output by tissues but it appears that NEFA flux, the ratio acetyl-CoA:CoA (Knowles *et al.*, 1974) or factors influencing ketogenesis may be important catalysts.

1.2.3 *Acetate Entry Rate and Techniques for Studying Acetate Metabolism*

Acetate entry rate represents the sum of the inflows of acetate (that is the exogenous and endogenous sources) into the body pool of acetate. A measure of entry rate is obtained from studies which investigate acetate metabolism in specific tissues using a

combination of arterial-venous (A-V) differences and isotope dilution. Such experiments involve the continuous infusion of labelled acetate to achieve constant specific radioactivity of acetate in the circulation. The use of [^{14}C] acetate permits the fate of acetate in the organ or tissue under study and in the whole animal to be traced (Bergman & Wolff, 1971; Pethick *et al.*, 1981; Pethick & Lindsay, 1982). Alternatively an *in vitro* approach may be used to study acetate metabolism, which involves incubating either isolated cells or tissue slices or perfusing an organ (either *in situ* or isolated) with [^{14}C] acetate media (Yang & Baldwin, 1973; Snoswell *et al.*, 1982; Jessop *et al.*, 1986).

Acetate entry rate is highly correlated with arterial acetate concentration in sheep, irrespective of their physiological or nutritional status and the equation is described,

$$y = 2.06x - 0.10 \quad (r^2 = 0.94; P < 0.001)$$

where y = acetate entry rate (mmol. h^{-1}. kg^{-1} bodyweight), and x = arterial acetate concentration (mM) (Pethick & Lindsay, 1982).

1.2.4 *Acetate Utilisation*

Few studies have investigated the tissues both supplying and utilising acetate on a whole animal basis. Such experiments must allow for the simultaneous uptake and release of acetate. Bergman & Wolff (1971) and Pethick *et al.* (1981) have conducted experiments which do consider these aspects using a combination of isotope dilution and A-V difference. Pethick *et al.* (1981) studied the simultaneous utilisation and production of acetate by the gut, liver and muscle in insulin stabilised diabetic sheep. All three tissues were found to utilise and produce acetate. Acetate utilisation by all tissues was found to be linearly related to arterial supply and highly significant regression equations were obtained for all tissues.

It was found that acetate had the potential to supply approximately 40% of the

energy needs of the fed sheep, but this was not directly oxidised since only 26% of respiratory CO_2 was derived from acetate (Pethick *et al.*, 1981). Acetate was a significant fuel for all tissues, theoretically accounting for 29-50% of the O_2 consumption.

A range of studies, both *in vitro* (Leng & Annision, 1963; Mayfield *et al.*, 1966) and *in vivo* (Bergman & Wolff, 1971; Thompson *et al.*, 1975) have shown that liver metabolises relatively little acetate. However Pethick *et al.* (1981) found the liver to remove 17% of the acetate entry rate. Hence there is contrasting evidence as to whether or not the liver utilises acetate and it must be noted that Pethick *et al.* (1981) found little of the acetate utilised by the liver to be oxidised. As the authors indicate this was equivocal and it may reflect errors associated with the method used in calculating the proportion oxidised. It is recognised that large quantities of acetate may enter non-oxidative pathways, for example, 3-hydroxybutyrate synthesis and non-essential amino acid synthesis, but the amount entering fatty acid synthesis may be limited since it has reported that the latter is minimal in ruminant liver (Bauman & Davies, 1975).

In the gut, large quantities of acetate are extracted during the passage of blood from the artery to the portal vein. Both Bergman & Wolff (1971) and Pethick *et al.* (1981) agree that approximately 30% of absorbed acetate is directly utilised by the gut. The latter study found the acetate utilised by the gut to be directly oxidised which is contrary to the findings of Bergman & Wolff (1971), who suggested that much acetate is used for lipogenesis. These authors appreciated that acetate utilisation by the gut could be a multiplicity of factors including lipogenesis, oxidation by smooth muscle, net conversion to other compounds, simple exchange or even possibly diffusion into the lumen of the digestive tract.

Adipose tissue is the major site of fatty acid synthesis in ruminants and forms a dynamic store of energy (Ballard *et al.*, 1969). Acetate is the dominant precursor of fatty acids in ruminants compared to glucose in non-ruminants. Since Pethick *et al.*

(1981) found the combined uptake by muscles, gut and liver to account for 96% of whole body turnover, the majority of the remaining acetate (4%) is presumably used for fatty acid synthesis which supports findings that the absolute rate of lipogenesis in non lactating ruminants is low (Van der Walt, 1984). Lipogenesis will be discussed further in a later section.

Arterial-venous and isotope dilution procedures are particularly suitable for the study of skeletal muscle metabolism since only minor surgery is required and measurements are made on a relatively large muscle mass. Approximately 30% of the energy metabolism of muscle is derived from acetate (Pethick *et al.*, 1981). This acetate is largely oxidised although some acetate may be used for intermuscular fat synthesis. Pethick & Lindsay (1982) have found that muscle extracted less acetate in the lactating animal and the amount spared was comparable to that extracted by the udder.

It is clear from this discussion that acetate is a major metabolite in sheep as a substrate both for oxidation and fat synthesis.

1.2.5 *Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase*

The endogenous production of acetate is catalysed by acetyl-CoA hydrolase (EC 3.1.2.1), an enzyme first detected in a preparation of pig heart (Gergely *et al.*, 1952). The enzyme hydrolases acetyl-CoA producing stoichiometric amounts of acetate and CoA:

$$\text{Acetyl-CoA} + H_2O \rightleftharpoons \text{Acetate} + \text{CoA}$$

The existence of a true acetyl-CoA hydrolase has been questioned by Costa & Snoswell (1975) who suggested acetyl-CoA hydrolysis in rat liver was solely an artifact of acetyl-CoA carnitine acetyltransferase and acetylcarnitine hydrolase. Subsequently Snoswell & Tubbs (1978) discarded this theory because they could not detect acetylcarnitine hydrolase in either rat or sheep liver, and agreed with the observations

of Knowles *et al.* (1974) that a true acetyl-CoA hydrolase did exist.

Acetyl-CoA synthetase (EC 6.2.1.1) is the enzyme responsible for the activation of acetate in tissues:

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Acetyl-CoA} + \text{AMP} + \text{PP}_i$$

The activities of acetyl-CoA synthetase and acetyl-CoA hydrolase in a tissue are indicators of the maximum capacity of that tissue to utilise and to produce acetate respectively. Many experiments have investigated the activities of these enzymes in both herbivores and non-herbivores (Quaraishi & Cook, 1972; Knowles *et al.*, 1974). Table 1.3 presents the activities of the enzymes in various tissues of sheep. In most tissues the rate of synthetase activity is greater than hydrolase with the notable exception of the liver. The heart and kidney have particularly high activities of acetyl-CoA synthetase reflecting the importance of acetate metabolism in these tissues.

Quaraishi & Cook (1972) found acetyl-CoA synthetase to be located predominantly in the mitochondria whereas Knowles *et al.* (1974) found it to be mostly cytosolic. Studies by Snoswell & Koundakjian (1972) support the latter view. The cytosolic location of this enzyme is presumably important in the incorporation of acetate into fatty acid synthesis (although the latter is reported to be low in ruminant liver). The mitochondrial location of acetyl-CoA synthetase in the heart presumably reflects the importance of acetate as a precursor of acetyl-CoA for further oxidation in the TCA cycle.

The activity of acetyl-CoA synthetase in all the muscles examined by Knowles *et al.* (1974) was low, (except the masseter muscle). The activity in the leg muscle of sheep was only 5% that of heart tissue, but even so half of the acetate presented to the tissue was removed in a single passage of blood. Knowles *et al.* (1974) have suggested that more of the enzyme is catalytically active at the physiological concentrations of acetate

Table 1.3 *Activities of Acetyl CoA Synthetase and Acetyl CoA Hydrolase in various sheep tissues (in nmol converted per s per g of tissue protein). From Knowles et al., (1974).* Data are presented as mean±S.E.M., for between 4 and 6 animals.

Tissue	Acetyl-CoA Synthetase	Acetyl-CoA Hydrolase
Liver	67±8	167±13
Heart	826±82	57±10
Kidney Cortex	545±32	62±7
Brain	47±14	35±7
Soleus muscle	32±14	113±17
Masseter muscle	750±63	122±18
Perirenal adipose	262±90	60±23

in muscle compared to heart tissue.

Acetyl-CoA hydrolase is important in ruminants for maintaining the endogenous plasma acetate fraction and in rodents it is likely to be responsible for most of the plasma acetate since the exogenous supply is low (depends on diet). The enzyme is located predominantly in the mitochondrial fraction of the liver and heart, the intracellular location for the production of acetyl-CoA from either pyruvate or fatty acids. Palmquist (1972) demonstrated that acetate could be produced from palmitate in intact sheep or cattle, and Knowles *et al.* (1974) suggested that any tissue that has the capacity to form acetyl-CoA from palmitate may produce acetate provided acetyl-CoA hydrolase is present.

Under certain conditions and by certain tissues acetyl-CoA hydrolase may serve to produce acetate from acetyl-CoA when the entry of acetyl-CoA into the TCA cycle is restricted (i.e. as a result of a lack of ADP). In this situation the enzyme serves to reduce "acetyl pressure" (Snoswell & Koundakjian, 1972; Knowles *et al.*, 1974). In support of this, Knowles *et al.* (1974) investigated the effects of exposure of rats to a 100% N_2 atmosphere for 20min periods on blood and liver acetate. Anoxia resulted in increasing concentrations of blood and liver acetate which probably reflects an inhibition of the TCA cycle while the formation of acetyl-CoA continued.

The enzyme may also be involved in controlling the levels of acetate and CoA in the cell. The degree of control may vary from one tissue to another depending on their metabolic roles (Quraishi & Cook, 1972). For example in the liver acyl carnitine derivatives are important in metabolism and hence the hydrolase could be involved in transfer reactions. Alternatively in the brain the enzyme might serve to maintain optimum levels of CoA needed for glucose oxidation, the primary fuel for this tissue.

Studies using rat and sheep tissues have demonstrated that starvation and induced diabetes influence the activities of acetyl-CoA hydrolase and acetyl-CoA synthetase. Withdrawal of food from sheep for 120h resulted in a decrease in blood acetate from 630 to 90μM and the activity of hepatic acetyl-CoA hydrolase increased by approx.

60%, with no alteration in the activity of acetyl-CoA synthetase (Knowles *et al.*, 1974). The activity of the hydrolase in rat liver was also increased on starvation (24h) with no effect on the synthetase. Seufert *et al.* (1974) found the activity of the synthetase to decrease approx. 50% after 2 days starvation, with only a small rise in hydrolase activity. Seufert *et al.* (1974) showed that the ratio of acetyl-CoA synthetase to acetyl-CoA hydrolase was altered in the liver of rats when diabetes was induced. The production of acetate increased four-fold. The concentration of blood acetate increased 27 fold between 120h starvation and alloxan-diabetic sheep (Knowles *et al.*, 1974). This is probably the combination of an inhibition of acetate utilisation by tissues (as demonstrated by the hind limb study in Knowles's work) as a result of insufficient glucose and an increase in acetate production due to increased acetyl pressure.

1.2.6 *Cytoplasmic ATP-Stimulated Acetyl-CoA Hydrolase*

In addition to the "mitochondrial" acetyl-CoA hydrolase, Prass *et al.* (1980) have reported the existence of an extramitochondrial acetyl-CoA hydrolase of high activity in rat liver (20 μmol per min per g wet wt.). The enzyme is cold-labile, which explains why it had not been detected previously under standard procedures for assaying enzyme activity (i.e. homogenising tissue at 4°C; using isotonic buffers). The enzyme was stable when incubated in 1.3M sucrose at a 4°C or in 0.3M sucrose at 20°C, but a high loss of activity may be observed in 0.3M sucrose at 4°C (Prass *et al.*, 1980).

In contrast with the "mitochondrial" acetyl-CoA hydrolase, the cytoplasmic enzyme was activated by ATP and inhibited by ADP (Prass *et al.*, 1980; Matsunaga *et al.*, 1985; Söling & Rescher, 1985). It is inhibited by CoA and long chain acyl-CoAs, but acetate has no influence (Isohashi *et al.*, 1983).

It has been proposed that cytoplasmic acetyl-CoA hydrolase may be involved in a substrate cycle between acetate and acetyl-CoA (Rabkin & Blum, 1985; Jessop *et al.*, 1986) and results of Crabtree *et al.* (1989) would confirm this hypothesis.

Cytoplasmic acetyl-CoA hydrolase is thought to regulate the CoA levels in the cytosol. Matsunaga *et al.* (1985) propose that through this function the enzyme regulates the opposing metabolic states of fatty acid oxidation and fatty acid synthesis. When ß-oxidation of fatty acids in both mitochondria and peroxisomes is elevated, cytosolic CoA, acetyl-CoA and acyl-CoA pools are known to be increased 2-4 times (Greenbaum *et al.*, 1971; Broekhoven *et al.*, 1981). Hence CoA supplies must be increased. Peroxisomes do not oxidise acetyl-CoA or form ketone bodies and they have low hydrolase activities (see Matsunaga *et al.*, 1985). But they do transfer acetyl-CoA to the cytosol. Matsunaga *et al.* (1985) suggest that the cytosolic hydrolase is responsible for the hydrolysis of acetyl-CoA and hence the provision of CoA. In support of this hypothesis they report that the rate of this enzyme may double when peroxisomal oxidation is elevated and they note that the enzyme is inhibited by CoA but not by acetate.

On the other hand when fatty acid synthesis is elevated in rat liver, cytosolic acetyl-CoA hydrolase increases (Matsunaga et al., 1985). The authors propose that in this situation the enzyme controls the levels of CoA which is a substrate in the cleavage of citrate to acetyl-CoA by ATP-citrate lyase. Acetyl-CoA is carboxylated to malonyl-CoA by the enzyme acetyl-CoA carboxylase. The authors emphasise that malonyl-CoA does not inhibit the cytosolic enzyme (Prass *et al.*, 1980) and CoA is an important regulator of fatty acid synthesis, activating acetyl-CoA carboxylase.

Söling & Rescher (1985) carried out a kinetic investigation of the mitochondrial and cytoplasmic enzymes in rat liver and calculated that, at the cellular concentrations of the salient activators and inhibitors, the cytoplasmic enzyme would express only 10% of its activity *in vivo*. This has been confirmed by other studies (Rabkin & Blum, 1985; Jessop *et al.*, 1986).

1.2.7 *Lipogenesis in the Ruminant Animal*

It has been suggested that the efficiency of utilisation of acetate for lipogenesis may explain part of the increased heat increment associated with feeding fibre diets. Before proceeding to discuss this theory in greater detail this section will emphasis the major precursors and pathways involved in fatty acid synthesis by the ruminant animal.

In non-lactating ruminants more than 90% of long chain fatty acid synthesis occurs in adipose tissue (Bauman & Davies, 1975). Comparison of the rates of [1-^{14}C] acetate and of ^{3}H from $^{3}H_2O$ incorporation into fatty acids in sheep adipose tissue slices (incorporation of ^{3}H from $^{3}H_2O$ represents the total rate of fatty acid synthesis from all precursors) confirmed that acetate is the major precursor of long chain fatty acids in the ruminant (Vernon, 1975). This is contrary to monogastrics where glucose is the major precursor and the cytoplasm of the liver is usually the dominant site.

In the rat liver acetyl-CoA is transferred from the mitochondria to the cytosol, the site of fatty acid synthesis by the citrate cleavage pathway (Fig.1.2). This pathway involves the translocation of acetyl units from the mitochondria to the cytosol as citrate, which then undergo ATP-dependent cleavage forming acetyl-CoA and oxaloacetate. Oxaloacetate is reduced to malate which may re-enter the mitochondria or be decarboxylated by the enzyme NADP-malate dehydrogenase (Fig.1.2). Ruminant tissues normally lack significant activities of ATP-citrate lyase and NADP-malate dehydrogenase accounting for the low incorporation of glucose into fatty acids by ruminants (Bauman, 1976). However doubt has been cast on the rate limiting role of these enzymes by the ability of L-lactate to serve as a carbon precursor (Prior & Jacobson, 1979). This would suggest that ATP-citrate lyase and NADP-malate dehydrogenase may operate, perhaps even being involved in lipogenesis from acetate as well as from L-lactate as a carbon source (Prior *et al.*, 1981). The incorporation of pyruvate, propionate, methylmalonate, butyrate and 3-hydroxybutyrate by ruminant adipose tissue has been demonstrated *in vitro*, but it is unlikely that they contribute significantly *in vivo* (Vernon, 1981).

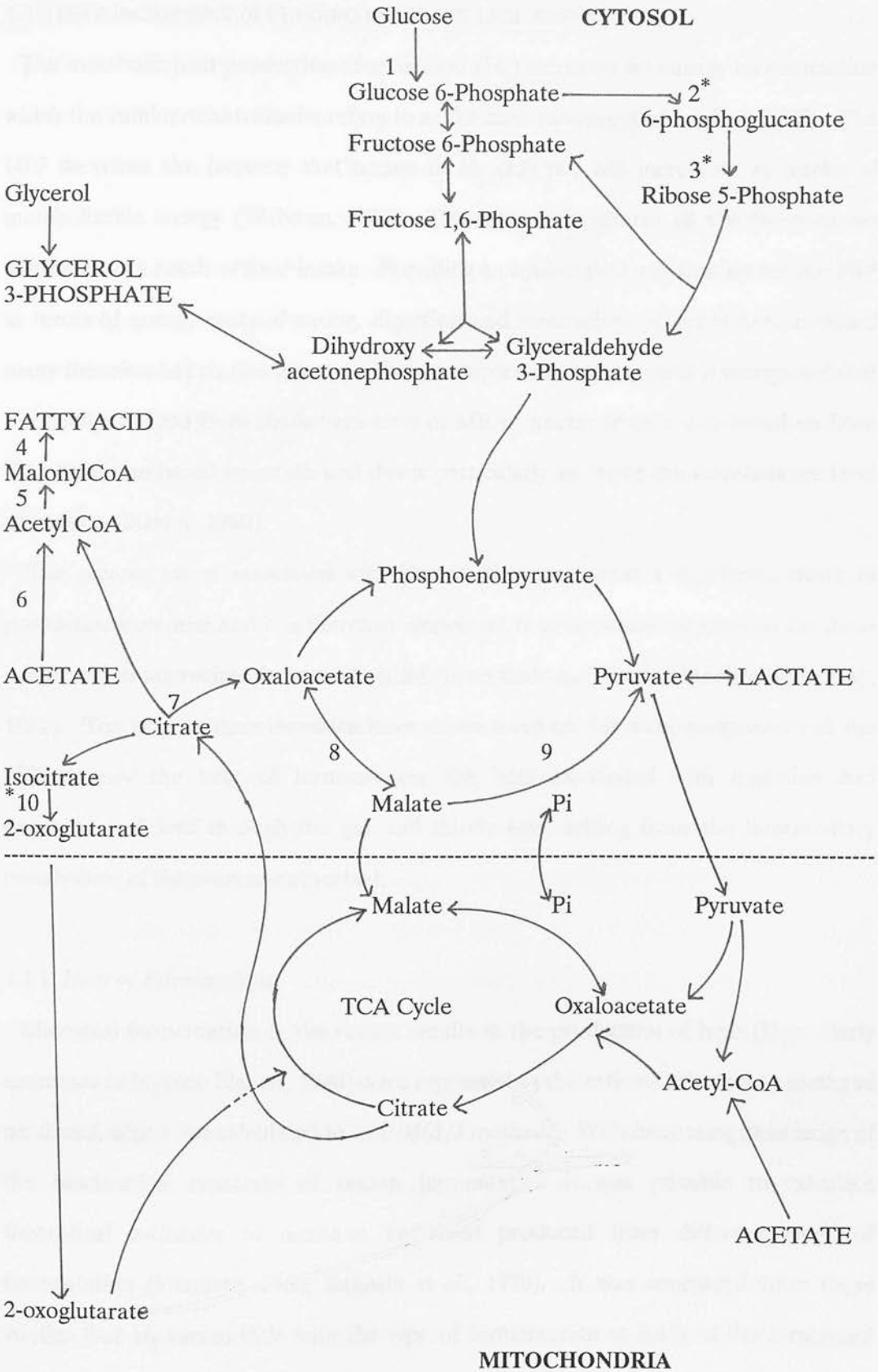
The synthesis of fatty acids requires considerable quantities of cytoplasmic NADPH. Theoretically fourteen molecules of NADPH are required for the synthesis of one molecule of palmitic acid from eight molecules of acetyl-CoA. In non-ruminants the primary source of NADPH is via the oxidation of glucose by the pentose cycle and the coupled operation of NAD-malate dehydrogenase and NADP-malate dehydrogenase (Fig.1.2). In ruminant tissues oxidation of glucose via the pentose cycle with the concomitant production of NADPH by the action of glucose 6-phosphate and 6-phosphogluconate dehydrogenases produces 50-80% of the NADPH required for fatty acid synthesis (Vernon, 1981). As the activity of the malate dehydrogenase cycle in ruminants may be limited, another NADP-linked dehydrogenase, isocitrate dehydrogenase, has been linked to NADPH production because of its high activities in adipose tissue. This stems from studies on fatty acid synthesis in the ruminant mammary gland in which it is thought this enzyme may provide up to 50% of the NADPH for fatty acid synthesis (Bauman & Davies, 1975).

The principal fatty acids synthesised from acetate by ruminant adipose tissue are palmitic, stearic and oleic (Vernon, 1981). Most of these fatty acids synthesised *de novo* are esterified to triacylglycerols.

The rates of fatty acid synthesis are influenced by many factors, including sex, breed and age of animal, site of synthesis within the animal (perirenal, subcutaneous etc), quantity and quality of the diet and the physiological state of the animal. These aspects are reviewed by Vernon (1981).

Fig. 1.2 Pathways for the synthesis of fatty acids and glycerol 3-phosphate in adipose tissue. Arrows indicate direction of flux. * - indicates major enzymes involved in NADPH production.

1. Hexokinase
2. Glucose 6-phosphate dehydrogenase
3. 6-phosphogluconate dehydrogenase
4. Fatty acid synthetase
5. Acetyl-CoA carboxylase
6. Acetyl-CoA synthetase (cytosolic)
7. ATP-citrate lyase
8. NAD-malate dehydrogenase
9. NADP-malate dehydrogenase
10. Isocitrate dehydrogenase


Glucose
CYTOSOL
1
Glucose 6-Phosphate
2*
6-phosphoglucanote
Fructose 6-Phosphate
3*
Ribose 5-Phosphate
Glycerol
Fructose 1,6-Phosphate
GLYCEROL
3-PHOSPHATE
Dihydroxy
acetonephosphate
Glyceraldehyde
3-Phosphate
FATTY ACID
4
MalonylCoA
5
Acetyl CoA
Phosphoenolpyruvate
6
ACETATE
Oxaloacetate
Pyruvate
LACTATE
7
Citrate
8
9
Isocitrate
*10
Malate
Pi
2-oxoglutarate
Malate
Pi
Pyruvate
TCA Cycle
Oxaloacetate
Acetyl-CoA
Citrate
ACETATE
2-oxoglutarate
MITOCHONDRIA

1.3 Heat Increment of Feeding and Fibre Utilisation

The metabolic heat production of an animal (H_t) increases on eating, an association which the ruminant nutritionist refers to as the heat increment of feeding (HIF). The HIF describes the increase that occurs in H_t (kJ) per MJ increment in intake of metabolisable energy (Webster, 1980). The term incorporates all the thermogenic responses as a result of food intake. Providing a physiological explanation for the HIF in terms of energy costs of eating, digestion and metabolism of feeds has produced many theories, but no one theory has been accepted. In ruminants it is recognised that the heat produced from similar amounts of ME is greater from a diet based on fibre than from one based on starch and this is particularly so above the maintenance level of feeding (Blaxter, 1980).

The greater losses associated with fibrous diets represents a significant waste of production potential and it is therefore important to understand the reasons for these losses and to appreciate factors which influence their magnitude (MacRae & Lobley, 1982). The various theories which have arisen focus on the main components of the HIF namely the heat of fermentation, the heat associated with ingestion and propulsion of feed through the gut and thirdly heat arising from the intermediary metabolism of the nutrients absorbed.

1.3.1 *Heat of Fermentation*

Microbial fermentation in the rumen results in the production of heat (H_f). Early estimates of H_f (see Blaxter, 1980) were expressed as the ratio of heat lost to methane produced, which was calculated to be 0.046J/J methane. With increasing knowledge of the biochemical reactions of rumen fermentation it was possible to calculate theoretical estimates of methane and heat produced from different types of fermentation (Hungate, 1966; Baldwin *et al.*, 1970). It was concluded from these studies that H_f varied little with the type of fermentation at 6.4% of the fermented energy. However methane production varied; fermentations giving rise to large proportions of propionate (starch based diets) were associated with lower methane

production compared to those producing lesser amounts of propionate (fibrous diets).

Webster *et al.* (1975) determined indirectly estimates of H_f *in vivo* by estimating the anaerobic heat production of the tissues drained by the portal vein using the technique of continuous thermodilution. Expressing results in terms of apparently digested energy (DE), H_f was determined as 0.06-0.076 J fermentation heat/ J DE in the case of fibrous diets and 0.022J/J for a pelleted barley diet. These results, the theoretical work of Hungate and Baldwin and a series of *in vitro* experiments (see Webster, 1980) suggest that the H_f is rather constant at about 6-7% of the digested energy. Blaxter (1980) concluded that this showed that H_f made up a significant proportion of the HIF below maintenance but not above, where large differences in HIF between fibrous and starch based diets are observed.

1.3.2 *The Work of Digestion*

Kellner (1926) observed that in steers HIF was proportional to the fibre content of the diet and he attributed these differences to variation in the energy expended in the mechanical and chemical processes involved in the preparation of ingesta for absorption. This "work of digestion" ("Verdaurungsarbeit") was greater for fibre diets, having a higher content of structural carbohydrate or crude fibre compared with starch diets. On logical grounds it is likely that the energy costs of prehending, masticating, ruminating, and propulsion through the gut would be greater for a fibre diet of low digestibility than for a starch diet. However, as Blaxter (1980) has discussed, the work of Webster and his colleagues (Osuji *et al.*, 1975; Webster *et al.*, 1975) suggest that the increments of heat associated with the work of digestion are only minor proportions of the total increment of heat either below or above maintenance.

1.3.3 *Heat of Metabolism*

In 1944 Barcroft, McAnally and Phillipson proved that the main source of energy for the ruminant consisted of the volatile fatty acids (namely acetate, propionate and butyrate) primarily derived during anaerobic fermentation in the rumen. It was soon

realised the VFA were major sources of energy for many tissues and various approaches were used to help elucidate the metabolic fate of the lower fatty acids. For example it was soon shown that acetate was the primary source for fatty acid synthesis and that propionate was a major precursor of glucose in the ruminant. Radioactive isotopes helped greatly in this work but at this stage little could be concluded about the energy expended in the process of product formation by following the fate of a carbon atom from a particular substrate into a product.

During the 1950's measurement of the efficiency of utilisation of the end products of ruminant digestion was a problem and much unequivocal evidence existed which suggested that the end products of digestion were responsible for a large proportion of the HIF and in particular it may be related to the proportion of acetic acid produced in the rumen (McClymont, 1952; see Blaxter, 1962). As a result Armstrong & Blaxter working at the Hannah Research Institute applied calorimetric techniques to intact animals given the end products as sole food or as a supplement to a basal diet, either singly or in mixtures. This approach allowed estimation of the extent to which the energy of the compounds absorbed could be used for replacing endogenous sources of energy in meeting maintenance and muscular energy requirements and in enabling productive processes (Blaxter, 1962).

1.3.4 *Utilisation of Energy for Maintenance*

Armstrong & Blaxter (1957a) and Armstrong *et al.* (1957) determined the efficiencies of utilisation for maintenance of some of the end products of ruminant digestion. When food is given to a fasted animal in amounts which do not result in a deposition of fat in the body, the energy provided spares the oxidation of endogenous sources of energy. This concept was used to compare foods or nutrients in their ability to replace endogenous sources of energy, and hence provide a measure of the ability of a nutrient to maintain an animal.

The technique involved fasting an animal until a state of steady metabolism was reached. The nutrient to be tested was infused into the gut via a permanent fistula and

the infusion continued until steady state was attained. The animal was again fasted. The experiment lasted about two weeks and throughout the experiment urine and faeces were collected daily. This permitted the determination of the amount of body fat and protein lost whilst fasting and during the infusion of the nutrient. The efficiency of nutrient utilisation for maintenance was therefore determined as:

$$\text{efficiency for maintenance} = 100 \times \frac{\text{heat of combustion of fat and protein lost from the body during fasting} - \text{heat of combustion of fat and protein lost from the body when nutrient is given}}{\text{heat of combustion of nutrient given}}$$

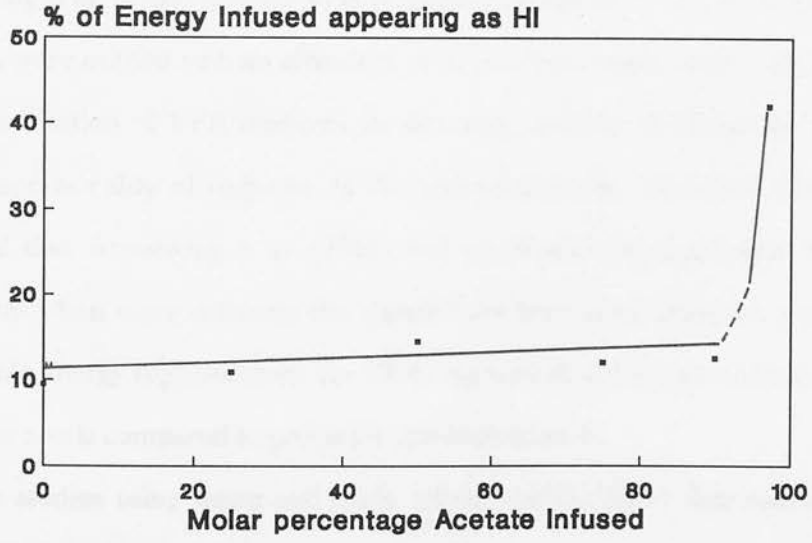
It was found that energy derived from the VFA's either given singly or in mixtures was used less efficiently for maintenance than glucose. Acetic acid, infused alone, was poorly utilised (calorimetric efficiency = 0.59) and resulted in a major metabolic disturbance. Acetic acid accumulated in the peripheral blood, resulting in severe acidosis; blood sugar fell; a slight ketosis occurred and a marked increase in protein breakdown took place. The depletion of sugar reserves and the increased protein breakdown (stimulation of gluconeogenesis) confirmed other results which suggested that a carbohydrate source was necessary for the oxidation of acetic acid. The efficiencies of utilisation of propionic and butyric acids were 0.87 and 0.76 respectively compared to body fat.

Propionic acid and butyric acid when infused together were used very efficiently, which Armstrong & Blaxter (1957a) described as a synergism between the propionic and butyric acids. However no such synergism was found between acetate and butyrate. Acetate was used efficiently in the presence of propionate which agrees with the studies of Davies *et al.* (1960).

When the proportions of acetate, propionate and butyrate were infused in proportions reflecting those produced in the rumen under a wide variety of dietary circumstances, the efficiency of energy utilisation for maintenance varied very little

(Fig. 1.3). It was concluded that provided a small amount of propionate was present in the mixture, the major VFA were utilised with an equal efficiency for maintenance and over the range 0-90% molar proportion of acetate the efficiency varied so little that it could be neglected. This has been confirmed by other studies (see Blaxter, 1962; Ørskov *et al.*, 1979). However Blaxter & Boyne (1978) in a comprehensive analysis of eighty calorimetric experiments demonstrated that the efficiency of utilisation of gross energy for maintenance was related to the metabolisability (q) of the diet.

Fig. 1.3 *Effect of varying the molar percentage of acetic acid in a mixture on its heat increment, as determined in fasting sheep (Blaxter, 1962).*



1.3.5 *Utilisation of Energy for Production*

Armstrong & Blaxter (1957b) studied the efficiency with which some of the end products of digestion were used for lipogenesis by infusing animals which were precisely maintained on a basal ration with nutrients, either singly or together and determined the heat increment by indirect calorimetry. It was assumed that the animal was maintained as a result of the basal diet and any increase in energy retention on supplementation was attributed to the nutrient or nutrients.

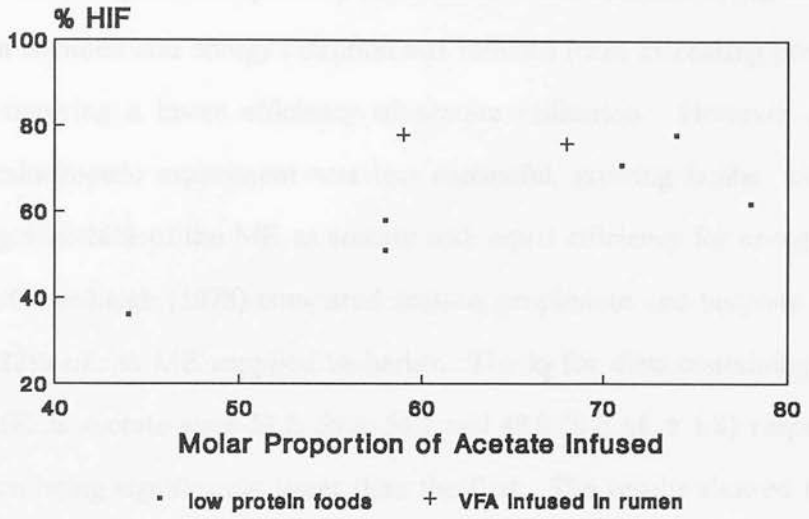
The heat increments above maintenance were considerably greater than those obtained below maintenance. Infusing acetic, propionic and butyric acid as an increment adding approximately 15% ME to a basal ration of dried grass, the observed heat increments for acetate, propionate and butyrate were 0.671, 0.437 and 0.381, respectively. Hence acetate was used with an efficiency considerably less than that of propionic or butyrate (Armstrong & Blaxter, 1957b).

Armstrong *et al.* (1958) infused VFA mixtures containing 59 or 14% energy as acetic acid, they were utilised with an efficiency of 32 and 58% respectively. They concluded that the utilisation of VFA mixtures for fattening could be predicted on the basis of direct proportionality of response to the individual acids. However Ørskov (1965) illustrated that Armstrong *et al.* (1958) had predicted heat increments from molar proportions when more correctly this should have been done from the proportions of combustible energy supplied from the VFA. Nevertheless the heat increment was still greater for acetic compared to propionic and butyric acids.

Further studies using sheep and cattle (Blaxter *et al.*, 1966) demonstrated a close relationship between the amount of acetate produced and the HIF of the diet (Fig. 1.4).

From this work a theory explaining the increased heat increment associated with fibrous diets developed. Marked differences exist in the molar proportions of acetate, propionate and butyrate produced from diets fermented in the rumen and the ratio, acetate:propionate increases with increasing fibre content in the diet. The heat increment is positively correlated with the molar

Fig. 1.4 *Relationship between HIF and the molar percentage of acetate in rumen liquor (see MacRae &* Lobley, *1982)*



percentage of acetate produced in the rumen, and the latter is influenced by the fibre content of the diet. A synergism existed between 2 and 3 carbon acids, the provision of 3 carbon acids (propionate) facilitated the utilisation of 2 carbon acids (acetate), which is simply explained by the fact that the oxidation of acetyl-CoA in the TCA cycle requires the continuous regeneration of oxaloacetate, which may be generated from propionate.

Experiments using growing ruminants testing the theory have tended to give contradictory evidence. Tyrell *et al.* (1979) have found inefficient use of acetate (<35%) infused into steers fed on an all-hay diet, but more efficient (70%) in animals given hay plus concentrate. Eskeland *et al.* (1973) investigating protein deposition in lambs infused VFA directly into the blood and found lower nitrogen retention with acetate than with either propionate or butyrate. In contrast no difference could be found between the three acids when infused into the rumen (Rook *et al.*, 1963) or as sodium or calcium salts mixed into the diet (Ørskov & Allen, 1966a,b,c; Ørskov *et al.*,

1966). In a comparative slaughter experiment, Bull *et al.* (1970) supplemented basal diets with triacetin and were unable to find differences in utilisation. Hovell *et al.* (1976) determined the utilisation of acetate salts by comparative slaughter replacing 14-19% of the ME provided by barley with salts of acetic acid and it was found that fat deposition declined and energy retention was reduced (with increasing proportions of acetate), implying a lower efficiency of acetate utilisation. However a follow-up indirect calorimetric experiment was less successful, growing lambs utilised diets containing 4 or 16% of the ME as acetate with equal efficiency for energy retention. Hovell & Greenhalgh (1978) compared acetate, propionate and butyrate which each replaced 22% of the ME supplied by barley. The k_f for diets containing 0, 7, 15 or 22% of ME as acetate were 57.2, 59.6, 54.1 and 48.8 (S.E.M. ± 1.8) respectively, the latter value being significantly lower than the first. The results showed that acetate was utilised with different efficiencies depending on the level of incorporation. It was concluded that the efficiency of acetate utilisation for energy retention is not a constant, but varies with its contribution to ME.

Blaxter (1980) in a review of the literature until the late seventies concluded that *"the major reason for the difference between heat increments still resides in differences in the mixture of end products of digestion formed and particularly in the ratio of acetate and butyrate to propionate".*

Supplementation of a basal diet with either VFA's or salts of VFA's has many disadvantages (Hovell *et al.* (1976), Ørskov *et al.* (1979), Ørskov & MacLeod (1990). Problems of inappetence and digestion were frequently observed when VFA were infused above 0.15 of ME. Addition of VFA or their salts to a basal diet inevitably interferes with the metabolism of the basal diet to an extent which is very difficult to assess. As an extension of the intragastric infusion techniques used by Armstrong & Blaxter, Ørskov and colleagues (Ørskov *et al.*, 1978) devised techniques which were more advanced. Lambs could be sustained entirely by intragastric infusion of VFA, protein, minerals and vitamins for long periods of time. Hence they were able to study the utilisation of VFA without the problems of interference with digestion and

metabolism of the basal diet (Ørskov *et al.*, 1979).

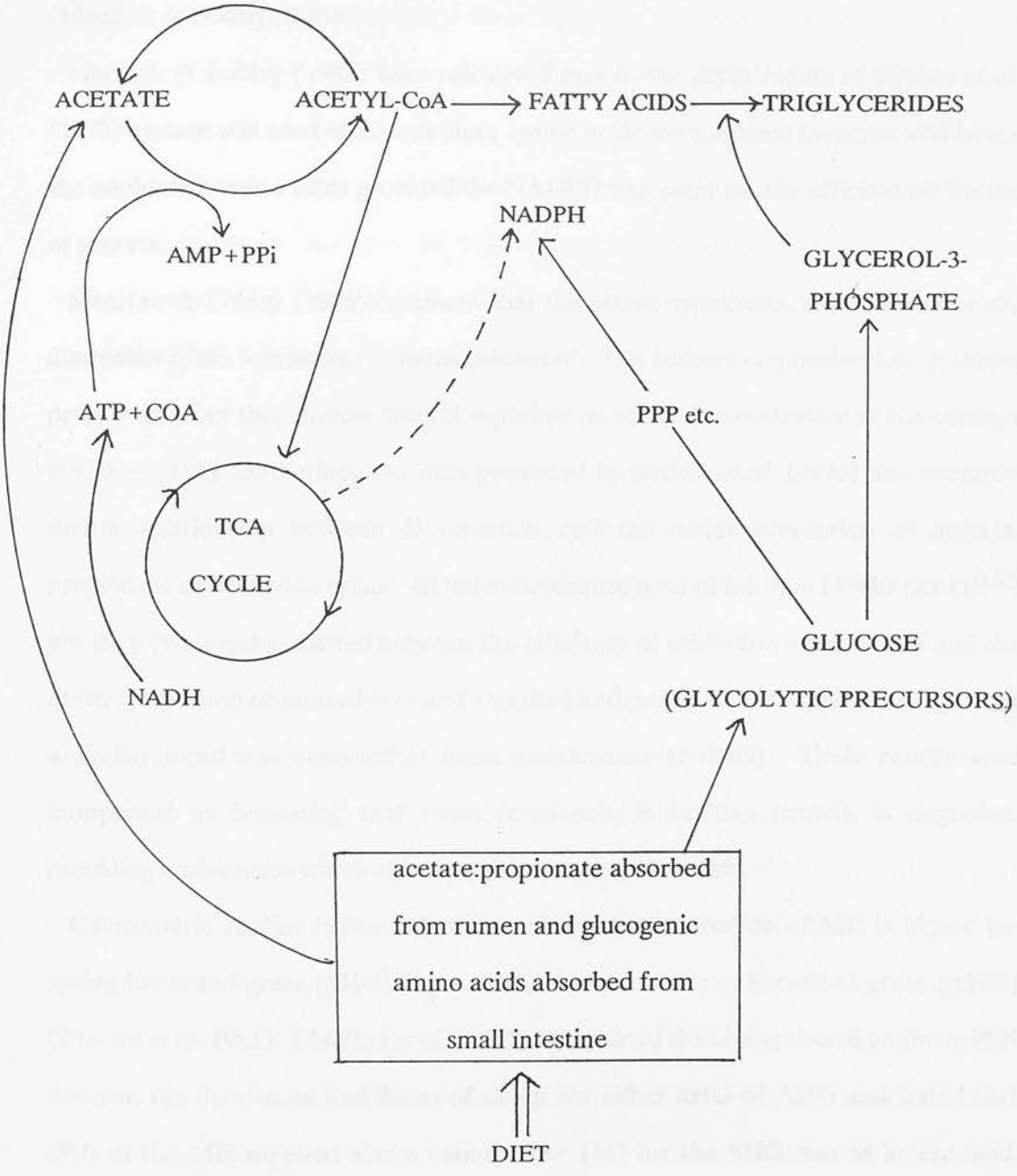
These animals were used in calorimetric studies to measure the heat increments when different proportions of the energy intake were derived from acetate. The heat increment from a mixture containing 35% molar proportion of acetate was significantly lower, but over the range 45-85% molar proportion of acetate the heat increments were not significantly different from each other. It was concluded that the differences in k_f normally given to ruminants cannot be attributed to differences in utilisation of VFA's.

The inability of ruminants to utilise acetate efficiently may result from a restricted rate of lipogenesis from acetate (MacRae & Lobley, 1982). These authors emphasised that the anabolism of acetate to fat has an obligatory requirement for both NADPH and glycerol 3-phosphate. As discussed in section 1.2.7) reduced NADP is derived either from glucose metabolism, via the pentose phosphate pathway or via TCA cycle intermediates (the isocitrate dehydrogenase shuttle). Since ruminants absorb little glucose from the digestive tract, glucose and the glycolytic intermediates must be synthesised. The primary precursors are propionate and glucogenic amino acids. If the latter are limiting then in turn NADPH supply will be inadequate. Consequently conversion of acetate to fat is reduced.

MacRae & Lobley (1982) suggested that in those experiments where evidence for the efficient utilisation was obtained, there were ready supplies of either propionate and/or protein to provide, indirectly, sufficient NADPH for fatty acid synthesis from acetate. Conversely when NADPH is inadequate (as might be the case for fibre diets), acetate is oxidised in the TCA cycle providing a limited amount of NADPH (permitting limited fat synthesis) and the excess ATP is lost as heat in some form of futile cycle, lowering the overall efficiency of energy utilisation.

This theory is diagrammatically illustrated in Fig.1.5. It may be that the substrate cycle between acetate and acetyl-CoA, which has been shown to operate in rodent

Fig. 1.5 *Effect of Diet on Acetate Utilisation*



tissues, would burn up the excess ATP, lowering the efficiency of acetate utilisation (MacRae & Lobely, 1986).

MacRae & Lobley (1982) have calculated that in the experiments of Ørskov *et al.* (1979) acetate was used efficiently since amino acids were infused in excess and hence the additional amino acids provided the NADPH necessary for the efficient utilisation of acetate.

MacRae & Lobley (1986) comment that the above hypothesis "*presents a dramatic illustration of the interactions between substrates*". The authors emphasise that no direct proof exists, but they discuss several experiments which demonstrate that the concept is viable. They reexamined the data presented by Ørskov *et al.* (1979) and conclude that a relationship between N retention and the molar proportion of acetate, propionate and butyrate exists. At the maintenance level of infusion (450kJ per $kg^{0.75}$ per d), a relationship existed between the efficiency of utilisation of infused N and the molar proportion of infused acetate (negative) and propionate (positive) ($r=0.97$) and a similar trend was observed at twice maintenance ($r=0.65$). These results were interpreted as indicating that when propionate is limiting protein is degraded, providing amino acids which enhance the utilisation of acetate.

Calorimetric studies indicate that the efficiency of utilisation of ME is higher for spring harvested grass (SHG) (k_f = 0.58) than for autumn harvested grass (AHG) (Ribeiro *et al.* 1981). MacRae *et al.* (1985) determined the absorption of amino acid N between the duodenum and ileum of sheep fed either SHG of AHG and found that 29% of the ME supplied above maintenance (M) for the SHG was as amino acid, compared with 13% for the AHG. In a follow up calorimetric study sheep were fed AHG, at 2 levels of intake M & 1.5M, and received abomasal infusions of either water or casein (30 g per d). The infusion of casein increased the efficiency of utilisation of the additional ME supplied from AHG ($P < 0.025$). As the ME intake of AHG was increased from M to 1.5M, the heat produced was 18% lower for the casein infused animals compared to those infused with water. This suggested that the provision of extra amino acids may be associated with increased efficiency of utilisation of ME.

Ortigues *et al.* (1989) reported the effects of fishmeal on growth and calorimetric efficiency in heifers fed straw based diets. Fishmeal supplementation increased total amino acid flow to the small intestine and was associated with increased growth rates. The energetic efficiency of growth increased up to 14.8% crude protein (CP), but higher levels (18.4% CP) resulted in a reduction in energetic efficiency. Amino acid supply to the small intestine was increased by the fishmeal and the results were interpreted in terms of a reduction of the wasteful oxidation of acetate and enhanced protein deposition, whilst at the higher level of CP it was suggested that protein deposition had reached a maximum and/or acetate had become limiting.

It is evident that much support may be found in the literature which would suggest that the additional heat increment associated with fibrous diets may be related to the VFA's produced in the rumen and the availability of glucose precursors. However Ørskov & MacLeod (1990) in a review of dietary induced thermogenesis present results which oppose the theory. Using the technique of intragastric nutrition and measuring heat production using indirect open circuit calorimetry, cattle were infused with a VFA mixture in which the proportion of acetic and propionic acids varied from 32-92 and 0-60 molar percent respectively, and the proportion of butyric acid was held constant at 8%. Energy was infused at an estimated 1.5 x M (675 kJ/kg $wt^{0.75}$). Although no indication of variation was given, the data suggested that heat production did not increase with increasing molar percent of acetate in the infusate. The authors reported that the N excretion in the urine increased at 75-80 molar % acetate in the infusate, suggesting an increase in the oxidation of amino acids, and this is followed by an elevation in the plasma levels of 3-hydroxybutyrate (indicating glucose deficiency). Finally at 85% acetate a metabolic crisis occurred resulting in a reduction in heat production and the excretion of acetic acid in the urine.

However it is clear from the graphs that an elevation in urinary nitrogen excretion occurred from approximately 45% molar acetate infused and a similar increase may be observed in plasma 3-hydroxybutyrate. Failure to comment on these points, the manner in which the results have been presented and whether or not casein was

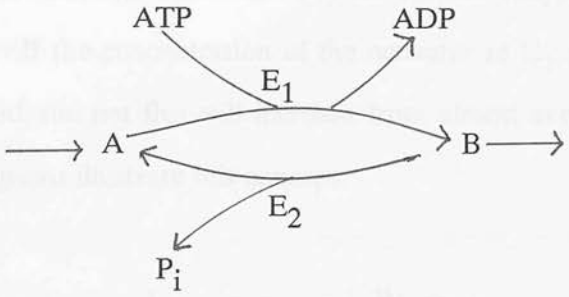
infused into the abomasum are serious omissions by the authors. The increase in urinary N excretion would support the view that amino acids may facilitate the utilisation of acetate.

As Ørskov *et al.* (1979), using animals maintained entirely by infusion, found no differences in the efficiencies of utilisation VFA, they suggest that an explanation for the differences in the efficiency of utilisation of starch and fibrous diets must lie in the other components of the heat increment. The authors suggested that differences in k_m and k_f are dubious. Determination of the efficiency of use of nutrients below maintenance is dependent upon a measure for fasting metabolism. The latter is related to the level of feeding received before fasting and on organ size (Armstrong & Blaxter, 1984) and it is also associated with large increases in nitrogen excretion, indicating a deficiency of glucose precursors. Ørskov & MacLeod (1990) discuss recent evidence which suggests that the demand for glucose precursors may be met by the infusion of small amounts of glucose or VFA's (KuVera *et al.*, 1987). It is proposed that the concept of differences in efficiency of utilisation of ME below and above maintenance ought to be reconsidered and that one should use one third of the estimated energy for maintenance as a starting point, so that the demand for glucose precursors is likely to be met. Since the calculation of k_f is dependent on a value for maintenance Ørskov & MacLeod (1990) suggest that unless maintenance is determined in the experiment, "*it becomes possible to be some what subjective as to the desired results*". These authors conclude that differences in efficiency of ME use may be explained in terms of the energy costs of eating, rumination and digestion and that the studies conducted over the last 70 years have merely confirmed the deduction of Kellner (1926).

It is clear from this review that the precise reasons for the large heat increments associated with fibre diets is uncertain. The literature contains research which supports the various theories but as yet no one theory has been entirely accepted.

1.4 Substrate Cycles and Intermediary Metabolism

At certain key points in intermediary metabolism non-equilibrium reactions exist. Where such a reaction is paired with another non-equilibrium reaction, providing a reverse flux, cycling between intermediates exists. This is referred to as a substrate cycle (Newsholme & Crabtree, 1976). Consider the substrate cycle between A and B, catalysed by the enzymes E_1 and E_2, respectively.



If both enzymes are catalytically active, A will be converted to B (forward reaction) which can be converted back into A (reverse reaction). Hence a cyclic flux exists and since the reactions are opposing, an input of free energy is required, which is usually provided by the hydrolysis of ATP. The cycle need not be restricted to a set of simple opposing reactions in a metabolic pathway as above, but may involve several reactions, and may even extend over two or more tissues (Newsholme & Crabtree, 1976).

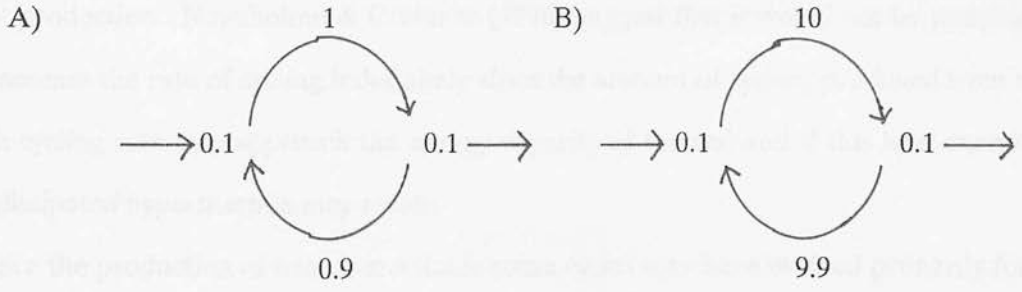
The first evidence for a substrate cycle was found by Steinberg (1963) for the triacylglycerol-fatty acid cycle of adipose tissue. Other examples include glucose/glucose-6-phosphate, fructose 6-phosphate/fructose bisphosphate, glycogen/glucose 6-phosphate, glutamate/glutamine (see Newsholme *et al.* 1984). It is also possible to view protein turnover as a substrate cycle (Reeds *et al.* 1985).

These cycles are proposed as having two roles (Newsholme & Crabtree, 1976) which are intimately linked:

1) metabolic regulation and 2) heat production.

Originally it was thought that cycling arose simply because metabolism could not entirely inhibit one enzyme while the other was active, and as a consequence ATP was

hydrolysed wastefully. Newsholme & Crabtree (1976) proposed that substrate cycles improve metabolic control by increasing the sensitivity of a pathway to changes in the concentrations of effectors (i.e. hormones, substrates, ATP, cyclic AMP). The higher the cycling rate relative to the flux through the pathway, the greater a proportional change in the forward or reverse rates will alter the flux. Consider the reversible scheme A-B above. As the product of the forward enzyme (E_1) is produced, most of it could be converted back into substrate A by the reverse enzyme E_2. Hence the net flux (A-B) is low. If the concentration of the activator of E_1 is increased, whilst E_2 remains unchanged, the net flux will increase from almost zero to a moderate rate. The following diagrams illustrate this concept,



In A, a 10% increase in the rate of the forward reaction increases the flux by a factor of 2. In B, the cycling rate is eleven fold greater, a 10% increase in the forward reaction results in an eleven fold increase in flux.

In general,

$$\frac{\text{Fractional increase in flux}}{\text{Fractional increase in rate of forward reaction}} = 1 + \frac{\text{Cycling rate}}{\text{Flux}} = \text{SENSITIVITY}$$

Hence in A the sensitivity is $1 + 0.9/0.1 = 10$, and B is $1 + 9.9/0.1 = 100$

It is clear that substrate cycles provide a unique mechanism for controlling sensitivity at non-equilibrium reactions in intermediary metabolism. As mentioned many

substrate cycles have been demonstrated but obviously to fulfil their role in metabolic regulation cycling is expected to vary from one condition to another, since the requirement for sensitivity will vary from one condition to another. Newsholme *et al.* (1984) discuss several experiments which demonstrate such variations for a number of cycles. For example it has been shown that the rate of cycling between fructose 6-phosphate and fructose 1,6-diphosphate, in the isolated epitrochlearis muscle of the rat, may be increased up to ten fold by the hormone adrenaline or other β-adrenergic agents.

Increasing cycling results in additional energy expenditure and consequently heat production. Gains in sensitivity are proportional to the rate of cycling such that the higher the rate of cycling the greater the response to regulators but the greater the heat production. Newsholme & Crabtree (1976) suggest that it would not be possible to increase the rate of cycling indefinitely since the amount of cycling produced from a high cycling rate may approach the energy capacity of the cell and if this heat cannot be dissipated hyperthermia may result.

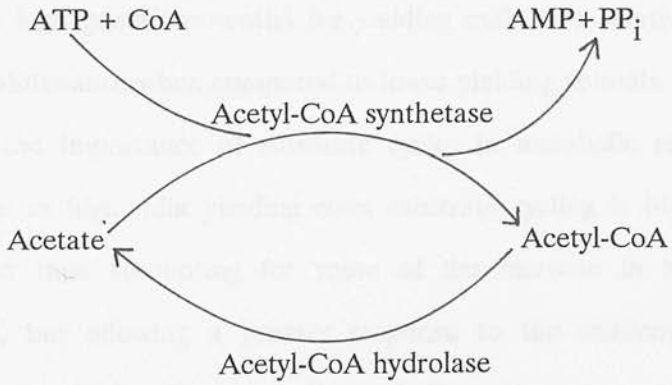
Since the production of heat is inevitable some cycles may have evolved primarily for heat production, for example the uncoupling of oxidative phosphorylation occurring in the mitochondria of brown adipose tissue is related to increasing rates of fatty acid oxidation and TCA cycle activity (see Newsholme & Crabtree, 1976).

A substrate cycle between acetate and acetyl-CoA has recently been identified in rat hepatocytes (Jessop *et al.*, 1986). Due to the importance of acetate to the energy economy of ruminant animals this cycle is of greater potential importance in these animals compared to non-ruminants. However it has been stated that substrate cycles make little contribution to the energy expenditure of the ruminant (Baldwin *et al.*, 1980), but in the light of recent results this may not be the case. Jessop *et al.*, (1986) report high rates of hepatic acetate-acetyl-CoA cycling in rats and the activity of five substrate cycles (including that between acetate-acetyl-CoA) accounted for up to 26% of ATP expenditure by isolated hepatocytes of rats (Rabkin & Blum, 1985). Substrate

cycling, including the steps of glycolysis, triglyceride turnover and the Cori cycle was considered to account for approximately 7.5% of ATP turnover in the young pig (Reeds *et al.*, 1985).

The rate of substrate cycling between acetate and acetyl-CoA (Fig. 1.6) in rat hepatocytes was correlated with increasing concentrations of acetate. The cycle results in the hydrolysis of two molecules of ATP and it has been speculated that its physiological role may be to dissipate excess fuel, hence producing heat and/or restrict weight gain (Jessop *et al.*, 1986). If this substrate cycle responded similarly in ruminants it may help to explain why the efficiency of utilisation of ME

Fig. 1.6 *The substrate cycle between acetate and acetyl-CoA.*



is lowered by feeding diets that give rise to large quantities of acetate (see section 1.3.5). MacRae & Lobley (1986) suggested that this phenomenon may result from a restricted rate of fatty acid synthesis from acetate and hence increased acetate oxidation, perhaps in a "futile cycle". This role could be fulfilled by the substrate cycle operating between acetate and acetyl-CoA.

Crabtree *et al.* (1987) investigated the effects of infusing acetate upon the rate of acetate cycling across the hindlimb of sheep. They concluded that this cycle operating

in hindlimb would account for only 0.5% of the total heat produced by the animal and suggested that it would make very little contribution to the HIF associated with feeding diets producing large amounts of acetate. The authors appreciated that dietary manipulations could change the rate of cycling indirectly (i.e. by hormonal action), but it must also be noted that muscle may not be the primary site for increased acetate cycling, associated with feeding fibrous diets.

In section 1.1.2 it was emphasised that there is a real need to obtain quantitative measurement of ancillary energy expenditures and factors influencing their activity. Gill *et al.* (1984) found that one problem associated with their model attempting to simulate the growth of sheep, was a build up of ATP. This suggests that the model fails to take into account certain ATP utilising processes. The authors have implicated variations in the energy costs of substrate cycling or other "maintenance processes" as possible reasons for current inconsistencies. Taylor *et al.* (1986) have suggested that cattle with a high genetic potential for yielding milk have relatively high metabolic needs for maintenance when compared to lower yielding animals. The above section emphasised the importance of substrate cycles in metabolic regulation and it is probable that in high milk yielding cows substrate cycling is higher than in lower yielding cows thus accounting for some of the increase in heat production at maintenance, but allowing a greater response to the challenge of lactation by increasing the sensitivity of key steps in metabolic pathways to regulators.

The literature is lacking in information relating to substrate cycling in ruminant tissues and the substrate cycle between acetate and acetyl-CoA would appear to be of most interest.

CHAPTER TWO

STUDIES on the ACTIVITIES of ACETYL-CoA HYDROLASE and ACETYL-CoA SYNTHETASE in RUMINANT TISSUES.

Abstract

1. The activities of ATP-stimulated cytosolic acetyl-CoA hydrolase and "mitochondrial" acetyl-CoA hydrolase were investigated in ovine muscle (semi-membranosus), rumen epithelium (dorsal), perirenal adipose tissue and liver.

2. Scottish Blackface lambs (approx. 12 weeks old) were fed on either plain sugarbeet pulp (U), molassed sugarbeet pulp (M) or barley (B), at one of 3 levels of intake, low (L), medium (M) or high (H), calculated to achieve growth rates of 30, 100 or 200g per d respectively. All diets were of equivalent ME concentration. At slaughter (after 18 weeks), samples of liver and perirenal adipose tissue were removed for the determination of acetyl-CoA hydrolase and acetyl-CoA synthetase activities.

3. ATP-stimulated cytosolic acetyl-CoA hydrolase was detected in muscle, rumen epithelium and liver but not in perirenal adipose tissue. This enzyme was unaffected by cold ($4^{o}C$).

4. Diet but not feeding level significantly influenced the activities of ATP-stimulated ($0.06<P<0.07$) and "mitochondrial" ($P<0.05$) acetyl-CoA hydrolase. In general total hydrolase activity was higher on barley diets with no significant difference between unmolassed and molassed sugarbeet pulp. Diet but not feeding level significantly influenced acetyl-CoA synthetase ($P<0.01$). The main effect was sugarbeet/barley, sugarbeet having higher activities.

5. Feeding level influenced both acetyl-CoA hydrolase ($P<0.05$) and synthetase ($P<0.001$) in perirenal adipose tissue. Both enzymes increased with feeding level in the order H, M, L.

6. It was estimated that the substrate cycle between acetate and acetyl-CoA in ovine liver may account for 2.5% of basal heat production.

Key Words: Acetyl-CoA hydrolase Acetyl-CoA synthetase Substrate Cycle Energy.

Publications: Jessop *et al.* (1990): Scollan *et al.* (1988)

2.1 INTRODUCTION

A large proportion of the energy requirement of a ruminant is derived from the oxidation of acetate, produced by microbial fermentation primarily in the rumen. Acetyl-CoA synthetase (EC 6.2.1.1) is the enzyme responsible for the activation of acetate by tissues.

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Acetyl-CoA} + \text{AMP} + \text{PP}_i$$

Many tissues also contain acetyl-CoA hydrolase (EC 3.1.2.1), which catalyses the hydrolysis of acetyl-CoA to acetate.

$$\text{Acetyl-CoA} \rightleftharpoons \text{Acetate} + \text{CoA}$$

This enzyme is predominantly mitochondrial (Knowles *et al.*, 1974). However the liver of several mammals (notably rodents) contains an additional, cytoplasmic activity which differs from the mitochondrial enzyme in being activated by ATP, inhibited by ADP, and, in some species, cold labile (Prass *et al.*, 1980; Matsunaga *et al.*, 1985).

The role of the ATP-stimulated enzyme is uncertain, although its presence with acetyl-CoA synthetase raises the possibility of it being involved in the operation of a substrate cycle between acetate and acetyl-CoA. Such a cycle has been shown to occur in rat hepatocytes (Rabkin & Blum, 1985; Jessop *et al.*, 1986), and in sheep muscle and liver *in vivo* (Pethick *et al.*, 1981; Crabtree *et al.*, 1987). In ruminants it has been proposed that this substrate cycle may help explain part of the increased heat increment associated with fibrous diets which produce large quantities of acetate, compared to starch based diets (Crabtree *et al.*, 1987).

This study reports the activities of ATP-stimulated acetyl-CoA hydrolase and "mitochondrial" acetyl-CoA hydrolase in various ovine tissues, and further investigates

the influence of temperature on the ATP-stimulated enzyme. As part of an ongoing lamb growth trial in which lambs were given diets likely to result in differing supplies of acetate as a nutrient, the activities of acetyl-CoA hydrolase and synthetase were investigated in liver and adipose tissue samples obtained from these lambs after slaughter. From these enzyme rates the potential for substrate cycling between acetate and acetyl-CoA can be determined.

2.2 MATERIALS and METHODS

ATP, ADP, oxaloacetate, coenzyme A, dithiothreitol (DTT), DTNB, MOPS, EDTA, EGTA, sucrose, acetic anhydride, sodium azide, benzamidine hydrochloride, triton X-100 and acetyl-CoA synthetase (EC 6,2,1,1) were purchased from Sigma Chemical Company, Poole, Dorset; trichloroacetic acid, magnesium chloride and potassium dihydrogen orthophosphate were obtained from BDH, Poole, Dorset; citrate synthase (EC 4,1,3,7) was from Boehringer Corporation, Lewes, E. Sussex.

Acetyl-CoA was prepared from acetic anhydride and CoA by the method of Simon & Shemin (1953).

2.2.1 *Animals*

The animals used in this study were part of a larger growth trial (Emmans *et al.*, 1989). Scottish Blackface wether lambs, approximately 12 weeks old, were fed on diets based on either plain sugarbeet pulp (U), molassed sugarbeet pulp (M) or barley (B) (Table 2.2.1). All diets were of equivalent ME concentration and were given at one of 3 levels of intake (low, medium or high), calculated to achieve growth rates of approximately 30, 100, or 200g per d respectively. Each lamb received its ration once daily at 08:00h and had free access to drinking water. Animals received their treatment for 18 weeks.

Table 2.2.1 Diet Formulation (kg per tonne: DM basis)

Constituent	Barley	Plain SBP	Molassed SBP
Barley	581.5	-	-
Plain SBP	-	587.5	-
Molassed SBP	-	-	593.5
Grassmeal	200	200	200
Soyabean Meal	110	110	110
Fishmeal	70	70	70
Vitamin/Mineral Mix	2.5	2.5	2.5
Salt	15	15	15
Limestone	21	5	-
Dicalcium Phosphate	-	10	9

2.2.2 *Tissue Sampling and Preparation*

Samples of liver, hind limb muscle (semi-membranosus), rumen epithelium (dorsal region), and perirenal adipose tissue were removed within 5min of death. For transport to the laboratory, liver, muscle and rumen epithelium were placed in homogenising medium containing sucrose 0.3M; potassium phosphate, 50mM, pH 7.4; EDTA 5mM; benzamidine-Cl 5mM; sodium azide 0.02% (w/v); ATP 0.2mM ($15^{o}C$) (Söling & Rescher, 1985). Adipose tissue was transported in a self-sealing plastic bag containing homogenising medium ($37^{o}C$).

Blotted samples of liver and muscle were cut into small pieces and homogenised in a glass-teflon Potter homogeniser at room temperature with a two-fold (w/v) volume of homogenising medium. Blotted samples of adipose tissue and rumen epithelium (approximately 2.5g and 2g respectively) were homogenised using an Ultra-Turrax T25 (Janke & Kunkel), in a two-fold (w/v) volume of the above buffer. Rumen epithelium

was prepared first by rinsing several times in homogenising buffer (15°C) to remove traces of rumen contents. Connective tissue associated with the body side of the epithelium was removed using forceps. The epithelium was then rerinsed several times in homogenising buffer and homogenised.

Liver and muscle homogenates were centrifuged at 2,000xg (15°C) for 10min using an MSE High Speed 18 centrifuge. The resulting pellets were discarded and the supernatants were spun for a further 30min at 20,000xg (15°C). The supernatants representing the cytosolic fraction were used to determine enzyme activity. The mitochondrial pellet (of the liver only) was resuspended in a two-fold (w/v) volume of "mitochondrial medium" consisting of sucrose 0.25M; triethanolamine-Cl 3.5mM; EGTA 1mM; pH 7.4 and centrifuged at 20,000xg for 30min (15°C). The supernatant was discarded and the pellet resuspended in a two-fold (w/v) volume of mitochondrial medium to which Triton X-100 (1% final concentration; w/v) was added.

Crude liver preparations were prepared by centrifuging homogenised fractions at 13,000xg for 3min using a Beckman Microfuge. Assays were conducted on the supernatant.

Homogenates of adipose tissue and rumen epithelium were centrifuged at 10,000xg for 3min. Assays were conducted on the supernatant.

2.2.3 Enzyme Assays

a) *Acetyl-CoA Hydrolase*

The method used was that of Söling & Rescher (1985). The liberation of free CoA from acetyl-CoA was assayed spectrophotometrically through the use of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412nm. DTNB is reduced by the sulphydryl groups of free CoA.

100µl of homogenate (50µl liver homogenate) were added to 1.4ml buffer (final concentrations: MOPS 100mM, pH 7.4; DTNB 2mM), containing either ATP (2mM) or ADP (2mM). The reaction was started by the addition of acetyl-CoA (0.5mM, final

concentration) and was measured in a PYE Unicam SP8-100 spectrophotometer at 412nm, 25^{o}C, against a reference cuvette containing no acetyl-CoA.

Cytosolic acetyl-CoA hydrolase is stimulated by ATP and inhibited by ADP, while "mitochondrial" acetyl-CoA hydrolase is largely unaffected by nucleotides. The difference between the ATP and ADP activities were therefore assumed to represent the activity of cytosolic, ATP-stimulated acetyl-CoA hydrolase. The ATP used was of the highest purity available, since any ADP contamination would partially or wholly inhibit the ATP-stimulated enzyme.

b) *Acetyl-CoA Synthetase*

Acetyl-CoA synthetase was assayed as described by Knowles *et al.*, (1974). The assay determines the rate of incorporation of [1-^{14}C] acetate into citrate in the presence of excess ATP, CoA, oxaloacetate and citrate synthase.

150μl of buffer (Tris 66mM; $MgCl_2$ 13.4mM; acetic acid 6.6mM; DTT 2.6mM; ATP 6.6mM; citrate synthase 0.88U; pH 8.0)* were added to 50μl of distilled water in each of three Eppendorff centrifuge tubes and incubated at 37^{o}C. After temperature equilibration, 100μl of homogenate were added to each tube and incubated for 10min. The reaction was stopped by the addition of 50μl of 10% trichloracetic acid. After centrifugation at 10,000xg (Beckman Microfuge) for 3min (to remove precipated proteins), 15μl portions of supernatant were spotted onto glass fibre filter paper strips. The strips were dried in an oven (120-130^{o}C) for 15min. 15μl of M HCl were spotted onto each strip on top of the original spot and the strips were left in the oven for a further 15min. This allowed unreacted acetate to volatilise, so that the remaining specific radioactivity was as citrate (produced in proportion to acetyl-CoA synthetase activity). Blanks were conducted, in triplicate, using distilled water in place of homogenate for each batch of strips in the oven. The radioactivity of the filter paper strips was determined in vials containing 3ml of xylene based scintillation cocktail (Beckman Ready Micro) in a Beckman LS 5000CE liquid scintillation counter. The

* - plus oxaloacetate (34mM), 37kBq [1-14C] sodium acetate

addition of excess oxaloacetate and citrate synthase removes acetyl-CoA as citrate. This prevented product inhibition and minimised acetyl-CoA hydrolase activity. Under these conditions a linear reaction was observed with enzyme up to 15nmole acetyl-CoA synthetase and with time up to 20min.

c) *Lactate Dehydrogenase*

Lactate dehydrogenase activity was assayed by the method of Kornbergh (1955). The assay measures the reduction of NAD^+ during the oxidation of lactate to pyruvate. The reaction mixture contained 200µl NAD^+ (10mM), 150µl lactate (1M), 2.675ml Tris/HCl (100mM) (pH 7.0) and 25µl of liver homogenate (total volume 3ml). The incubation was carried out at 25°C and the increase in optical density at 340nm was measured continuously in a Pye Unicam SP8-100 spectrophotometer. Blank incubations were carried out in the absence of lactate to determine non-specific oxidation of NADH and reduction of NAD^+.

d) *Citrate Synthase*

Citrate synthase was assayed by the method of Ochoa (1955). The assay measures the reduction of DTNB. The medium contained 100mM Tris/HCl (pH 8.0), 0.2mM DTNB, 0.1mM acetyl-CoA and 1mM oxaloacetate. The reaction conducted at 25°C, was initiated by the addition of 10µl of liver homogenate to 1ml of assay medium, and the change in optical density at 412nm was assessed in a Pye Unicam SP8-100 Spectrophotometer. Blank incubations were carried out in the absence of oxaloacetate to determine non-specific reduction of DTNB.

e) *Protein Assays*

The protein content of each tissue homogenate was determined by the method of Bradford (1976), using bovine serum albumin as a standard. A test kit was used, (Bio-Rad Protein Assay Kit II, Bio-Rad Laboratories, Caxton Way, Watford).

2.3 EXPERIMENTS

a) *The Activity of Acetyl-CoA Hydrolase in various ovine tissues.*

The activities of ATP-stimulated acetyl-CoA hydrolase and "mitochondrial" acetyl-CoA hydrolase were investigated in muscle, perirenal adipose tissue, rumen epithelium and liver. Tissue samples were obtained from lambs which were being slaughtered as part of other investigations at The University of Edinburgh.

Samples of liver were fractionated in mitochondrial and extramitochondrial fractions as described in section 2.2.2. The activity of both ATP-stimulated hydrolase and "mitochondrial" hydrolase was investigated in each fraction, with lactate dehydrogenase and citrate synthase being used as marker enzymes to assess the degree of cross contamination.

b) *Effect of Cooling on Acetyl-CoA Hydrolase Activity*

The effect of temperature upon the activity of ATP-stimulated acetyl-CoA hydrolase was assessed by maintaining liver homogenate on ice and withdrawing aliquots at frequent intervals for the determination of hydrolase activity. *

c) *Effect of P^1,P^5 - di(adenosine-5') pentaphosphate (Ap5A) on the rate of "mitochondrial" Acetyl-CoA Hydrolase*

The activity of ATP-stimulated acetyl-CoA hydrolase may be expressed in incubations with ADP as a result of ATP being formed from ADP by adenylate kinase. Ap5A is a potent inhibitor of adenylate kinase (Lienhard & Secemski, 1973), so to check for this Ap5A was added to incubations containing 2mM ADP (see section 2.2.3).

* For comparison the effect of temperature on this enzyme was also investigated in mouse liver.

d) *Effect of Diet and Feeding Level on the Activities of Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase in ovine liver and perirenal adipose tissue.*

Liver - the influence of diet (plain SBP, molassed SBP, barley) and feeding level (low or high) on the activities of acetyl-CoA hydrolase and acetyl-CoA synthetase in crude liver homogenate preparations were studied.

Perirenal Adipose tissue - the influence of diet (plain SBP, molassed SBP, barley) and feeding level (low, medium or high) on the activities of acetyl-CoA hydrolase and acetyl-CoA synthetase were studied.

Statistical Analysis

Data were analysed using two-way analysis of variance to test for significant effects of diet or feeding level. Least significant differences were calculated for pairs of individual treatment means.

2.4 RESULTS

Tables 2.4.1 & 2.4.2 present the activities of ATP-stimulated and "mitochondrial" acetyl-CoA hydrolase in various ovine tissues. The ATP-stimulated hydrolase was present in muscle, rumen epithelium and liver but not in perirenal adipose tissue. "Mitochondrial" acetyl-CoA hydrolase was present in all tissues and its activity was greater than ATP-stimulated acetyl-CoA hydrolase.

In liver the ATP-stimulated hydrolase was located only in the cytosol, but acetyl-CoA hydrolase was located in both cytosolic and mitochondrial compartments, although the activity was approximately 2.2 fold greater in the mitochondrial fraction. Lactate dehydrogenase and citrate synthase activities suggest that contamination of the cytosol by the mitochondrial fraction and vice versa was very low.

The activity of the ATP-stimulated hydrolase from ovine liver was unaffected by cold ($4^{o}C$) for up to 60min. Indeed samples of homogenate could be stored at $-20^{o}C$ for up to 14d without any loss of activity. In comparison approx. 50% of mouse liver ATP-stimulated acetyl-CoA hydrolase was lost in 8min, and no activity was detected after 45min (Table 2.4.4).

Addition of 20nM P^1,P^5-di(adenosine-5') pentaphosphate to incubations containing 2mM ADP did not reduce the rate of hydrolysis of acetyl-CoA.

Table 2.4.1 *The activities of Acetyl-CoA Hydrolase in various ovine tissues. Activities are expressed as nmol of acetyl-CoA hydrolysed per min per mg protein at 25°C and expressed as means ± S.E.M. for the number in parenthesis. N.D. - not detected.*

Tissue	ATP-stimulated Hydrolase	Mitochondrial Hydrolase
Muscle	0.2±0.10 (4)	4.0±2.30 (4)
Perirenal Adipose	N.D.	6.9±1.39 (4)
Rumen Epithelium	0.3±0.07 (6)	1.6±0.33 (6)

Table 2.4.2 *Intracellular distribution of acetyl-CoA hydrolase in liver from Blackface sheep. Activities are expressed as nmol of acetyl-CoA hydrolysed per min per mg protein at 25°C and expressed as means ± S.E.M. for four sheep.* N.D. - not detected.

	Crude Homogenate	Cytosol	Mitochondria
Acetyl-CoA hydrolase			
(+ 2mM ADP)	2.3±0.14	2.6±0.17	5.8±1.60
(+ 2mM ATP)	5.0±1.09	6.2±0.94	5.8±1.60
ATP-stim. hydrolase	2.6±1.10	3.6±0.84	N.D.
Lactate dehydrogenase	50.0±3.40	80.0±8.90	0.9±0.06
Citrate synthase	2.7±0.46	0.1±0.01	41.4±12.29

Table 2.4.3 *Effect of temperature on ATP-stimulated acetyl-CoA hydrolase activity in sheep liver. Activities were determined at 25°C in homogenates that had been maintained at two temperatures and are expressed as a percentage of the initial activity (2.41 nmol acetyl-CoA hydrolysed per min per mg protein).*

	4°C	20°C
time (min):		
0	100	100
15	93	-
30	91	98
45	97	-
60	97	100

Table 2.4.4 *Effect of cooling on ATP-stimulated acetyl-CoA hydrolase activity in mouse liver. Activities were determined at 25°C and are expressed as a percentage of the initial activity (3.63 nmol acetyl-CoA hydrolysed per min per mg protein).*

time (min)	Activity
0	100
3	77
7	52
13	48
21	36
32	11
45	0

2.4.1 *Effect of Diet and Feeding Level on the Activities of Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase in liver.*

Two-way analysis of variance indicated that diet significantly affected both ATP-stimulated ($0.06 < P < 0.07$) and "mitochondrial" acetyl-CoA hydrolase ($P < 0.05$) (Table 2.4.5a). However, there is no obvious trend across diets for the ATP-stimulated hydrolase, whereas the activity of the "mitochondrial" hydrolase increased across diets, in the order plain SPB < molassed SBP < barley. Total acetyl-CoA hydrolase activity was significantly influenced by diet ($P < 0.01$). Little difference was observed between plain SBP and molassed SBP, but in comparison total hydrolase activity was higher on barley based diets. Hence the primary effect is SBP/barley. Feeding level had no effect on acetyl-CoA hydrolase.

Acetyl-CoA synthetase was significantly affected by diet ($P < 0.01$) (Table 2.4.5b). Plain SBP had higher synthetase activities ($P < 0.05$) than either molassed SBP or barley. The latter two were not significantly different. Feeding level had no effect on acetyl-CoA synthetase.

Table 2.4.5a *Influence of Diet and Feeding Level on the rates of ATP-stimulated acetyl-CoA hydrolase and mitochondrial acetyl-CoA hydrolase in liver. Activities are expressed as nmol acetyl-CoA hydrolysed per min per mg protein and are the mean ± S.E.M. for the number of animals in parenthesis. Means within a column with differing superscripts differ significantly ($P<0.05$).*

TRT	ATP-stim. Hydrolase	Mitochondrial Hydrolase	Total Hydrolase
UL	2.1±0.16^{a} (5)	4.0±0.65^{a} (5)	6.1±0.66^{a} (5)
UH	2.5±0.30^{a} (5)	4.7±0.62 (5)	7.2±0.35^{a} (5)
ML	1.4±0.25^{a} (6)	4.6±0.41^{a} (6)	6.0±0.51^{a} (6)
MH	2.4±0.38 (6)	5.3±0.56 (6)	7.7±0.64^{a} (6)
BL	4.3±0.76^{b} (5)	5.9±0.58 (5)	10.2±0.73^{b} (5)
BH	2.2±0.33^{ab} (9)	7.0±0.92^{b} (9)	9.3±0.86^{b} (9)

UL - Plain Sugarbeet/low level of feeding

UH - Plain Sugarbeet/high level of feeding

ML - Molassed Sugarbeet/low level of feeding

MH - Molassed Sugarbeet/high level of feeding

BL - Barley/low level of feeding

BH - Barley/high level of feeding

Table 2.4.5b *Influence of Diet and Feeding Level on the rates of acetyl-CoA synthetase in liver. Activities are expressed as nmol acetyl-CoA synthesised per min per mg protein and are the mean ± S.E.M. for the number of animals in parenthesis. Means with differing superscripts differ significantly (P<0.05).*

TRT	Acetyl CoA Synthetase
UL	2.9 ± 0.38^{a} (4)
UH	3.6 ± 0.65^{a} (5)
ML	$1.9\pm0.65^{a,b}$ (6)
MH	$2.0\pm0.49^{a,b}$ (5)
BL	$2.1\pm0.34^{a,b}$ (5)
BH	1.9 ± 0.13^{b} (8)

UL - Plain Sugarbeet/low level of feeding

UH - Plain Sugarbeet/high level of feeding

ML - Molassed Sugarbeet/low level of feeding

MH - Molassed Sugarbeet/high level of feeding

BL - Barley/low level of feeding

BH - Barley/high level of feeding

2.4.2 Effect of Diet and Feeding Level on the Activities of Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase in Perirenal Adipose Tissue.

Two-way analysis of variance indicated that diet affected acetyl-CoA hydrolase, but only at the 10% level of significance. The hydrolase rate tended to increase in the order barley, molassed SBP, plain SBP. Feeding level influenced hydrolase rates ($P<0.05$). Hydrolase activity increased with feeding level in the order high, medium, low (Table 2.4.6a).

The activity of acetyl-CoA synthetase was unaffected by diet, but was significantly influenced by feeding level ($P<0.001$). Synthetase activities increased with feeding level in the order, high, medium, low (Table 2.4.6b).

The molassed SBP data were omitted and a barley/plain SBP comparison was analysed using two-way analysis of variance. This was conducted primarily to analysis the barley/plain SBP effect on the activity of both enzymes. Acetyl-CoA hydrolase was influenced by both diet and feeding level ($P<0.05$). Hydrolase activities were higher on plain SBP compared to barley and activites were higher at the low level of feeding compared to the high level. Acetyl-CoA synthetase activity tended to be higher on plain SBP, but this was not significant.

Table 2.4.6a & b *Influence of Diet and Feeding Level on the rates of (a) acetyl-CoA hydrolase and (b) acetyl CoA synthetase in perirenal adipose tissue. Activities are expressed as nmol per min per mg protein and are the mean ± S.E.M. for the number of animals in parenthesis. Means with differing superscripts differ significantly (P<0.05).*

a) *Acetyl-CoA Hydrolase*

D/F	Low	Medium	High
U	16.0 ± 3.53^{a} (3)	16.2 ± 3.17^{a} (4)	6.7 ± 1.19^{b} (4)
M	11.6 ± 0.15^{a} (3)	14.4 ± 2.95^{a} (4)	6.4 ± 1.52^{b} (5)
B	13.2 ± 2.27^{a} (3)	4.4 ± 0.57^{b} (3)	6.3 ± 1.30^{b} (5)

b) *Acetyl-CoA Synthetase*

D/F	Low	Medium	High
U	12.9 ± 1.91^{a} (3)	13.5±2.34 (3)	7.3 ± 1.71^{b} (4)
M	12.4 ± 1.39^{a} (3)	11.4 ± 2.10^{ab} (4)	8.9±2.04 (5)
B	12.7 ± 0.98^{a} (4)	9.9±2.26 (5)	5.5 ± 0.70^{bc} (5)

U - Plain sugarbeet

M - Molassed sugarbeet

B - Barley

2.5 Discussion

Results in Tables 2.4.1 & 2.4.2 show that ATP-stimulated acetyl-CoA hydrolase activity is present in ovine muscle, rumen epithelium and liver, but not in perirenal adipose tissue. Activities in liver are much greater than in muscle or rumen epithelium (approx. 12 fold). As in other species, the activity was confined to the extramitochondrial fraction (Table 2.4.2), although the specific activity measured in sheep liver was much lower than that determined in the liver of rodents (Prass *et al.*, 1980) (3.6 v 65.0 nmol per min per mg protein). In addition this enzyme from sheep liver was not found to be cold-labile (Table 2.4.2).

The extramitochondrial acetyl-CoA hydrolase from rat liver has been shown to be a cold-labile oligome ric enzyme that undergoes a reversible conformational transition between a dimeric and a tetrameric form in the presence of ATP or ADP at 25-37^{o}C, and between a dimeric and a monomeric form at low temperature. Only ATP resulted in an increase in enzyme activity of the tetrametric and dimeric enzyme (to a similar extent) (Isohashi *et al.*, 1983; Söling & Rescher, 1985). However, the K_m values for acetyl-CoA of the dimeric and tetrameric enzyme were different, being 170μM and 60μM, respectively (Isohashi *et al.*, 1983). Hence ATP and ADP both appear to have effects on the catalytic properties of acetyl-CoA hydrolase and on the association-dissociation state of the enzyme. Söling & Rescher (1985) believe that the physiological role of control by nucleotides resides predominantly in their regulation of enzyme activity and to a lesser extent on their effects on subunit association.

Crabtree (see Jessop *et al.*, 1990) has also determined the activity of ATP-stimulated acetyl-CoA hydrolase in sheep liver. The mean activity observed for five animals was 0.8 μmoles per min per g liver (wet wt.). This is only slightly greater than the mean activity observed in this study - 0.6 (when expressed in the same units as Crabtree's).

From Table 2.4.2 it can be seen that a high percentage of total acetyl-CoA hydrolase activity is found in the presence of 2mM ADP. This is taken to represent the activity of "mitochondrial" acetyl-CoA hydrolase (see section 2.2.3). It is possible that this may be

due partly to ATP being formed from ADP by adenylate kinase thus preventing full inhibition of ATP-stimulated acetyl-CoA hydrolase activity. However, addition of P^1,P^5-di(adenosine-5') pentaphosphate, a potent inhibitor of adenylate kinase (Lienhard & Secemski, 1973), to incubations containing 2mM ADP did not reduce the rate of hydrolysis of acetyl-CoA.

It may be calculated from Table 2.4.2 that approx. 70% of the "mitochondrial" acetyl-CoA hydrolase is mitochondrial, which is in agreement with the studies of Knowles *et al.* (1974) and Snoswell & Tubbs (1978). The mean activity of the mitochondrial hydrolase observed in this study, 5.8 nmoles per min per mg protein (Table 2.4 .2) is similar to that reported by Knowles *et al.* (1974), but Snoswell & Tubbs (1978) reported an activity of 52 nmoles per min per mg protein. It is not apparent why the activity reported by Snoswell & Tubbs (1978) is so high.

Acetyl-CoA hydrolase is the enzyme responsible for the production of endogenous (or tissue) acetate in both non-ruminants and ruminants and its presence in any tissue indicates a potential of that tissue to produce acetate (Knowles *et al.*, 1974). Various reports suggest that the enzyme may be involved in controlling the "over production" of acetyl-CoA, by hydrolysing acetyl-CoA to acetate and hence reducing "acetyl pressure", particularly under conditions when the activity of the TCA cycle may be limited (Snoswell & Koundakjian, 1972; Knowles *et al.*, 1974). This may be particularly pertinent for the "mitochondrial" hydrolase. The physiological role of the "mitochondrial" hydrolase for the formation of free acetate is supported by its higher substrate specificity for acetate rather than butyrate compared to the ATP-stimulated acetyl-CoA hydrolase. The mitochondrial enzyme may also function to hydrolase acetyl-CoA during times of acute over production of acetyl-CoA, for instance during β-oxidation of fatty acids.

Both mitochondrial and ATP-stimulated cytosolic acetyl-CoA hydrolase may serve to control the levels of acetate and CoA in their respective compartments, the potential control may perhaps vary in each compartment dependent upon the metabolic roles

and state of each site. For example in liver, mitochondrial CoA is critical for many reactions, including a number of the TCA cycle intermediates and in the cytosol where acyl carnitine derivatives are important in metabolism, the hydrolase could be involved in transfer reactions (Quraishi & Cook, 1972).

Studies by Prass *et al.*, 1980 could find no other explanation for the hypothesis that the ATP-stimulated, ADP-inhibited, cytosolic enzyme has any other action other than catalysing the hydrolysis of acetyl-CoA to acetate and CoA. In addition to its complex regulatory properties, at least in rats, the K_m for acetyl-CoA is high relative to estimates of cytosolic concentrations (see Prass *et al.*, 1980), and hence substrate availability may play an important role in limiting enzyme activity. However the K_m for the ATP-stimulated hydrolase in sheep liver is not known, and it is therefore difficult to speculate upon the influence of acetyl-CoA upon this enzyme in the ruminant. The physiological role of this enzyme in the rat has received considerable attention and various ideas have been put forward. Matsunaga *et al.* (1985) suggested that the enzyme may be involved in the maintenance of the cytosolic acetyl-CoA concentration and CoA pool for both fatty acid synthesis and oxidation. The hydrolase may also serve to convert peroxisomally generated acetyl-CoA into acetate for subsequent oxidation in the mitochondria (Matsunaga *et al.*, 1985; Leighton *et al.*, 1989); it may be important in hydrolysing toxic acyl-CoA derivatives (Prass *et al.*, 1980); and it may enable ATP-citrate lyase to provide a continuous supply of oxaloacetate for gluconeogenesis (Prass *et al.*, 1980).

The hydrolase may also serve to control cytoplasmic CoA concentrations by serving as a "safety valve" to prevent excessive accumulation of cytoplasmic acetyl-CoA, thereby deflecting excess fuel from the liver to the extrahepatic tissues (Prass *et al.*, 1980; Matsunaga *et al.*, 1985). However, this is difficult to reconcile with the formation of acetate in rat hepatocytes despite a net uptake of acetate (Crabtree *et al.*, 1989). This would suggest that the ATP-stimulated enzyme may be involved in a substrate cycle between acetate and acetyl-CoA (Rabkin & Blum, 1985; Jessop *et al.*, 1986). Crabtree

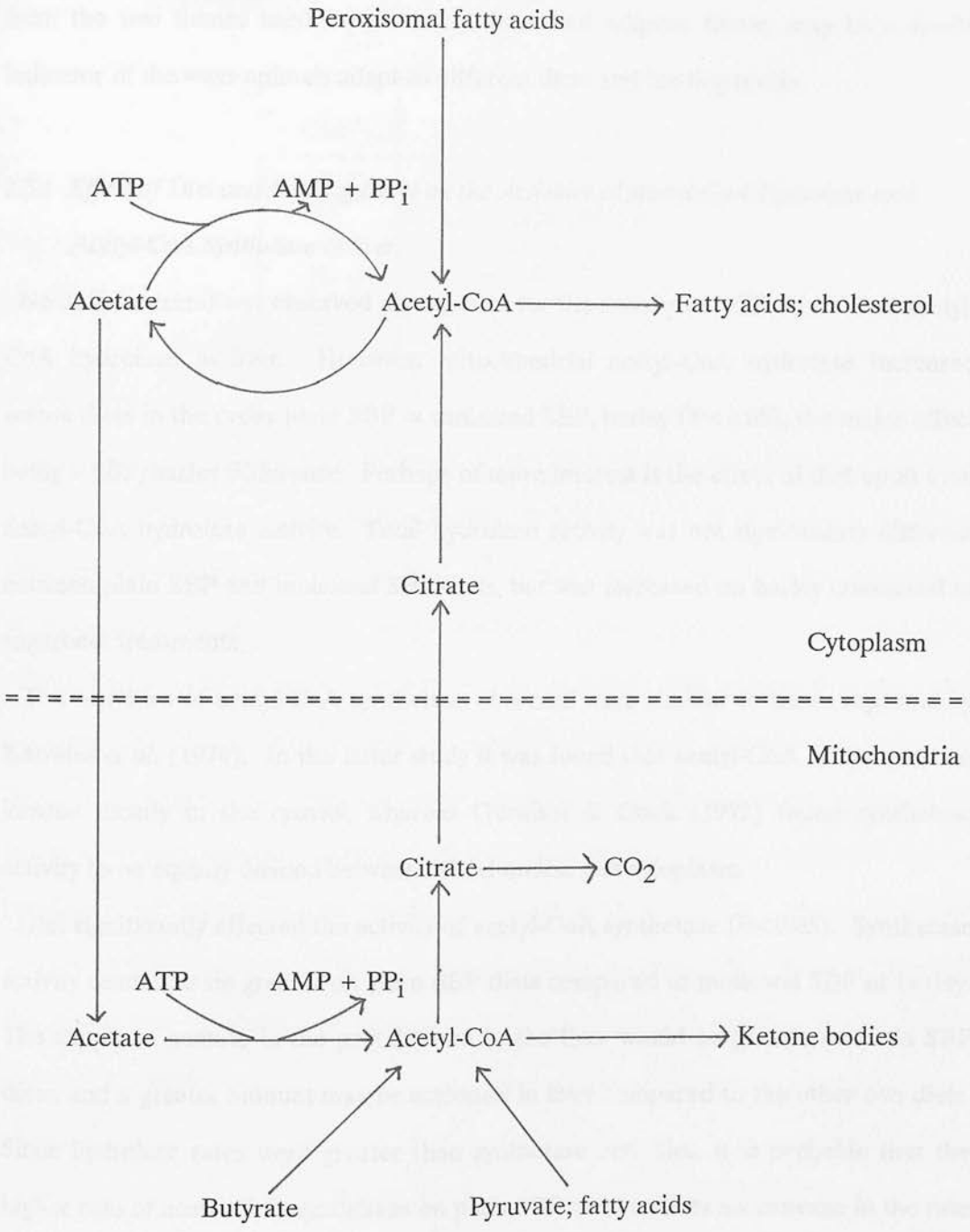
et al. (1989) measured the rate of acetyl-CoA hydrolysis in rat hepatocytes using a technique involving [1-^{14}C] butyrate. In this study it was found that when the hydrolysis of acetyl-CoA was inhibited, there was no corresponding increase in net acetate uptake, suggesting that the activity of acetyl-CoA synthetase was coupled to that of the hydrolase. This would support the hypothesis that ATP-stimulated acetyl-CoA hydrolase and acetyl-CoA synthetase act together to form a substrate cycle between acetate and acetyl-CoA, in liver cytoplasm.

However since acetate can serve as a significant fuel for oxidation in rat liver, mitochondrial activation must be of some importance. Consequently Crabtree *et al.* (1990) investigated the fluxes of acetate to acetyl-CoA and vice versa, in both cytosolic and mitochondrial compartments. It was found that, in contrast, with acetyl-CoA hydrolysis most of the acetyl-CoA synthesis occurred in the mitochondria. This is contrary to what one may have predicted from a consideration of the distribution of the maximum catalytic activities of acetyl-CoA synthetase (measured *in vitro*) which shows that more than 80% of the total synthetase activity is located in the cytoplasm (see Crabtree *et al.*, 1990). The authors propose that a comprehensive examination of the kinetics of liver acetyl-CoA synthetases and hydrolases is required. It was also concluded that the cytoplasmic substrate cycle between acetate and acetyl-CoA would contribute approx. 0.07% of the total heat produced by the animal and the degree of sensitivity conferred by this substrate cycle (determined by the ratio, rate of cycling/net flux; Newsholme & Crabtree, 1976) is low. Hence this cytoplasmic cycle alone is unlikely to be important in metabolic regulation. However, Crabtree *et al.* (1990) point out that this is only a tentative conclusion since it has been shown that many substrate cycles are under metabolic or hormonal control (Newsholme *et al.*, 1984) and many other factors may therefore influence the rate of cycling. It is also proposed that cytoplasmic acetyl-CoA hydrolysis could be involved in part of a larger substrate cycle between the mitochondria and cytoplasm, in which acetate forms acetyl-CoA in the mitochondria which is transferred to the cytoplasm and is there hydrolysed to acetate.

It was calculated that a substrate cycle such as this could account for approx. 1% of the total heat production by the animal. The system proposed is depicted in Fig. 2.5.1. It is not a conventional substrate cycle but Crabtree *et al.* (1990) suggest it is equivalent to one in providing sensitivity and feedback. It is proposed that such a cycle may serve to control the sensitivity of hepatic acetate uptake to its regulators.

The activities of ATP-stimulated cytosolic acetyl-CoA hydrolase and "mitochondrial acetyl-CoA hydrolase were also determined in bovine liver (samples obtained from a local slaughter house). The activity of ATP-stimulated acetyl-CoA hydrolase was 2.3 ± 0.64 and "mitochondrial acetyl-CoA hydrolase" was 5.2 ± 0.9 nmoles per min per mg protein (mean ± S.E.M., for four animals). These activities are similar to those detected in ovine liver (Table 2.4.2).

Fig. 2.5.1 *The scheme proposed by Crabtree et al. (1990) for acetate substrate cycling in rat hepatocytes.*



It is appreciated that the activities of the enzymes determined *in vitro* may not reflect the situation *in vivo*, since the effect of activators, inhibitors or competitive substrates were not investigated. However, differences observed between the enzyme activities from the two tissues used in this study, liver and adipose tissue, may be a useful indicator of the ways animals adapt to different diets and feeding levels.

2.5.1 *Effect of Diet and Feeding Level on the Activities of Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase in liver.*

No definite trend was observed across diets for the activity of ATP-stimulated acetyl-CoA hydrolase in liver. However, mitochondrial acetyl-CoA hydrolase increased across diets in the order plain SBP = molassed SBP, barley ($P<0.05$), the major effect being a SBP/barley difference. Perhaps of more interest is the effect of diet upon total acetyl-CoA hydrolase activity. Total hydrolase activity was not significantly different between plain SBP and molassed SBP diets, but was increased on barley compared to sugarbeet treatments.

The activities of acetyl-CoA synthetase observed were similiar to those reported by Knowles *et al.* (1974). In the latter study it was found that acetyl-CoA synthetase was located mostly in the cytosol, whereas Quraishi & Cook (1972) found synthetase activity to be equally divided between mitochondria and cytoplasm.

Diet significantly affected the activity of acetyl-CoA synthetase ($P<0.05$). Synthetase activity tended to be greater on plain SBP diets compared to molassed SBP or barley. The supply of acetate in the portal blood to the liver would be greater on plain SBP diets, and a greater amount may be activated in liver compared to the other two diets. Since hydrolase rates were greater than synthetase activities, it is probable that the higher rate of acetyl-CoA synthetase on plain SBP diets reflects an increase in the rate of substrate cycling between acetate and acetyl-CoA.

Table 2.5.1 *Influence of Diet on Net Acetate Flux in liver, determined as the rates of acetyl-CoA synthetase minus total acetyl-CoA hydrolase. Activities are expressed as nmol acetate converted to acetyl-CoA per min per mg protein and are expressed as mean ± S.E.M. for the number of animals in parenthesis.*

Diet	Net Acetate Flux
U	-3.28 ± 0.66^{a} (9)
M	-5.0 ± 0.64^{a} (11)
B	-7.0 ± 0.72^{b} (13)

Interesting observations may be made if one analyses the net utilisation of acetate across the three diets, simply by subtracting the total activity of acetyl-CoA hydrolase from the corresponding activity for acetyl-CoA synthetase. The results indicate that on all three diets the net effect is the production of acetate, but this varies across diets (Table 2.5.1). Acetate production was greater on barley diets compared to plain and molassed SBP diets ($P<0.01$), with no significant difference between the latter two diets. This suggests that there is a greater need to hydrolyse acetyl-CoA to acetate on barley when compared to sugarbeet pulp diets. It is probable that there may be an over production of acetyl-CoA on the barley based diets, since the supply of glucose precursors will be greater. The hydrolysis of acetyl-CoA to acetate may be increased if the production of acetyl-CoA is greater than the metabolic capacity of the cell to metabolise acetyl-CoA.

2.5.2 *Effect of Diet and Feeding Level on the Activities of Acetyl-CoA Hydrolase and Acetyl-CoA Synthetase in Perirenal Adipose Tissue.*

The effect of diet and feeding level upon the activities of acetyl-CoA hydrolase and acetyl-CoA synthetase are presented in Table 2.4.5a & b. Across all three diets, diet

only effected acetyl-CoA hydrolase ($P<0.1$) (higher on plain SBP diets), but when the molassed SBP data were omitted and a plain SBP/barley analysis conducted this significance increased ($P<0.05$) and the effect on acetyl-CoA synthetase also tended more toward significance ($P<0.14$) (activities higher on plain SBP diets). If these two enzymes form a substrate cycle *in vivo*, this may reflect an increased rate of substrate cycling between acetate and acetyl-CoA.

In ruminants it is well recognised that the heat produced is greater on a diet based on fibre and consequently the efficiency of ME use is lower, than from one based on starch. Differences also exist in the composition of nutrients available for absorption from the gut. Fibre based diets give rise to larger quantities of acetate and it has been proposed that the lower efficiency of ME use may be related to a restricted rate of fat synthesis from acetate (Blaxter, 1962, Hovell & Greenhalgh, 1976, MacRae & Lobley, 1982). The conversion of acetate into fatty acids has an obligatory requirement for reduced NADP, and this is derived from both the products of glucose metabolism, via the pentose phosphate pathway, and from acetate oxidation, via the isocitrate dehydrogenase shuttle (Bauman & Davies, 1975). The ruminant animal must synthesise it's glucose requirement from precursors, predominantly propionate and certain amino acids. Consequently if the latter are limiting, NADPH supply will be inadequate and the conversion of acetate to fat reduced. Diets producing larger quantities of acetate produce less glucose precursors and in order to maintain fat synthesis it has been proposed that more acetate is oxidised in the TCA cycle, resulting in the production of NADPH and ATP. MacRae & Lobely (1982) proposed that the ATP produced may be used up by increased activity of non-productive processes, contributing to the heat increment. It has been demonstrated that the activity of the substrate cycle between acetate and acetyl-CoA in rat hepatocytes is increased with increasing acetate concentrations (Jessop *et al.*, 1986). The increased activities of both acetyl-CoA hydrolase and acetyl-CoA synthetase on plain SBP, in this study, would tend to support the hypothesis that the substrate cycle between acetate and acetyl-CoA

may provide the facility to utilise excess ATP.

The activities of acetyl-CoA hydrolase and acetyl-CoA synthetase decreased with increasing level of feeding ($P<0.05$ and $P<0.001$, respectively). It is reasonable to suggest that substrate cycles act as a switching mechanism allowing metabolic pathways to be turned on or off rapidly. Generally the higher the rate of cycling relative to the flux along the metabolic pathway the greater the sensitivity of the switching mechanism (Newsholme *et al.* 1984). The results obtained suggest that the substrate cycle between acetate and acetyl-CoA may be acting as such a switching mechanism. At lower feeding levels, with once daily feeding, the animal would switch from periods of energy surplus to energy deficit during each day. Such variations are demonstrated in Chapter 3, in blood acetate, on the once daily fed animal. At the low level of feeding blood acetate increases rapidly after feeding (Fig. 3.4.1a), but the peak is sustained for a much shorter period compared to animals receiving a high level of feeding (Fig. 3.4.1b).

The activity of lactate dehydrogenase was also measured in the perirenal adipose samples. This was a simple test to demonstrate that the effects observed upon acetyl-CoA hydrolase and acetyl-CoA synthetase were real and not simply differences, for example, in the amount of protein in each sample. The results obtained suggest that neither diet or feeding level influence lactate dehydrogenase (Fig. 2.5.2).

The activities of acetyl-CoA synthetase observed compare well with those of Knowles *et al.* (1974). However acetyl-CoA hydrolase activities reported by Knowles *et al.* (1974) were lower (<2 nmoles per min per mg protein). This is perhaps a reflection of the different techniques used to assess the activity of acetyl-CoA hydrolase. The method used by Knowles *et al.* (1974) allows free CoA to accumulate, which is then assayed after the reaction has been stopped at a set time. These conditions would permit acetyl-CoA to be resynthesised by acetyl-CoA synthetase, thus reducing the accumulation of free CoA and hence under estimating the activity

Fig. 2.5.2 *Influence of Diet and Feeding Level on the activity of Lactate Dehydrogenase in perirenal adipose tissue. Activities are expressed as nmol lactate oxidised per min per mg protein and are the mean ± S.E.M. for the number of animals in parenthesis.*

D/F	Low	Medium	High
U	307±20.0 (3)	322±21.0 (2)	300±15.0 (4)
M	282±2.0 (2)	289±9.0 (3)	330±12.0 (3)
B	300±17.0 (3)	282±12.0 (2)	286±10.0 (4)

of acetyl-CoA hydrolase. In this study free CoA reacts with DTNB, so that it is not available as a substrate for acetyl-CoA synthetase.

2.5.3 *The Potential Contribution of Substrate Cycling between Acetate and Acetyl-CoA to Heat Production.*

The potential heat produced as a result of substrate cycling between acetate and acetyl-CoA may be calculated from the in vitro activities of acetyl-CoA hydrolase and acetyl-CoA synthetase obtained in this study.

Assuming that a substrate cycle between acetate and acetyl-CoA operates in sheep liver cytoplasm, its maximum capacity would be approx. 0.5 μmoles acetate cycled per min per g liver at 25^{o}C (see appendix 1). If the activity doubles for each 10^{o}C rise in temperature, the maximum activity of the cycle would be 1 μmole acetate cycled per min per g liver at 37^{o}C. Since this cycle hydrolyses ATP to AMP, it effectively hydrolyses two molecules of ATP per revolution; and each μmole of ATP hydrolysed (and subsequently resynthesised) *in vivo* is accompanied by the release of 0.077J of heat (Gill *et al.*, 1984). So the maximum rate of heat production by this cycle is 2 x 0.077, ie. 0.015 J per min per g liver and assuming 0.75 kg liver per animal, would be equivalent to 160kJ per day. This approximates to 2.5% of the basal metabolic rate.

CHAPTER THREE

EFFECT of DIET, FEEDING LEVEL and PROTEIN LEVEL on SELECTED PLASMA CHARACTERISTICS and ACETATE METABOLISm of OVINE PERIRENAL ADIPOSE TISSUE *in vitro*.

Abstract

1. Expt.1. 24 Blackface wether lambs (approx. 25kg) were fed on either barley (B), sugarbeet pulp (S) and grass/ rice husks (G), at one of 2 feeding levels (L, 1.2xM; H, 2xM). Lambs were blood sampled over a 22h period and plasma was assayed for acetate, glucose and insulin. After slaughter the influence of acetate, glucose and insulin upon the incorporation of acetate into CO_2 and lipid was studied.

2. Expt.2. 16 Blackface wether lambs (25-27kg) were fed on diets based on either barley (B) or sugarbeet pulp (S), at one of 2 protein levels (PL, 11.5% CP; PH, 20.1% CP). Level of feeding was 2xM and constant level of ME intake was given. In both expts lambs remained on treatment for 9 weeks. Animals were fed semi-continuously and blood sampled over a 24h period. Plasma was assayed for acetate, glucose and insulin. After slaughter the metabolism of acetate to CO_2 and lipid was investigated in perirenal adipocytes.

3. Observing diet and feeding level effects on plasma acetate, glucose and insulin was obscured by variation, probably as a result of irregular food intake. Barley treatments resulted in higher plasma insulin ($P<0.05$) concentrations when compared to sugarbeet pulp and grass/rice husks. Feeding level had no effect. Daily variation in plasma acetate and insulin was evident.
Acetate incorporation into CO_2 and lipid was influenced by acetate, glucose and insulin. Difficulties were experienced with adipocyte incubations, which were related to the number of adipocytes in each incubation vessel.

4. Semi-continuous feeding helped to achieve near-steady-state conditions. Diet did not significantly effect plasma acetate, glucose or insulin. Level of protein in the diet influenced plasma acetate, higher concentrations of acetate were associated with lower levels of protein ($P<0.05$).
Diet influenced acetate incorporation into CO_2 ($P<0.05$), whereas protein level influenced acetate incorporation into lipid ($P<0.05$).

5. The results suggest that the efficiency of utilisation of ME is influenced by carbohydrate source and protein level in the diet. It is proposed that the efficiency of ME is related to the maintenance of glucose homeostasis, involving VFA metabolism and protein turnover.

Key Words: Lamb Fibre Starch Protein Adipocyte Energy

Data from expt 2 have been presented to the Nutrition Society (Scollan & Jessop, 1990, in press).

3.1 INTRODUCTION

In ruminants it is recognised that the efficiency of utilisation of equivalent amounts of ME is lower on a diet based on fibre than from one based on starch. Various explanations have arisen but no one hypothesis has been universally accepted.

Volatile fatty acids, which are the end-products of anaerobic fermentation in the rumen, were first demonstrated by Barcroft *et al.* (1944) to be the major energy sources for the ruminant animal and it was suggested that variations in the principal acids acetic, propionic and butyric would explain the additional heat associated with fibre diets (McClymont, 1952). Diets with differing composition have different fermentation patterns in the rumen. The ratio of acetate:propionate tends to increase as the fibre content of the diet increases (Blaxter, 1962, Annision & Armstrong, 1970). As the proportion of energy derived from acetate increases the efficiency with which the ME is used declines (Blaxter, 1962). A series of experiments showed that the heat produced as a result of infusing acetate, propionate or butyrate into the rumen of fasted sheep was similar, whilst above maintenance the heat produced from infused acetate (67kJ/100kJ total VFA) was greater than with propionic and butyric acids (44 and 38 kJ/100kJ respectively) (Armstrong & Blaxter, 1957a,b; Armstrong *et al.*, 1957, 1958). In the same experiments it was shown that the infusion of 3-carbon acids (propionic) facilitated the utilisation of 2-carbon acids (acetic).

It was proposed that the ability of ruminants to utilise acetate depended on the availability of glucose precursors (Blaxter, 1962, Hovell *et al.*, 1976) and the efficiency of ME utilisation for growth was related to the amount of acetate produced.

Subsequent experiments which investigated the efficiency of VFA utilisation for growth tended to give contradictory evidence. Studies by Tyrell *et al.* (1979), Eskeland *et al.* (1973) and Hovell *et al.*, (1976) supported the theory whilst Rook *et al.* (1963), Ørskov & Allen (1966a,b,c), Ørskov *et al.* (1979) and Bull *et al.* (1970) did not. MacRae and Lobley (1982) attempted to explain these observations. They emphasised the point made by Armstrong & Blaxter (1970) that the anabolism of acetate to fat has an obligatory requirement for both NADPH and glycerol-3-

phosphate. Reduced NADP is derived either from glucose metabolism in the pentose phosphate pathway or from a TCA-related reaction, the isocitrate dehydrogenase shuttle (Bauman, 1975). Hence adequate supplies of these intermediates or glucose precursors are required. In ruminants the dominant glucose precursors are propionate and glycogenic amino acids (usually non essential). MacRae and Lobley (1982) suggested that diets resulting in high acetate levels would be limiting in glucose (as a result of less propionate and less amino acids produced from fibrous diets), and hence the incorporation of acetate into lipid would be impared. Some NADPH may be produced from acetate (via NADP linked isocitrate dehydrogenase) permitting limited fatty acid synthesis. Concomitant with the production of NADPH from acetate, ATP is produced. It is suggested that this excess ATP is dissipated by means of a substrate cycle possibly between acetate and acetyl-CoA (MacRae & Lobely, 1986).

This is a very attractive hypothesis but opposition continues, in particular from Ørskov and his colleagues (Ørskov *et al.*, 1990). No direct evidence exists which supports this theory but much indirect evidence may be found in the literature (MacRae and Lobley, 1986). Of prime interest is the relationship which has been observed between nitrogen retention and the molar proportions of infused acetate, propionate and butyrate. This relationship is highly correlated for acetate (negative) and propionate (positive) ($r = 0.97$). The authors interpret these results as indicating that when propionate is limiting amino acids are required to enhance the utilisation of acetate.

Recently an alternative hypothesis has been reported which suggests that the efficiency of energy retention is related to the energy costs of protein synthesis rather than lipid synthesis. Emmans *et al.* (1989) using a comparative slaughter approach determined the efficiency of ME use of barley and sugarbeet pulp, of equivalent metabolisability, for maintenance, protein and lipid gain, in lambs. These authors found that there was no difference in the efficiency with which ME was used for maintenance and lipid gain, but that there was a substantial difference in the metabolic cost of protein gain. This indicates that the energy costs of protein synthesis/turnover

may be related to the efficiency of ME use. Several studies report that the rate of protein degradation is increased in animals fed diets which result in an acetate fermentation (Bryant & Smith, 1982; Abdul-Razzaq & Bickerstaff, 1989). Abdul-Razzaq & Bickerstaff (1989) concluded that the proportion of ruminal VFA's influences protein turnover, protein synthesis and the efficiency of protein retention.

Evidence therefore exists which suggests that the rumen VFA influence the efficiency of ME use either via fatty acid or protein metabolism. In either case the VFA effect is not direct and is probably mediated by hormones.

High starch diets increase the levels of insulin and glucose in the plasma (Trenkle, 1970; Walker & Elliot, 1973; Jenny & Polan, 1975; Abdul-Razzaq *et al.*, 1988). In turn both insulin and glucose influence the rates of lipogenesis and protein synthesis (Emery, 1973; Rao *et al.*, 1973; Abdul-Razzaq & Bickerstaff, 1989).

The objective of this study was to investigate the plasma levels of acetate, glucose and insulin in lambs fed on isoenergetic diets likely to result in differing patterns of rumen fermentation, at differing levels of feed intake or protein level. In addition, after slaughter, an investigation of acetate metabolism by perirenal adipose tissue obtained from these lambs would be conducted.

3.2 *METHODS and MATERIALS*

Laboratory chemicals used were of AnalaR grade and were supplied by BDH Chemicals Limited, Poole Dorset. Biochemicals were supplied by Sigma Chemicals Company Limited, Poole, Dorset. Bovine Serum Albumin (Fraction V) was defatted according to the procedure of Chen, (1967).

3.2.1 *Animals*

Expt 1. Twenty four Scottish Blackface wether lambs were kept in individual pens. Initially they received a basal diet (Table 3.2.1) and when a liveweight of 25kg was attained, they were randomly allocated to one of three diets, diet B (barley), diet S (sugarbeet pulp) or diet G (grass/rice husks) (Tables 3.2.2 & 3.2.3), offered at one of

two feeding levels L & H (level L - calculated to be 1.2 x maintenance; level H- 2.0 x maintenance). Hence there were six dietary treatments (BL, SL, GL, BH, SH, GH), and four animals per treatment.

Expt 2. Sixteen Scottish Blackface wether lambs (25-27kg) were kept in individual pens. Initially the lambs received a basal diet (Table 3.2.1). Once four lambs had reached a daily intake of 936g they were randomly allocated to one of two diets, diet B (barley) or S (sugarbeet pulp) at one of two protein levels (PL or PH) (Table 3.2.4 & 3.2.5). Hence there were four dietary treatments BPL and SPL (11.5% crude protein; 11.6 MJ/kg DM); BPH and SPH (20.1% crude protein; 11.9 MJ/kg DM). Lambs were fed at a level calculated to supply twice maintenance $\hat{ME}_i$ energy requirement. The sequence was repeated until all 16 lambs had been allocated to a treatment, with four lambs per treatment.

The calculated ME of the low protein treatments were 0.3MJ/kg DM lower than the high protein treatments. To maintain a constant level of ME intake per day the low intake lambs received an extra 27g/d.

In both experiments each lamb was given its ration once daily at 08:00h and had free access to drinking water. The animals were subjected to the experimental treatment for 9 weeks, after which time they were slaughtered.

Table 3.2.1 *Basal Diet Used For Acclimatisation (kg/tonne DM)*

Barley	584.0
Dried Grass Meal	200.0
White Fishmeal	60.0
Soyabean Meal	70.0
Molasses	50.0
Salt	15.0
Ground Limestone	15.0
Trace Elements/Vitamins*	1.0
Ammonium Chloride	5.0

* Norvite Supplement No.317

Table 3.2.2 *Diet Formulations - Expt. 1 (kg/tonne DM)*

Constituent	B	S	G
Barley	553	-	-
Plain SBP	-	503	-
Grassmeal	200	230	595
Rice Husks	-	-	266
Soya	110	126	44
Fishmeal	70	80	28
CMS 20	35	35	35
Salt	15	15	15
Limestone	15	1	15
Dicalcium Phosphate	-	8	-
PH 8164 Ewe/Lamb	2	2	2

Table 3.2.3 *Calculated Composition of diets - Expt. 1 (per kg DM)*

	B	S	G
CP (g)	206	206	154
RDP (g)	131	112	90
UDP (g)	75	94	64
ME (MJ)	12.1	12.0	9.0
CF (g)	82	162	243
MADF (g)	98	198	300
NDF (g)	243	387	550
Starch & Sugar (g)	333	88	100
Oil (g)	25	22	27
Ash (g)	87	102	146
Ca (g)	12.6	13.0	12.5
P (g)	5.9	6.0	3.6
Mg (g)	1.6	1.8	3.3
Na (g)	7.0	7.2	7.6
CP/ME (g/MJ)	17.0	17.2	17.1
RDP/ME (g/MJ)	10.8	9.3	10.0

Table 3.2.4 Diet Formulations - Expt. 2 (kg/tonne DM)

	BPL	BPH	SPL	SPH
Barley	714.4	542.8	-	-
SBP	-		712.9	517.0
Grassmeal	191.7	192.6	193.6	189.6
Soyabean Meal	-	133.8	-	129.2
Fishmeal	-	70.0	-	77.6
Urea	-	-	4.9	-
Molasses	52.3	52.5	52.8	51.7
Limestone	18.6	13.1	1.6	1.6
Dicalcium Phosphate	6.8	-	18.5	18.1
Salt	14.5	13.3	13.9	13.6
PH8164	1.7	1.8	1.8	1.7

Table 3.2.5 *Calculated Composition of the diets - Expt. 2 (per kg DM)*

	BPL	BPH	SPL	SPH
CP (g)	115.1	200.0	114.9	201.0
RDP (g)	85.8	122.8	61.9	105.2
UDP (g)	29.3	77.2	53.0	95.8
ME (MJ)	11.6	11.9	11.6	11.9
CF (g)	82.0	81.7	184.4	159.8
MADF (g)	96.0	97.1	224.2	194.9
NDF (g)	259.6	241.0	444.0	381.7
Starch & Sugar (g)	449.6	367.2	87.5	91.3
Oil (g)	18.8	23.7	14.2	20.3
Ash (g)	84.0	88.5	101.2	101.1
Calcium (g)	12.6	12.6	12.6	12.6
Phosphorous (g)	5.0	6.0	5.0	5.0
Magnesium (g)	1.3	1.7	5.0	5.0
Sodium (g)	7.1	7.1	7.1	7.1
CP/ME (g/MJ)	9.9	16.8	9.9	16.8
RDP/ME (g/MJ)	7.4	10.3	5.3	8.8

3.2.2 *Blood Sampling*

In both experiments all lambs had been on trial for at least 21 days. 10ml blood samples were taken from the jugular vein into heparinised tubes, by venepuncture. During each sampling period collected blood was kept on ice until sampling was complete. The samples were then centrifuged at 2,500xg for 15min, using an MSE benchtop centrifuge. The supernatant (plasma) was divided into three aliquots of 1.5ml and stored at -20°C until analysed for glucose, acetate and insulin.

Expt 1. Lambs received their daily aliquot of food at 08:00h. Each animal was sampled over a period of 22h, commencing at 08:00h and every hour thereafter until 13:00h, and subsequently at 18:00, 22:00, 02:00 and 06:00h.

Expt 2. The objective of this expt. was to subject the lambs to a period of semi-continuous feeding sufficient to achieve near-steady-state conditions.

Commencing on a Tuesday morning at 08:00h the lambs received one quarter of their daily ration every 6h. At 08:00h - Thursday the lambs were given one sixth of their daily ration every 4h. From midnight Thursday feeding frequency was increased so that each lamb received one twelfth of its daily ration every 2h. Blood sampling commenced at 7 am on Friday. Over the following 24h the lambs were fed every 2h and blood sampled every 4h. Feeding then reverted to the once daily regime.

3.2.3 *Analysis of rumen VFA*

800μl of strained rumen liquor were mixed with 200μl of 25% (w/v) metaphosphoric acid in 0.5M H_2SO_4 and 40mM 2-ethylbutyric acid (as an internal standard). The mixture was centrifuged at 13,000xg (Beckman Microfuge) for 3min. 1μl of supernatant was injected onto a 6ft x 4mm internal diameter glass column packed with 10% SP-1200, 1% H_3PO_4 on Chromosorb WAW support, mesh size 80-100. The oven temperature was 125°C. The carrier gas was nitrogen at a flow rate of 30ml/min. The instrument used was a Pye Unicam Series 104GLC.

3.2.4 *Determination of Plasma Glucose*

The concentration of plasma glucose was determined spectrophotometrically (Beckman DU 62 spectrophotometer) using a commercial test kit (Glucose (HK) Sigma Diagnostics Kit, Sigma Chemical Company Limited, Poole, Dorset). The assay measures the reduction of NAD^+ during the oxidation of glucose 6-phosphate to 6-phospho-gluconate.

$$\text{Glucose} + \text{ATP} \xrightarrow[E_1]{} \text{Glucose 6-Phosphate} + \text{ADP}$$

$$\text{Glucose 6-Phosphate} + NADP^+ \xrightarrow[E_2]{} \text{6-Phospho-Gluconate} + \text{NADPH} + H^+$$

E_1 = Hexokinase
E_2 = Glucose 6-Phosphate Dehydrogenase

3.2.5 *Determination of Plasma Acetate*

Plasma acetate was determined by the method of Guynn & Veech (1974). The following sequence of reactions are involved:

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow[E_1]{} \text{Acetyl-CoA} + \text{AMP} + PP_i$$

$$\text{Acetyl-CoA} + \text{Oxaloacetate} \xrightarrow[E_2]{} \text{Citrate} + \text{CoA}$$

$$\text{Malate} + NAD^+ \xrightarrow[E_3]{} \text{Oxaloacetate} + \text{NADH} + H^+$$

E_1 = Acetyl-CoA Synthetase
E_2 = Citrate Synthase
E_3 = Malate Dehydrogenase

The reduction of NAD^+ to NADH, catalysed by malate dehydrogenase gives an indirect measure of the amount of acetate present. The reduction of NAD^+ is

measured spectrophotometrically at 340nm. To enable malate dehydrogenase to respond linearly both NADH and NAD^+ must be present in the incubation medium (Guynn & Veech, 1974). This ensures proportionality between NADH production and the amount of acetate present in the test sample.

The reaction used 1ml of medium (pH 7.4) containing 75mM Tris-HCL, 3mM $MgCl_2$, 1mM malate, 0.5mM NAD^+, 2mM ATP, 0.13mM CoA, 1 unit/ml citrate synthase, 5 units/ml malate dehydrogenase and 0.06 units/ml acetyl-CoA synthetase. 10μl of NADH (10mg/ml) were added to 1ml of media in a cuvette and warmed to 37^{o}C, for 5min. 50μl of plasma were added and the mixture was incubated for 15-20min at 37^{o}C. The increase in absorption was assessed (340nm) using a Beckman DU 62 spectrophotometer. Blank incubations were conducted (using water) to determine non-specific oxidation of NADH and reduction of NAD^+.

3.2.6 *Determination of Plasma Insulin*

Plasma insulin concentrations were determined as immunoreactive insulin in a radio-immunoassay using charcoal separation. Assays were conducted by the Rowett Research Institute, Aberdeen.

3.3 Adipocyte Preparation and Incubation

3.3.1 *Composition of Buffers*

Four buffers were associated with this study, transport (T), digestion (DB), washing (W) and incubation (I) buffers (Table 3.3.1).

3.3.2 *Tissue Sampling*

Samples of perirenal adipose tissue (approx. 15g) were collected within 2min of death and placed in thermos flasks containing 50ml of transport medium at 37^{o}C.

Table 3.3.1 *Composition of the Buffers*

Constituent	T	DB	W	I
KRB*	+	+	+	+
BSA % (w/v)	1	3	3	3
HEPES (mM)	5	5	5	5
Casein (mg/ml) Hydrolysate	300	300	300	300
Methionine (mg/ml)	10	10	10	10
Tryptophan (mg/ml)	10	10	10	10
Gentamycin sulphate (mg/ml)	0.05	0.05	0.05	0.05
Glucose (mM)	4.5	4.5	4.5**	4.5**
Acetate *** (mM)	0.8	0.8	0.8**	0.8**
Insulin (μU/ml)	20	35	35**	35**

* Krebs Ringer Bicarbonate buffer with one half of the recommended calcium concentration (1.27 mM $CaCl_2.6H_2O$)

** These may be omitted or different concentrations used depending on the objective of the incubation (see text).

*** Sodium Acetate

3.3.3 *Adipocyte Isolation*

The method was based on that of Yang & Baldwin (1973). Visible blood vessels were trimmed out and relatively clean fat lobes were used for cell preparation.

The trimmed adipose tissue was finely minced using scissors. The minced tissue was placed in a plastic flask with 35ml of dispersion medium. Cell dispersion was performed in an atmosphere of 95% O_2/5% CO_2 at 37°C in a shaking water bath (Grant Instruments, Cambridge, Ltd) set at 50 cycles/min. Dissociation of the tissue could be observed within 20min. After 1h of incubation, the flask was removed from the bath and the contents were stirred gently with a plastic rod, to facilitate cell release. The contents were passed through a plastic tea strainer into a 100ml plastic beaker. The filtrate was washed once with 10ml of washing medium (37°C). The cell suspension did not require centrifugation since the adipocytes rose readily to the top of the suspension. Medium below the fat cells was removed using a 20ml syringe fitted with a long needle. The fat cells were resuspended in 20-25ml of washing medium. Washing was repeated 3 times. After the final wash an aliquot of cell suspension was placed on a counting chamber (0.2mm deep; 1/16mm^2; Gallenkamp) and an approximation of the cell number was made. This was done simply by counting the number of cells as viewed through the eyepiece of the microscope (Nikon Optiphot HFX II, Nikon U.K. Ltd). If 15-20 cells were counted it was judged that the cell suspension did not require any dilution or concentration, since previous calculations indicated this would result in adipocytes numbers ranging between 200,000-500,000 cells per ml. This is well within the optimum number of cells per incubation (10^4-10^5, Dr P. Sinett-Smith, personal communication). Ten photographs per aliquot of cell suspension were taken to determine accurate cell numbers.

All the work was conducted at 37°C. Isolated adipocytes are very fragile and it is important to minimise temperature fluctuations (a hot plate was used) and avoid excess physical trauma (such as vigorous shaking or stirring). All glassware was freshly siliconised.

3.3.4 *Adipocyte Incubation*

The method was based on that of Yang & Baldwin (1973). The incubations were performed in 20ml silconised glass scintillation vials. 1ml of cell suspension was added to 1ml of incubation medium, containing the desired concentrations of acetate, glucose and insulin. The vessels were flushed with 95% O_2/5% CO_2 for 10 seconds and fitted with self-sealing rubber septum stoppers (Subaseal, Gallenkamp). The stoppers were fitted with a plastic centre well (Kontes Glass Company) containing a piece of filter paper (0.75x1.5cm). The flasks were incubated for 2h in a shaking water bath (Grants Instruments Ltd) set at 37°C and 40 cycles/min.

At the end of the incubation the vessels were removed from the water bath and placed on a tray. 250µl of hydroxy-hyamine were injected into the centre well, CO_2 was liberated from the incubation medium by the addition of 50µl of 0.25M sulphuric acid. After a 30min period at room temperature the centre wells were removed and placed in 7ml scintillation vials to which 3ml Beckman Ready-micro scintillation fluid were added. The vials were capped and $^{14}CO_2$ determined (see next section).

3.3.5 *Lipid Extraction*

On removal of the centre wells the vials were capped and stored at 0°C until the lipids were extracted. Total lipids were extracted using the procedure outlined in Fig. 3.3.1, the method of Folch (1957). All the lipid was dried and total ^{14}C activity in the lipid (using 20ml scintillation vials) was determined.

A Beckman LS 5000CE liquid scintillation counter was used to measure the amount of ^{14}C as carbon dioxide and lipid.

3.3.6 *Adipocyte Incubations - Expt 1*

Adipocytes prepared from these lambs were incubated at the respective concentrations of acetate, glucose and insulin, determined *in vivo*. For reasons which will be explained fully in the results section, the precise details of these incubations will not be noted.

Fig. 3.3.1 *Lipid extraction*

1) Add to incubation vessel:
   4ml chloroform
   0.5ml 0.88% KCl
   2 drops 1M HCl

↓

2) Rinse sides of flask with 4ml of 1:1 chloroform:methanol

↓

3) Shake well and leave overnight

↓

4) Aspirate off upper layer (to waste)

↓

5) Re-extract cells with 5ml "Upper phase"* mixture. Leave overnight

↓

6) Transfer lower phase to another vessel and allow to dry down. This was achieved by leaving vessels in a fume cupboard, overnight

↓

7) Redissolve lipid in 2-3ml chloroform

↓

8) Filter lipid into a pre-weighed 20ml scintillation vial, dry down, reweigh, and add scintillant for counting

* "Upper Phase" (total volume 98ml):

Chloroform	3ml
Methanol	48ml
0.88% KCl	47ml

In order to test the response of adipocytes, in terms of their capacity to oxidise acetate to CO_2 and to incorporate acetate into lipid, to varying concentrations of acetate, glucose and insulin, three sets of incubations were set up, using adipocytes pooled from 3 lambs:

1) Effect of varying acetate concentration on acetate incorporation into CO_2 and lipid. Adipocytes were incubated in triplicate in incubation buffer (Table 3.3.1), [1-^{14}C] acetate (1.06 KBq/μmole), 1000μU/ml insulin, 6.5mM glucose and acetate at each of the following concentrations: 0, 1.0, 2.0, 2.5, 3.0, 3.5 and 4.0mM.

2) Effect of varying glucose concentration on acetate incorporation into CO_2 and lipid. Adipocytes were incubated in triplicate in incubation buffer (Table 3.3.1), 3.5mM acetate, [1-^{14}C] acetate (1.06 KBq/μmole), 1000μU/ml insulin, and glucose at each of the following concentrations: 0, 2.0, 3.0, 4.0, 4.5, 5.0 and 6.5mM.

3) Effect of varying insulin concentration on acetate incorporation into CO_2 and lipid. Adipocytes were incubated in triplicate in incubation buffer (Table 3.3.1), 3.5mM acetate, [1-^{14}C] acetate (1.06 KBq/μmole), 6.5mM glucose and insulin at each of the following concentrations: 10^0, 10^1, $10^{1.5}$, $10^{1.8}$, 10^2, 10^3 and 10^4 μU/ml.

3.3.7 *Adipocyte Incubations - Expt 2*

1) A range of incubations were set up to investigate the effect on acetate incorporation into CO_2 and lipid of acetate concentrations varying between 0 and 1.0mM. Adipocytes were prepared from pooled perirenal tissue samples from three animals. These adipocytes were incubated in triplicate in incubation buffer (Table 3.3.1), [1-^{14}C] acetate (1.85KBq/μmole), 35μU/ml insulin, 4.5mM glucose and acetate at each of the following concentrations: 0, 0.1, 0.25, 0.50, 0.75 and 1.0mM.

2) Perirenal adipocytes prepared from each lamb were incubated in triplicate in

incubation buffer (Table 3.3.1) and [1-^{14}C] acetate (1.16KBq/μmole).

3.4 *RESULTS*

In both experiments all the animals remained healthy and all daily aliquots of food were consumed. In expt 1 it was noted that lambs receiving a high level of feeding tended to consume their daily aliquot over the 24h period, whilst those on a low level of feed intake ate all their food immediately.

The extra food given to the lambs receiving the low protein treatments in expt 2 ensured that the calculated ME intake across all 4 treatments was similar.

3.4.1 *Plasma Acetate, Glucose and Insulin*

Expt 1. The mean (± S.E.M.) concentrations of plasma acetate, glucose and insulin are shown in Table 3.4.1 & 3.4.2. Plasma concentrations of acetate were higher for barley diets (but only at the 10% level of significance) compared to sugarbeet pulp and grass/rice husks. No difference in plasma acetate was detectable between sugarbeet pulp and grass/rice husks fed animals. There was a small tendency (not significant) for glucose levels to be greater in barley fed animals. Diet source was a major influence on plasma insulin. Lambs fed on barley diets had higher insulin concentrations ($P<0.05$, 0.01) when compared to lambs fed on sugarbeet pulp and grass/rice husks respectively.

There was little difference between the low and high levels of feeding, although there was a slight indication that plasma glucose and insulin increased on high levels, but this trend was not significant.

Daily variation in plasma acetate is shown in Fig. 3.4.1a & b. Acetate levels for all diets and levels reached maximum values 2-4h after feeding. Those lambs fed barley diets attained maximum values more rapidly and had a greater response compared to lambs receiving sugarbeet pulp or grass/rice husks. The plasma acetate values of lambs fed on the barley diet at the high level

Table 3.4.1 *Influence of Diet and Feeding Level on Acetate, Glucose and Insulin concentrations in Plasma. Data are presented as means ± S.E.M.*

Diet	Level	Glucose (mM)	Acetate (mM)	Insulin (μU/ml)
B	L	4.53±0.44	0.64±0.32	27.2±4.03
B	H	4.31±0.31	0.99±0.36	27.9±3.68
S	L	4.12±0.18	0.51±0.12	19.9±2.97
S	H	4.32±0.12	0.29±0.01	19.8±1.39
G	L	3.78±0.16	0.34±0.10	12.2±0.67
G	H	4.89±0.87	0.43±0.13	16.4±0.62

Table 3.4.2 *Effect of Diet on mean (± S.E.M.) Plasma Insulin (μU/ml). Means with differing superscripts differ significantly ($P<0.05$).*

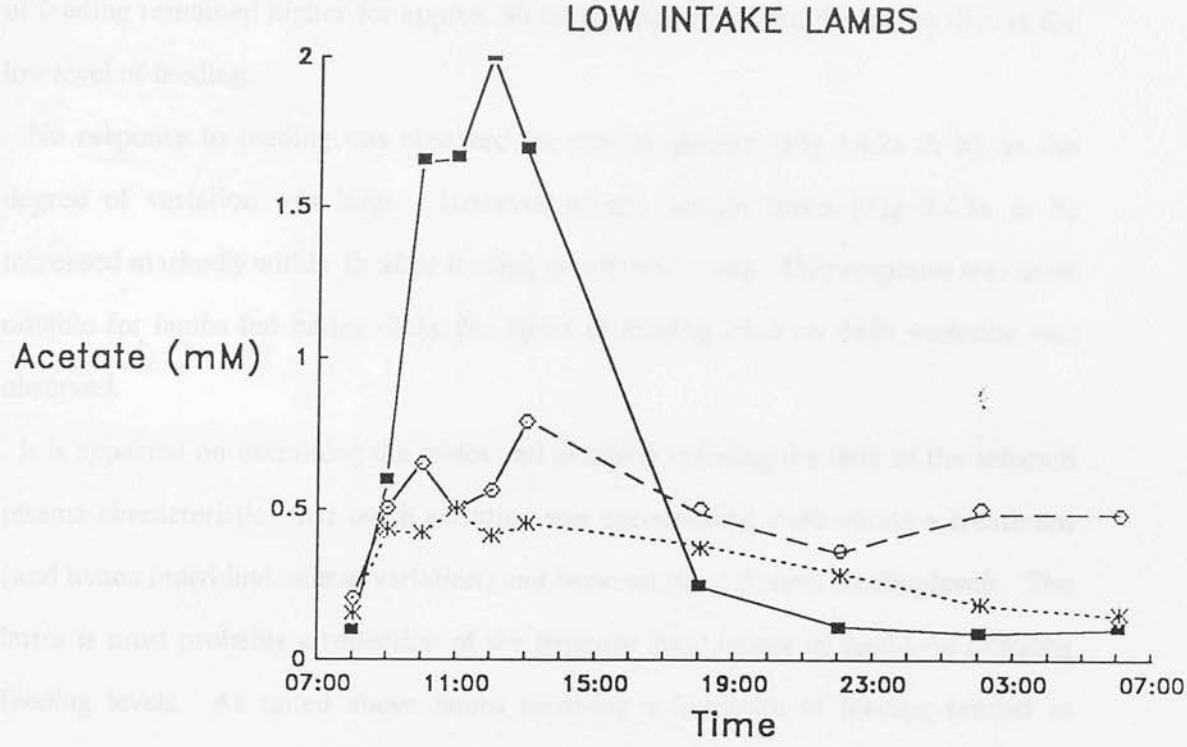
	B	S	G
Insulin	27.5^{a}±2.52 (8)	19.8^{b}±1.52 (8)	14.0^{b}±0.96 (7)

Fig. 3.4.1a & b *Daily variation in Plasma Acetate concentrations in lambs receiving low (a) and high (b) feeding levels. Where standard error bars are omitted, they were too large to fit on the diagram.*

Key: ——— - Barley Diet

— — — - Sugarbeet Pulp Diet

------ - Grassmeal/Rice Husks Diet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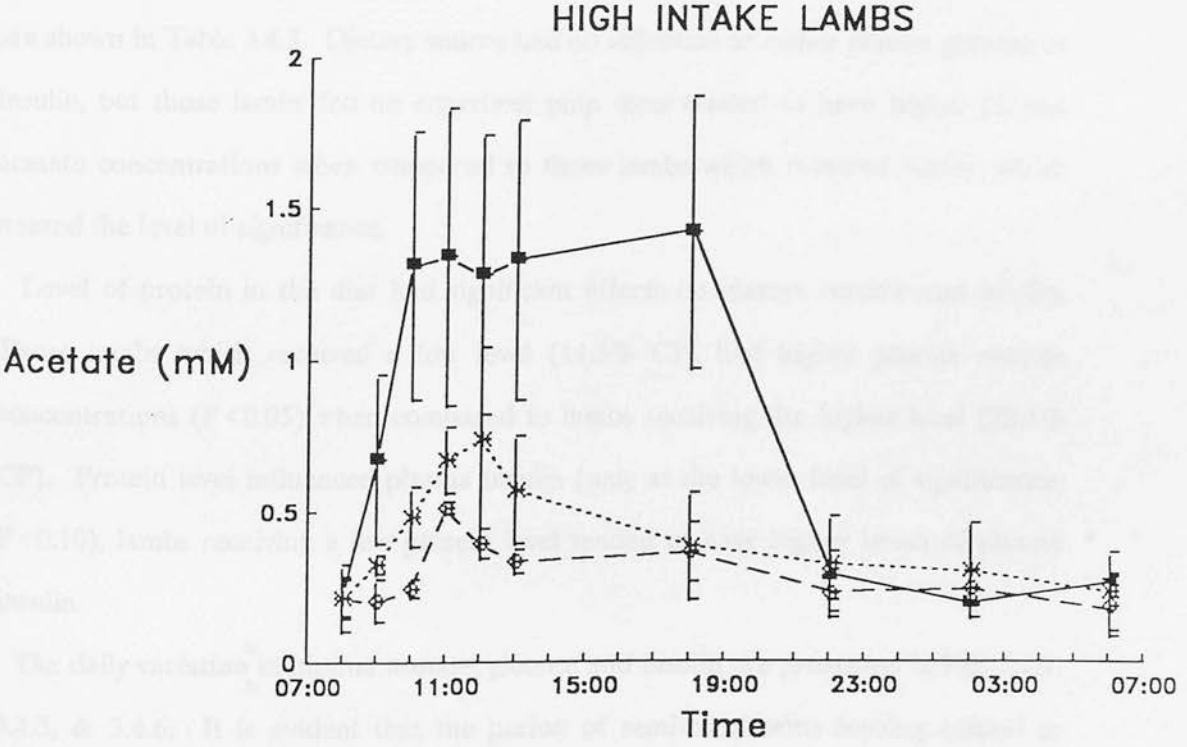
Time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Acetate (mM)
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Time

of feeding remained higher for approx. 4h longer than those fed the barley diet at the low level of feeding.

No response to feeding was observed for plasma glucose (Fig 3.4.2a & b), as the degree of variation was large. However plasma insulin levels (Fig 3.4.3a & b) increased markedly within 1h after feeding on all treatments. This response was most notable for lambs fed barley diets. No effect of feeding level on daily variation was observed.

It is apparent on examining the tables and graphs presenting the data of the selected plasma characteristics that much variation was encountered, both within a treatment (and hence individual animal variation) and between the different feeding levels. The latter is most probably a reflection of the irregular food intake of lambs on differing feeding levels. As noted above lambs receiving a low level of feeding tended to consume the majority of their food immediately upon receipt whilst those on the higher level consumed their ration over a much longer period.

Expt. 2. The mean (± S.E.M.) concentrations of plasma acetate, glucose and insulin are shown in Table 3.4.3. Dietary source had no influence on either plasma glucose or insulin, but those lambs fed on sugarbeet pulp diets tended to have higher plasma acetate concentrations when compared to those lambs which received barley, which neared the level of significance.

Level of protein in the diet had significant effects on plasma acetate and insulin. Those lambs which received a low level (11.5% CP) had higher plasma acetate concentrations ($P<0.05$) when compared to lambs receiving the higher level (20.1% CP). Protein level influenced plasma insulin (only at the lower level of significance, $P<0.10$), lambs receiving a low protein level tended to have higher levels of plasma insulin.

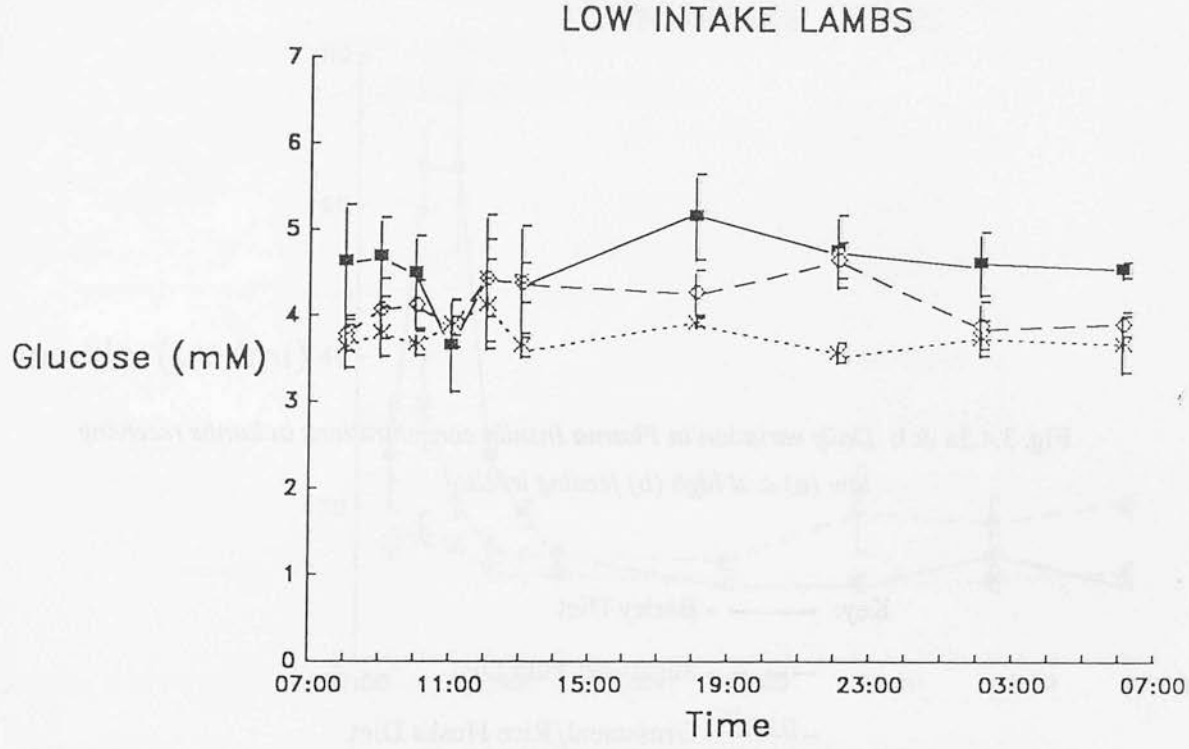
The daily variation in plasma acetate, glucose and insulin are presented in Fig. 3.4.4, 3.4.5, & 3.4.6. It is evident that the period of semi-continuous feeding helped to achieve near steady-state conditions. This is

Fig. 3.4.2a & b *Daily variation in Plasma Glucose concentrations in lambs receiving low (a) and high (b) feeding levels. Where standard error bars are omitted, they were too large to fit on the diagram.*

Key: ——— - Barley Diet

— — — - Sugarbeet Pulp Diet

------ - Grassmeal/Rice Husks Diet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Glucose (mM)
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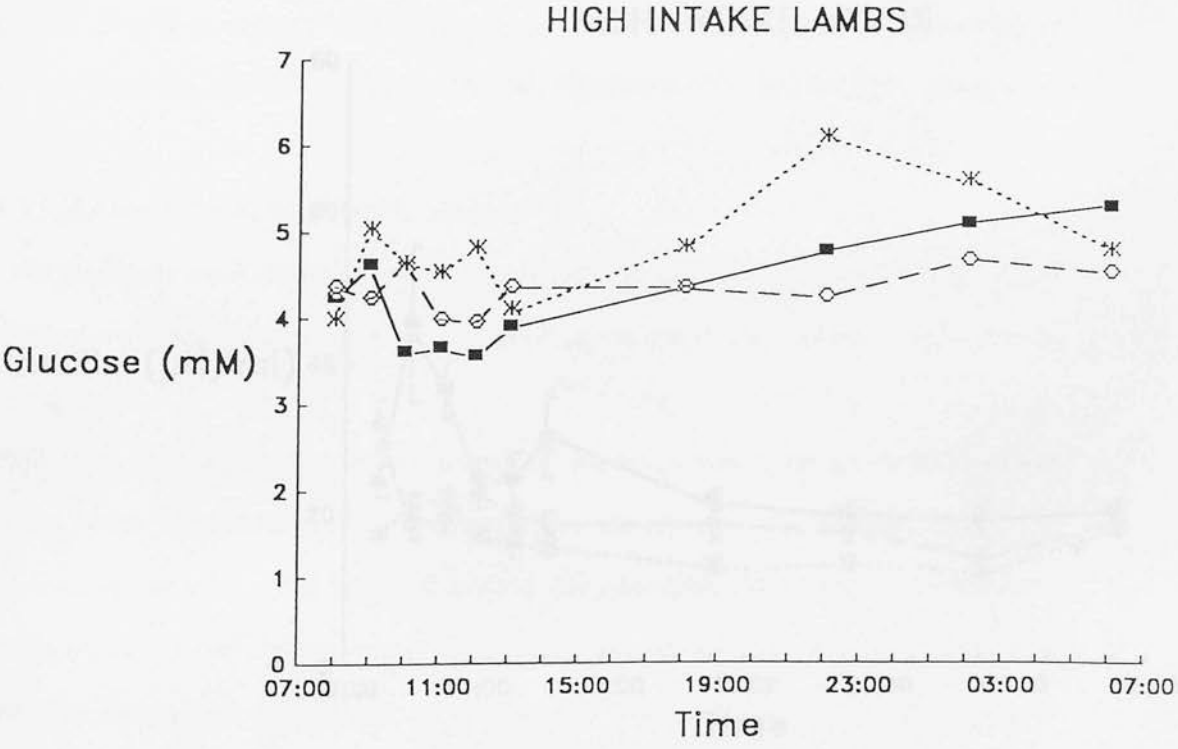
Time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Glucose (mM)
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07:00
11:00
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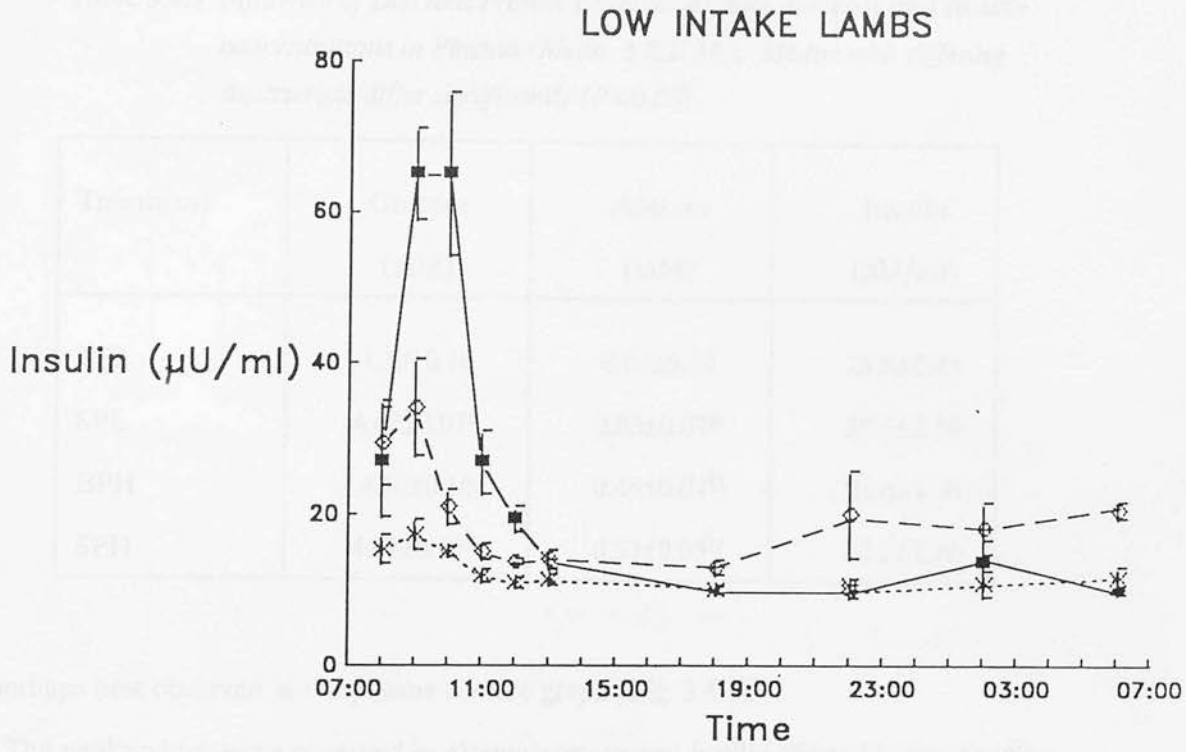
19:00
23:00
03:00
07:00
Time

Fig. 3.4.3a & b *Daily variation in Plasma Insulin concentrations in lambs receiving low (a) and high (b) feeding levels.*

Key: ——— - Barley Diet

— — — - Sugarbeet Pulp Diet

------ - Grassmeal/Rice Husks Diet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Insulin (μU/ml)
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60
40
20
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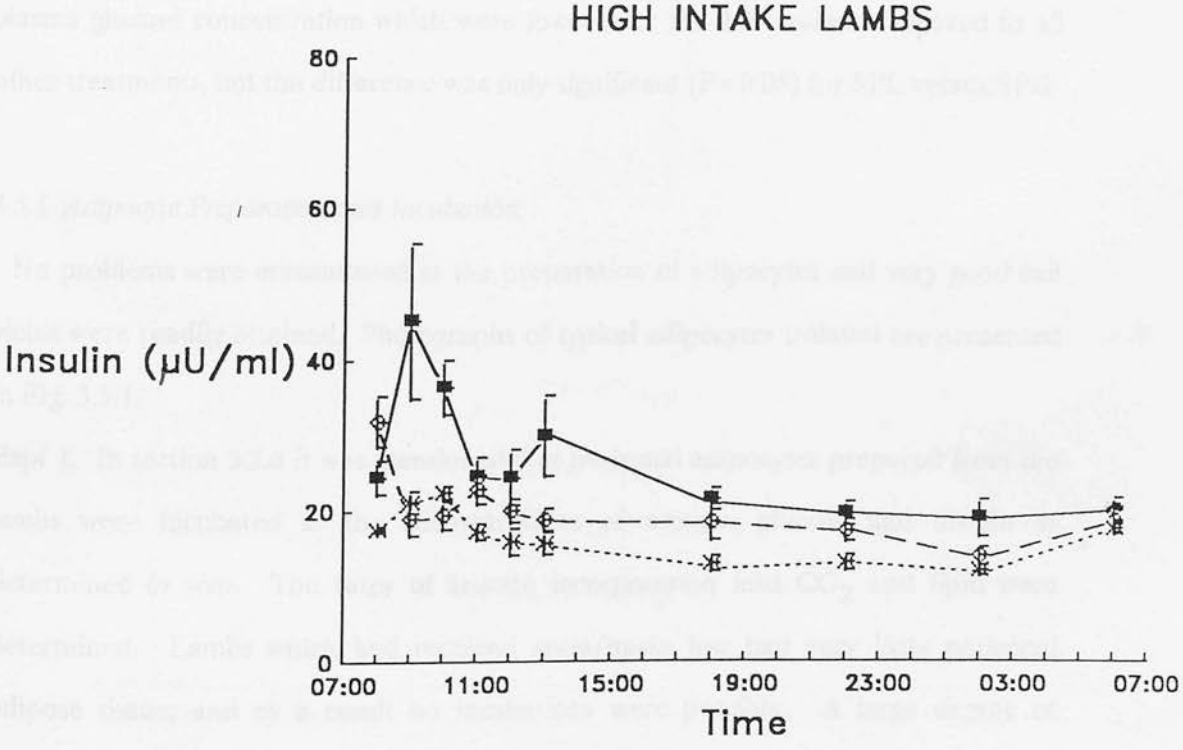
07:00
11:00
15:00
19:00
23:00
03:00
07:00
Time


HIGH INTAKE LAMBS
Insulin (μU/ml)
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60
40
20
0
07:00
11:00
15:00
19:00
23:00
03:00
07:00
Time

Table 3.4.3 *Influence of Diet and Protein Level on Acetate, Glucose and Insulin concentrations in Plasma (Mean ± S.E.M.). Means with differing superscripts differ significantly (P<0.05).*

Treatment	Glucose (mM)	Acetate (mM)	Insulin (μU/ml)
BPL	4.39±0.16	0.64±0.10	25.8±2.45
SPL	4.42±0.07^{a}	0.83±0.07^{a}	28.5±2.56
BPH	4.36±0.10	0.48±0.07^{b}	21.6±1.10
SPH	4.08±0.07^{b}	0.57±0.05^{b}	22.3±1.56

perhaps best observed in the plasma acetate graph (Fig. 3.4.4).

The peaks which were observed in plasma acetate and insulin (Expt 1) after feeding, were not detected in this expt. However it is notable that both insulin and glucose varied to a great extent over the 24h period, perhaps reflecting a circadian rhythm not related to feeding (Trenkle, 1978). Lambs receiving sugarbeet/high protein had plasma glucose concentration which were lower over the 24h period compared to all other treatments, but the difference was only significant ($P<0.05$) for SPL versus SPH.

3.5.1 *Adipocyte Preparation and Incubation*

No problems were encountered in the preparation of adipocytes and very good cell yields were readily attained. Photographs of typical adipocytes isolated are presented in Fig. 3.5.1.

Expt 1. In section 3.3.6 it was mentioned that perirenal adipocytes prepared from the lambs were incubated at the concentrations of acetate, glucose and insulin as determined *in vivo*. The rates of acetate incorporation into CO_2 and lipid were determined. Lambs which had received grass/husks low had very little perirenal adipose tissue, and as a result no incubations were possible. A large degree of variation was found in the rates obtained (Table 3.5.1) and no

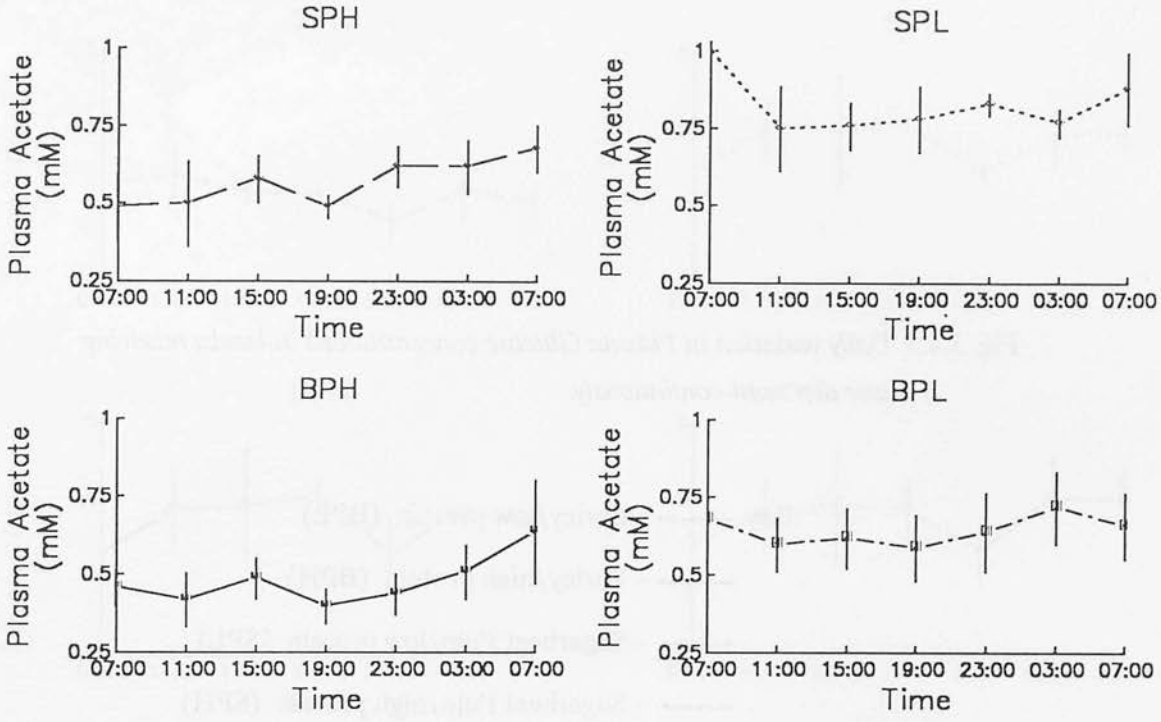
Fig. 3.4.4 *Daily variation in Plasma Acetate concentrations in lambs receiving their diet semi-continuously.*

Key —·—·— - Barley/low protein (BPL)

——— - Barley/high protein (BPH)

------- - Sugarbeet Pulp/low protein (SPL)

— — - Sugarbeet Pulp/high protein (SPH)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SPL
BPH
BPL
Plasma Acetate (mM)
1
0.75
0.5
0.25
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time

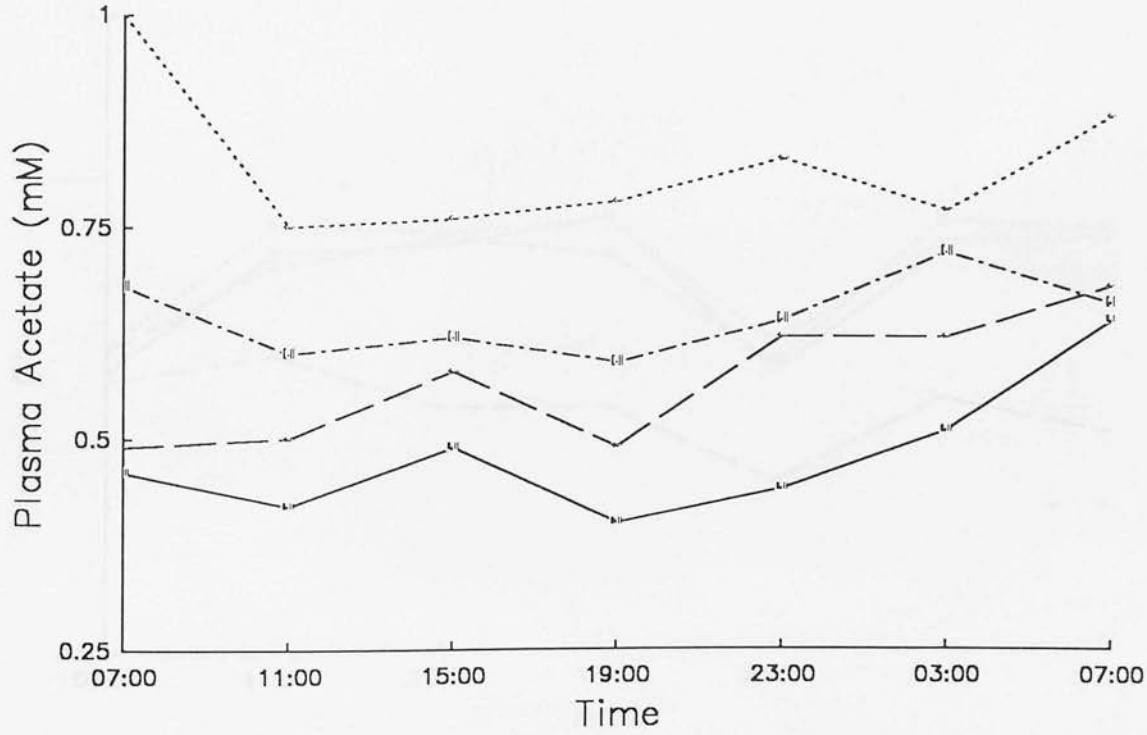

Plasma Acetate (mM)
1
0.75
0.5
0.25
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time

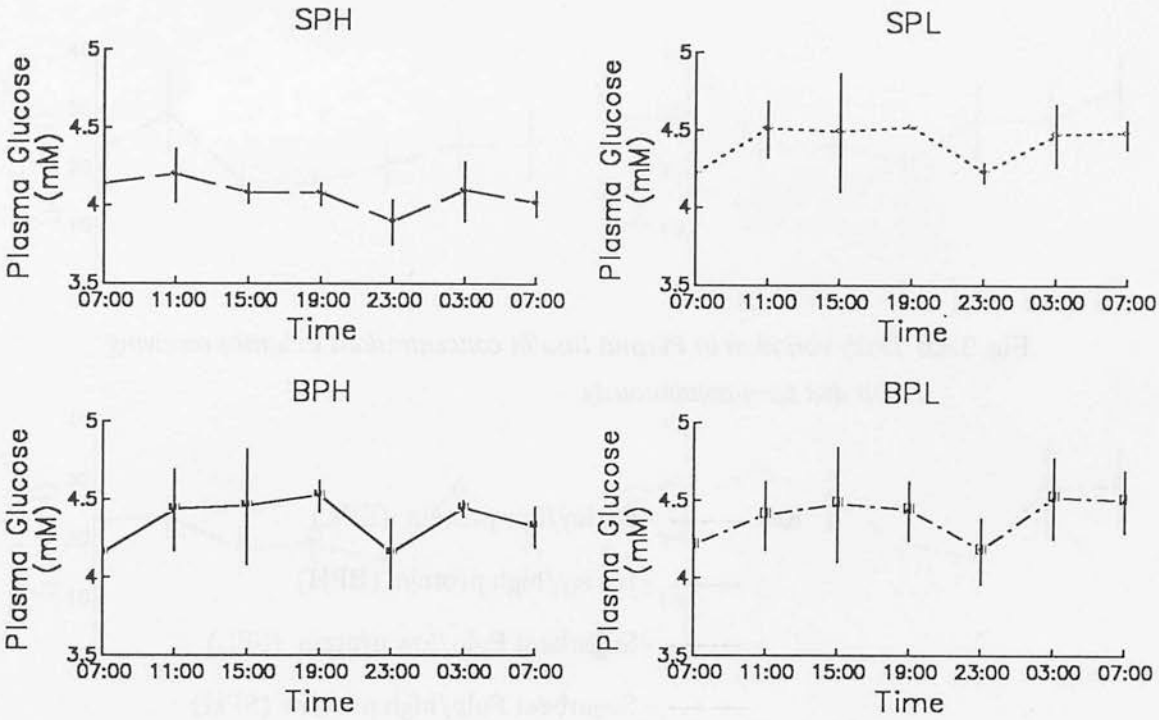
Fig. 3.4.5 *Daily variation in Plasma Glucose concentrations in lambs receiving their diet semi-continuously.*

Key —·—·— - Barley/low protein (BPL)

——— - Barley/high protein (BPH)

------ - Sugarbeet Pulp/low protein (SPL)

—— - Sugarbeet Pulp/high protein (SPH)


SPH
SPL
BPH
BPL
Plasma Glucose (mM)
5
4.5
4
3.5
07:00
11:00
15:00
19:00
23:00
03:00
07:00
Time

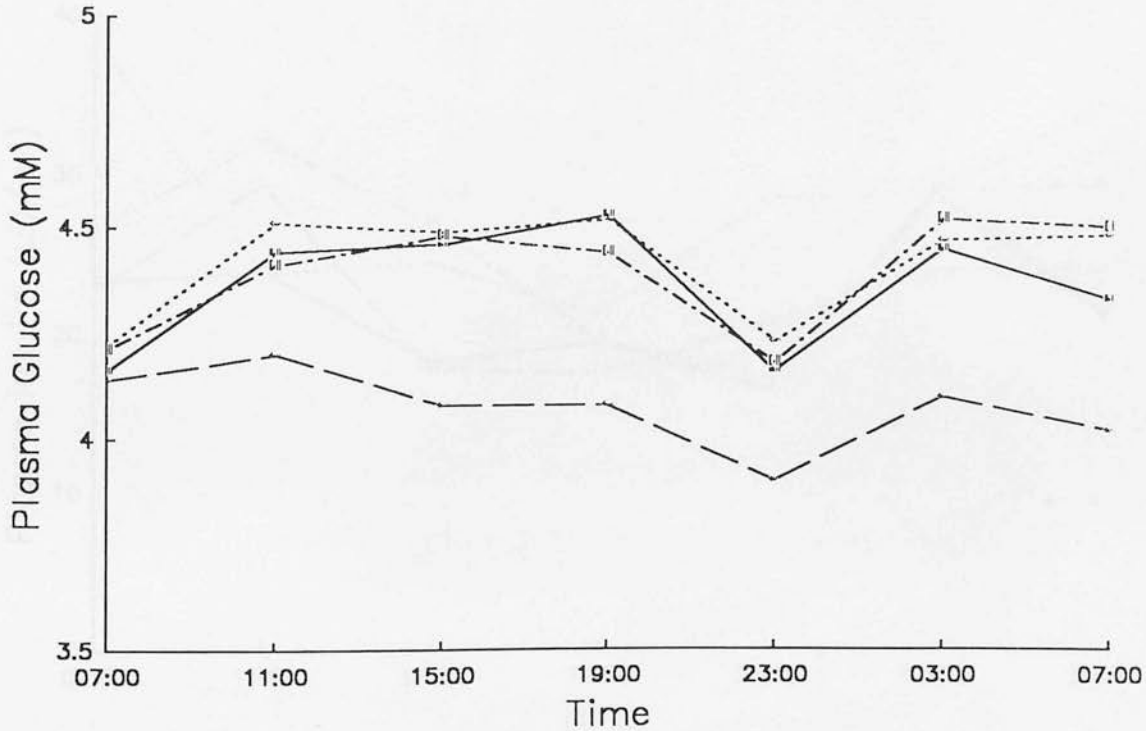

Plasma Glucose (mM)
5
4.5
4
3.5
07:00
11:00
15:00
19:00
23:00
03:00
07:00
Time

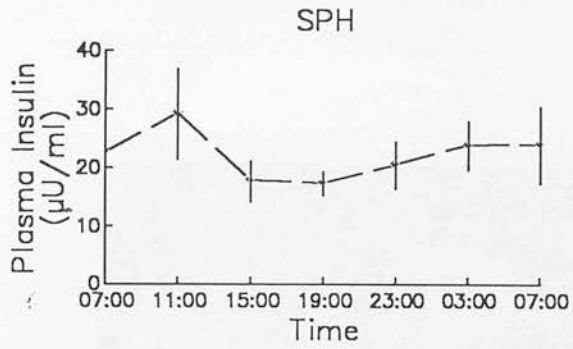
Fig. 3.4.6 *Daily variation in Plasma Insulin concentrations in lambs receiving their diet semi-continuously.*

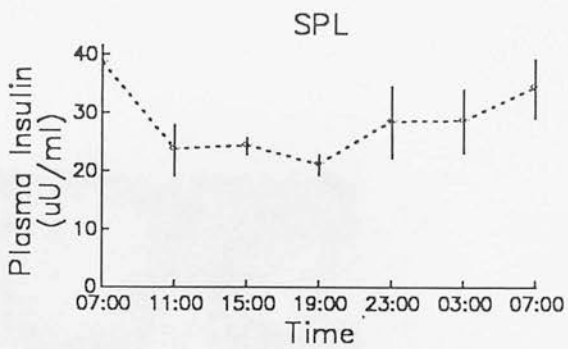
Key —·—·— - Barley/low protein (BPL)

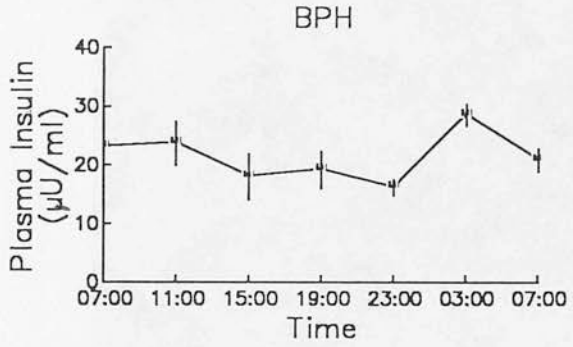
——— - Barley/high protein (BPH)

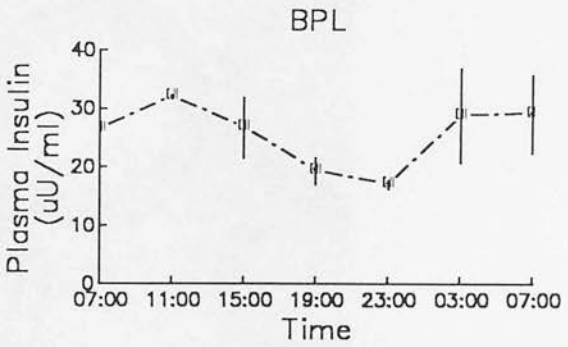
------- - Sugarbeet Pulp/low protein (SPL)

— — - Sugarbeet Pulp/high protein (SPH)


SPH
Plasma Insulin (μU/ml)
40
30
20
10
0
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time


SPL
Plasma Insulin (μU/ml)
40
30
20
10
0
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time


BPH
Plasma Insulin (μU/ml)
40
30
20
10
0
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time


BPL
Plasma Insulin (μU/ml)
40
30
20
10
0
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time

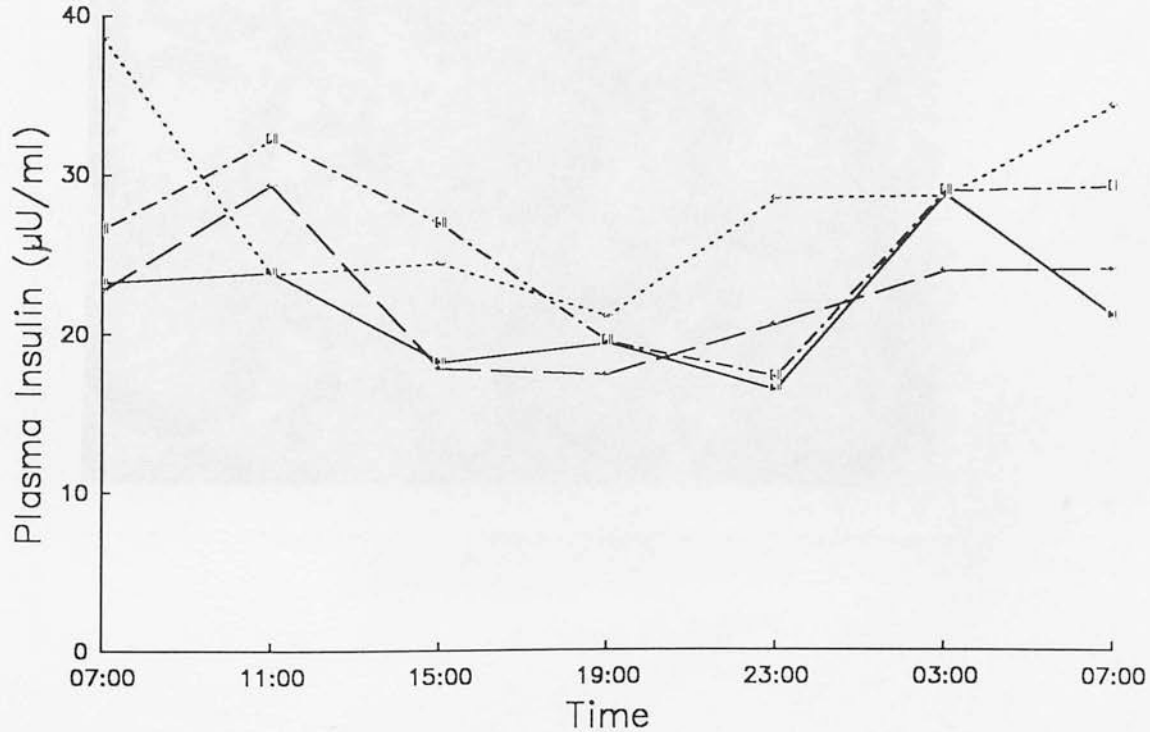

Plasma Insulin (μU/ml)
40
30
20
10
0
07:00 11:00 15:00 19:00 23:00 03:00 07:00
Time

Fig. 3.5.1 *Typical ovine perirenal adipocytes used in the incubations*

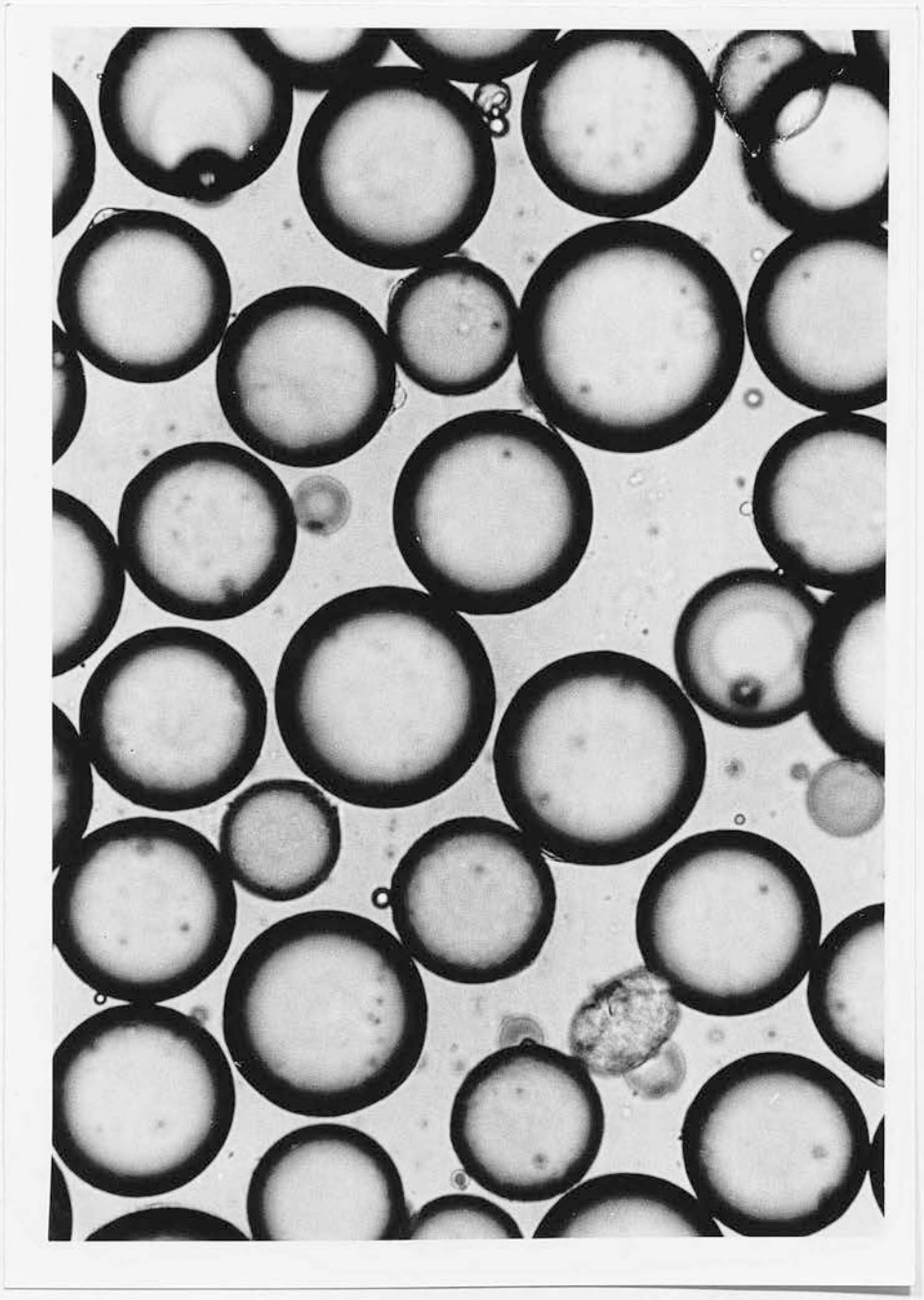


Table 3.5.1 *Expt.1. Individual data for the incorporation of acetate to CO_2 and lipid. Results are expressed as nmoles acetate incorporated per 2h per 10^6 cells. N.D. - not determined.*

TRT	Lamb No.	Acetate to CO_2	Acetate to Lipid
BL	46	81.0	969.6
BL	93	16.1	466.6
BL	38	70.0	2489.6
BL	127	19.3	1900.8
BH	31	39.0	1215.4
BH	96	17.5	2512.4
BH	25	34.9	1953.6
BH	124	7.28	945.6
SL	59	1.4	-.
SL	62	20.3	760.8
SL	80	10.5	271.4
SL	40	55.6	4824
SH	24	97.1	6854
SH	21	71.2	6648
SH	34	156.6	13015
GL	-	-	-
GH	74	38.8	667.2
GH	51	132.4	5179.2
GH	75	8.54	-.
GH	15	N.D.	2575.2

BL - Barley Low

BH - Barley High

SL - Sugarbeet Pulp Low

SH - Sugarbeet Pulp High

GL - Grass/Husks Low

GH - Grass/Husks High

meaningful analysis of the data was possible.

However since these were essentially the first adipocyte incubation conducted in this study, this exercise served to highlight some of the potential pitfalls which may be encountered with these incubations. It was concluded that too many cells were added to each incubation vessel. As indicated in section 3.3.3 the number of cells per vessel ought to range between 10^4-10^5 cells. Inexperience resulted in there being little control over the volume of the final cell suspension in this experiment, with the result that the number of cells per incubation vessel too great. Since fat cells float, many of the cells were not in contact with the incubation media. In this situation those cells not in contact with the media release substances which inhibit acetate oxidation and lipogenesis (Dr P. Sinett-Smith, personal communication). In future preparations the number of cells per ml was assessed prior to incubation (see section 3.3.3).

Fig. 3.5.2 presents the mean ± S.E.M. (3) of increasing the concentration of acetate in the incubation media on the rates of acetate incorporation into CO_2 and lipid. The result obtained in the presence of 0mM and [1-^{14}C] acetate was invalid, since the very small amount of acetate present as ^{14}C acetate would have been incorporated very quickly, resulting in an inaccurate measure of the rate of acetate incorporation. The rate of acetate incorporation into CO_2 increased above 1mM ($P<0.01$), but little effect was observed in the rate of acetate incorporation into lipid.

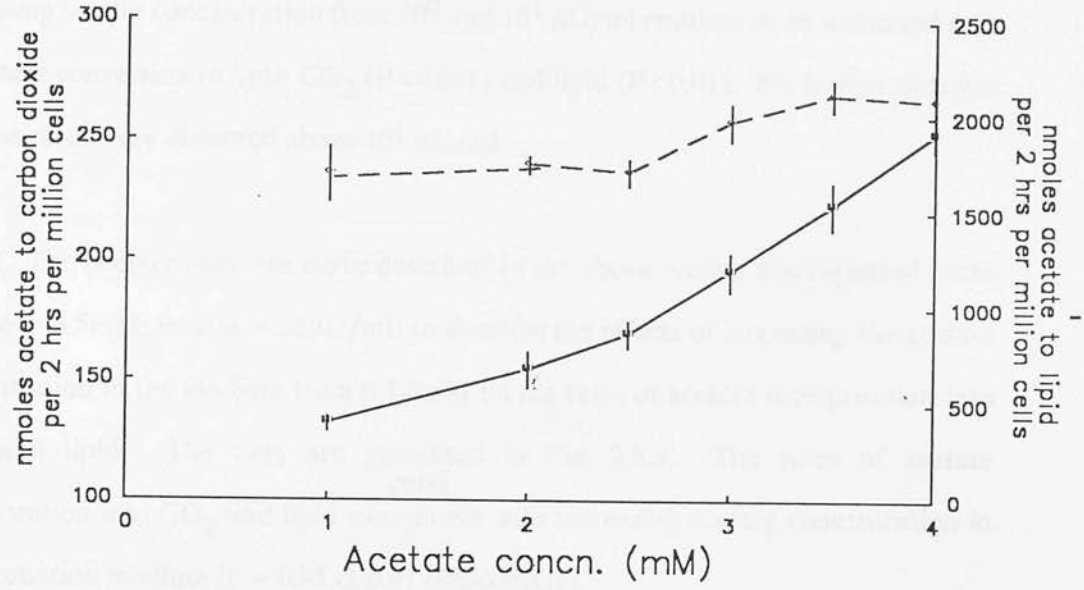
Fig. 3.5.3 describes the mean ± S.E.M. (3) of increasing the concentration of glucose in the incubation media on the rates of acetate incorporation into CO_2 and lipid. Increasing the glucose concentration from 0 to 2.0mM resulted in significant increases in both acetate incorporation into CO_2 ($P<0.001$) and lipid ($P<0.001$). Glucose concentrations greater than 2.0mM had no further effects.

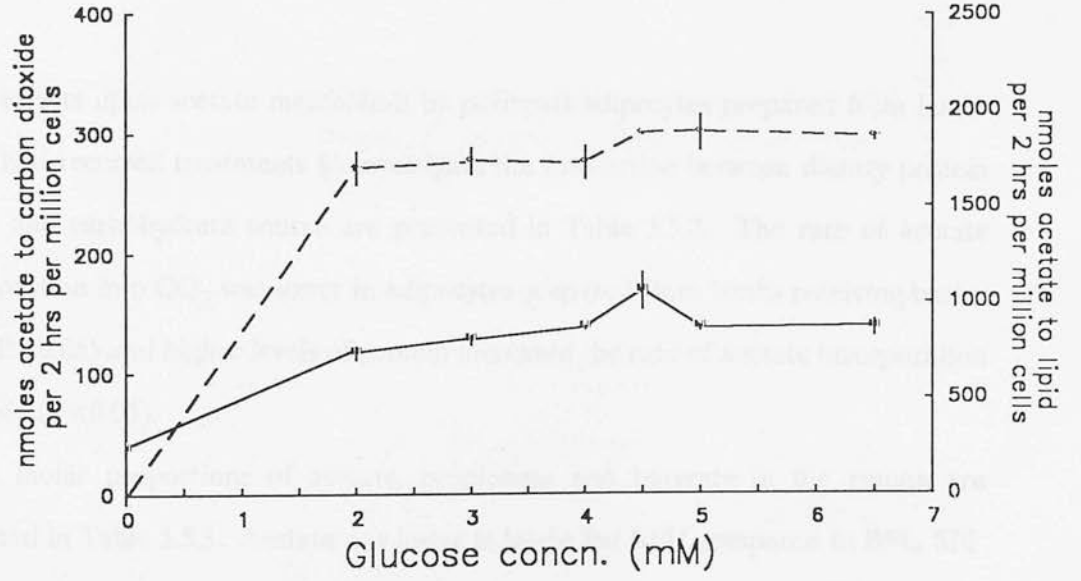
Fig. 3.5.4 presents the mean ± S.E.M. (3) of increasing the concentration of insulin in the incubation medium on the rates of acetate incorporation into CO_2 and lipid.

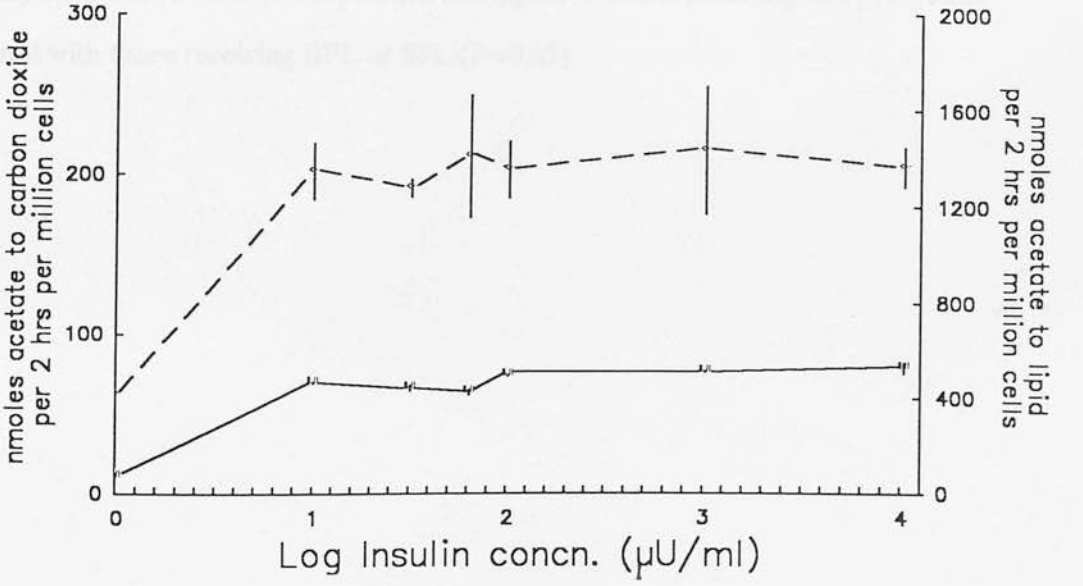
Fig. 3.5.2, 3.5.3, 3.5.4 *Acetate, Glucose and Insulin Response Curves for the incorporation of Acetate into CO_2 and Lipid, by ovine perirenal adipocytes.*

Key: ——— - Acetate to CO_2

– – – – - Acetate to Lipid


nmoles acetate to carbon dioxide per 2 hrs per million cells
nmoles acetate to lipid per 2 hrs per million cells
Acetate concn. (mM)


nmoles acetate to carbon dioxide per 2 hrs per million cells
nmoles acetate to lipid per 2 hrs per million cells
Glucose concn. (mM)


nmoles acetate to carbon dioxide per 2 hrs per million cells
nmoles acetate to lipid per 2 hrs per million cells
Log Insulin concn. (μU/ml)

Increasing insulin concentration from 10^0 and 10^1 μU/ml resulted in an increased rate of acetate conversion to both CO_2 ($P<0.001$) and lipid ($P<0.01$). No further changes in these rates were observed above 10^1 μU/ml.

Expt 2. The acetate response curve described in the above section was repeated (note glucose = 4.5mM; insulin = 35μU/ml) to describe the effects of increasing the acetate concentration in the medium from 0-1.0mM on the rates of acetate incorporation into CO_2 and lipid. The data are presented in Fig. 3.5.5. The rates of acetate incorporation into CO_2 and lipid were curvi linear with increasing acetate concentration in the incubation medium (r = 0.95 & 0.97 respectively).

The effects upon acetate metabolism by perirenal adipocytes prepared from lambs which had received treatments to investigate the interaction between dietary protein supply and carbohydrate source are presented in Table 3.5.2. The rate of acetate incorporation into CO_2 was lower in adipocytes prepared from lambs receiving barley diets ($P<0.05$) and higher levels of protein increased the rate of acetate incorporation into lipid ($P<0.05$).

The molar proportions of acetate, propionate and butyrate in the rumen are presented in Table 3.5.3. Acetate was lower in lambs fed BPH compared to BPL, SPL ($P<0.01$) and SPH ($P<0.05$). Propionate was higher in lambs receiving BPH ($P<0.05$) compared with those receiving BPL or SPL ($P<0.05$).

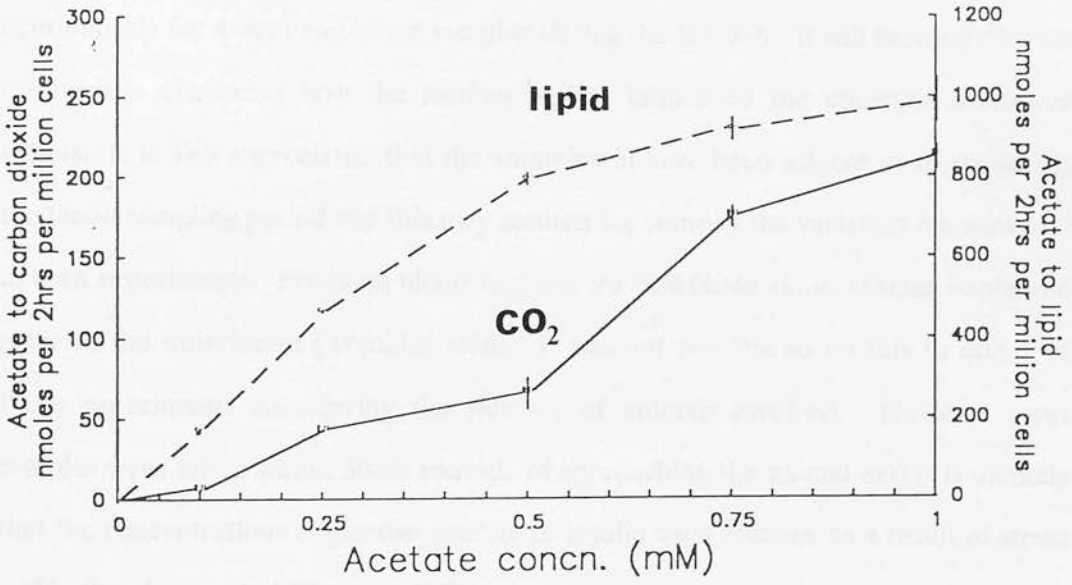
Table 3.5.2 *Effect of Carbohydrate Source and Crude Protein Level on the rates of Acetate incorporation into CO_2 and Lipid by perirenal adipocytes.* Results are expressed as *nmoles acetate incorporated per 2h per 10^6 cells. Means with differing superscripts differ significantly ($P<0.05$).*

Treatment	Acetate to CO_2	Acetate to Lipid
BPL	294±20.6^a	1306±101^a
SPL	421±40.0^b	1200±137^a
BPH	310±27.0^a	1632±101^b
SPH	355±27.0	1714±58

Table 3.5.3 Influence of Carbohydrate Source and Crude Protein Level on Rumen Molar Proportion of Acetate, Propionate and Butyrate. Means with differing superscripts differ significantly ($P<0.05$).

Treatment	Acetate	Propionate	Butyrate
BPL	0.67±0.01^b	0.20±0.02^b	0.11±0.02
SPL	0.65±0.01^b	0.23±0.01^b	0.09±0.01
BPH	0.54±0.03^a	0.33±0.03^a	0.08±0.01
SPH	0.64±0.01^b	0.25±0.02	0.08±0.01

Fig. 3.5.5 *Acetate response curve (0-1.0mM) for the incorporation of acetate into CO_2 and lipid.*



3.6 DISCUSSION

3.6.1 *Effect of Carbohydrate Source, Feeding Level and Protein Level on Plasma Concentrations of Glucose, Acetate and Insulin.*

The essential difference between experiment 1 and experiment 2 was the conditions under which the animals were blood sampled. In experiment 1 lambs were fed once daily and blood sampled over a 24h period whilst in experiment 2 lambs were fed semi-continuously for 4 days and blood sampled during the last 24h. It will become obvious during this discussion how the feeding regime influenced the observed treatment effects. It is also appreciated that the animals will have been subject to stress during the blood sampling period and this may account for some of the variation encountered in both experiments. Frequent blood samples are best taken via a catheter implanted prior to the experiment (Trenkle, 1978). It was not possible to do this in either of these experiments considering the number of animals involved. However most samples were taken within 30-60 seconds of approaching the animal and it is unlikely that the concentrations of glucose, acetate or insulin were affected as a result of stress within this time period (Trenkle, 1978).

The daily mean glucose concentrations ranged from 3.78-4.53mM in experiment 1 (Table 3.4.1) and 4.02-4.42 in experiment 2 (Table 3.4.3). These values are similar to published values for sheep (Trenkle, 1978, Galbraith, *et al.,* 1988). Feeding frequency, carbohydrate source, feeding level or protein level did not affect plasma glucose concentrations. This reflects the high degree of efficiency maintaining glucose homeostasis. The ruminant animal absorbs little or no glucose from the digestive tract and its glucose requirement must be synthesised, primarily from propionate and amino acids. Acute changes are required in the pattern of VFA formation in the rumen to significantly influence plasma glucose. Abdul-Razzaq *et al.* (1988), demonstrated that a 108% increase in rumen propionate resulted in only a 10% increase in plasma glucose. Jenny *et al.*, (1972) showed that cows fed a high starch diet had significantly higher plasma glucose compared to fibre fed animals. This study found that

demonstrating higher concentrations of glucose in the plasma on starch based diets is difficult to achieve, even if the animals are fed frequently as in expt. 2 (minimising variation as a result of irregular feed intake).

Plasma glucose was consistently lower for sheep fed on sugarbeet/high protein (Fig. 3.4.5), the mean daily concentration was 4.07 mM (Table 3.4.1), 7% lower than the mean of the other 3 diets (significant only, $P<0.05$ for SPL/SPH). A similar, non-significant effect may be observed in the data of Galbraith *et al.* (1988). In the rumen, the synchrony of energy (derived from CHO fermentation) and nitrogen supply is critical for the synthesis of microbial protein. On the SPH treatment, sugarbeet may be fermented slowly, limiting energy supply, whilst nitrogen derived from protein degradation may be plentiful. As a result the efficiency of nitrogen incorporation in microbial protein may be reduced. Excess nitrogen may be absorbed as NH_3 into the bloodstream, transported to the liver and converted to urea. Studies conducted in conjunction with this experiment demonstrated that the total oxygen consumption by the liver determined *in vitro* was approximately 30% greater for lambs fed on SPH when compared to all other treatments. It is possible that this increased O_2 consumption may in part reflect increased rates of urea synthesis and hence a greater demand for glucose.

Mean plasma acetate concentrations are typical of published values for ruminants (Madsen, 1983), ranging from 0.29-0.99mM in expt 1 (Table 3.4.1) and 0.48-0.83mM in expt 2 (Table 3.4.3). In expt 1 plasma acetate values were higher (but significant only at 10% level) for lambs fed on barley, when compared to sugarbeet and grass/rice husks. Feeding level had no effect. Ross & Kitts (1973) also reported higher plasma acetate concentrations in lambs fed on barley based diets. This effect is primarily a result of the rapid and more significant rise in the plasma acetate concentrations in lambs fed on barley diets immediately after feeding compared to the other diets (see below), because this starch based diet is rapidly fermentable. Hence when daily means are calculated barley diets tend to average higher than other less rapidly fermentable

diets. In expt 2, under a semi-continuous feeding regime, lambs fed on sugarbeet pulp diets had higher plasma acetate concentrations when compared to those given barley diets. However protein level in the diet had a more significant effect upon plasma acetate. A low level of protein (11.5% CP) was associated with higher plasma acetate concentrations ($P<0.05$).* It is possible that this reflects an effect on both the utilisation and production of acetate. The molar proportion of acetate in the rumen was lower on the BPH treatment but the value for SPH was no different from the other two treatments (SPL, BPL) (Table 3.4.3). Hence the lower plasma acetate concentration for BPH suggests either an increase in the rate of acetate production or increased utilisation. The SPH value most likely reflects increased acetate utilisation since the molar proportion in the rumen is similar to SPL & BPL. The higher plasma acetate concentrations for treatments SPL & BPL probably reflect increased acetate production and a lower rate of acetate utilisation. Presumably on high protein treatments the additional amino acids provided may act as glucogenic precursors facilitating the utilisation of acetate as proposed by MacRae & Lobley (1982). This is particularly pertinent in view of the fact that the concentrations of plasma acetate increase in the order BPH < SPH < BPL < SPL, demonstrating the protein effect.*

In expt 1 a post-prandial rise in plasma acetate was observed across all treatments (Figs 3.4.1a & b) and this was greater in the plasma of lambs fed on barley when compared to those fed on sugarbeet pulp or grass/rice husks. Similar trends were observed by Ross & Kitts (1973). The increase was more prolonged for lambs fed on the barley based diet at the high level of feeding, probably reflecting the high rate of acetate absorption from the rumen on fermentation of a highly digestible diet. In expt 2 the period of semi-continuous feeding resulted in near-steady-state conditions for plasma acetate.

This discussion has contrasted the mean acetate concentrations in terms of the rate of acetate production in the rumen and the rate of acetate utilisation by tissues. One can only speculate as to what these concentrations confer. Determination of acetate concentrations in the plasma are of little value for evaluation of nutrient supply unless

* Fig. 3.4.4

it is associated with a measure of acetate entry rate. However Annison *et al.* 1974 found plasma acetate concentrations to be 48% and acetate entry rate to be 77% higher in lactating cows fed a high fibre as compared with a low fibre diet. Using the equation of Pethick & Lindsay (1982) (see section 1.2.3), the acetate entry rates for SPL, BPL, SPH and BPH are 1.61, 1.22, 1.07, and 0.89 mmol. h^{-1}. kg^{-1} body-weight. These figures have been calculated using the mean acetate values presented in Table 3.4.1. It is appreciated that these entry rates are calculated from concentrations of acetate in venous blood and the equation of Pethick & Lindsay (1982) specifies the use of arterial acetate concentrations. Nevertheless it is likely that these acetate concentrations are similar to arterial, since the blood from which the samples were taken will be returning from the brain, which utilises very little acetate (Lindsay & Setchell, 1973). Unfortunately accurate body-weight figures are not available to obtain actual acetate entry rates.

Mean plasma insulin concentrations ranged from 12.2-27.9 μU/ml in expt 1 (Table 3.4.1 & 3.4.2) and 21.6-28.5 μU/ml in expt 2 (Table 3.4.3). These values fall within the normal range (Trenkle, 1970; Bassett, 1971; Galbraith *et al.*, 1988). In expt 1 feeding level had no effect on plasma insulin but diet did, plasma insulin being higher in sheep fed on barley diets compared to sugarbeet pulp or grass/rice husks, similar to the trend observed by Galbraith *et al.* (1988, 1989). In expt 2 analysis of variance indicated that protein affected plasma insulin levels. Mean insulin values were higher on low protein when compared to high protein treatments (but significant only at the $P<0.1$). This is contrary to the findings of Waghorn *et al.* (1987) and Galbraith *et al.* (1988) who reported that high protein levels were associated with increased levels of plasma insulin. A high correlation ($r=0.74$) has been demonstrated between the amount of protein digested in the intestine and plasma insulin (Bassett *et al.*, 1971). It is probable that the lower values obtained in this study on the high protein treatments are as a result of less carbohydrate in these treatments compared to the low protein treatments. It is unlikely to be related to the semi-continuous feeding regime to which

the animals were subjected during the sampling period, since the lambs in the study of Waghorn *et al.* (1987) were fed hourly for 40d.

In animals fed once per day an increase in plasma insulin is usually observed within one hour after feeding (if blood sampling is frequent), followed by a second peak 4-6h later (Trenkle, 1978). Such an initial peak was observed in those lambs fed on the barley diet in expt 1 (Fig. 3.4.3a & b, expt 1), but due to the high degree of variation the second peak was obscured. The first increase is not associated with an increase in plasma glucose (Figs 3.4.2a & b), although the second increase in plasma insulin normally occurs with an increase in plasma glucose (Bassett, 1974; Trenkle, 1978), suggesting that the absorption of glucogenic precursors may have stimulated insulin release. The secretion of insulin in response to the ingestion of feed is thought to be related to several factors (Trenkle, 1978). The initial response is probably a release of gastrointestinal hormones resulting from the passage of feed into the intestine and the second a combination of absorption of VFA from the rumen and gluconeogenic precursors from the intestine. All the factors which contribute to enhanced insulin secretion from the pancreas have been incorporated into one term, "*entero-insular axis*" (Morgan *et al.*, 1988).

Changes in the circulating levels of insulin in sheep have been shown to reflect changes in the rate of insulin secretion rather than changes in turnover (Trenkle, 1978). Hence in this study it is probable that changes in insulin concentration likewise reflect changes in the rate of secretion.

3.6.2 Acetate Metabolism by Perirenal Adipocytes

3.6.2.1 *Effect of varying glucose, acetate, and insulin on the rates of acetate incorporation into CO_2 and lipid in vitro.*

Glucose availability had significant effects on acetate utilisation, (Fig. 3.5.3). Acetate oxidation and conversion to lipid was low in the absence of glucose. Increasing the glucose concentrations from 0-2mM caused a 3-4 fold increase in the rate of acetate conversion to CO_2, and greatly stimulated the rate of acetate conversion to lipid.

Further increases in glucose concentration above 2mM had little effect. These observations are consistent with those of Yang & Baldwin (1973). The results demonstrate that acetate utilisation in ovine perirenal adipocytes is greatly enhanced by glucose. The low rate of acetate incorporation into lipid in the absence of glucose probably demonstrates a lack of NADPH and glycerol-3-phosphate. Acetate conversion to CO_2 in the absence of glucose suggests that acetate is oxidised, probably providing some NADPH (via isocitrate dehydrogenase) and ATP (via TCA cycle), but since glycerol-3-phosphate (synthesised from glucose) is limiting, little acetate is incorporated into lipid. At 2mM glucose maximal rates of acetate oxidation and conversion to lipid have been attained, and hence glucose availability is no longer a limiting factor.

Acetate availability had significant effects on acetate utilisation by perirenal adipocytes (Figs. 3.5.2 & 3.5.5). The rates of acetate incorporation into CO_2 and lipid increased linearly with increasing acetate concentrations in the incubation medium from 0.1-1.0mM (Fig. 3.5.5). At 1.0mM acetate the mean rates of acetate conversion to CO_2 and lipid were approximately 33 and 5.8 times greater respectively, than at 0.1mM acetate. Increasing acetate concentrations above 1.0mM significantly increased the rate of acetate oxidation ($P<0.01$), but had little effect on the rate of acetate incorporation into lipid. Similar results were observed by Yang & Baldwin (1973).

These results suggest that maximal rates of fatty acid synthesis from acetate was attained at approx. 1mM acetate. However the continued effects of acetate upon acetate oxidation above 1mM may represent a metabolic adaptation with physiological merit. Since the ruminant must synthesise the majority of its glucose requirement, it is in the animals interests to conserve glucose. The increased oxidation of acetate above 1mM acetate with little effect upon acetate incorporation into lipid, suggests that acetate may function to provide NADPH and ATP necessary for fatty acid synthesis. This would relieve the glucose demand, in terms of the provision of NADPH and ATP.

Tracer studies investigating the oxidation of labelled glucose to CO_2 by adipocytes might help to clarify the situation. One would expect to observe an effect upon the ratio [1-^{14}C] glucose/[6-^{14}C] glucose above 1mM acetate, reflecting less glucose metabolism via the pentose phosphate pathway. This might indicate a greater demand upon acetate for NADPH and ATP production. Yang & Baldwin (1973) incubated bovine adipocytes in the presence of glucose (2.5mM), either [1-^{14}C] glucose or [6-^{14}C] glucose and with or without acetate (5mM). The presence of acetate had little influence on the ratio [1-^{14}C] glucose/[6-^{14}C] glucose. However as discussed the critical situation is likely to be observed around 1mM acetate.

Insulin had a significant effect on acetate utilisation (Fig. 3.5.4). Increasing the insulin concentration in the incubation medium, increased acetate conversion to CO_2 and lipid. Increasing the insulin concentration from 10^0 to 10^1 μU/ml caused a 6 fold and a 3 fold increase in the rate of acetate conversion to CO_2 and lipid, respectively. Insulin concentrations greater than 10^1 μU/ml had little further effect.

In the ruminant published data on the effects of insulin on lipogenesis are not very convincing. Numerous reports have indicated that the synthesis of fatty acids from acetate and glucose was not markedly affected by insulin in short term incubations *in vitro* (1-3h) (see Vernon, 1981; Prior & Smith, 1982; Weekes, 1986). Longer term incubations have demonstrated that the decline in the rate of acetate incorporation into lipid could be prevented by the inclusion of insulin in the medium (Vernon, 1979). It has been suggested that lack of response to insulin may be related to the purity of the BSA used in the incubation medium. Etherton *et al.* (1984) found that certain types of BSA inhibit insulin binding to pig adipocytes and stimulated lipogenesis in the absence of insulin (Walton *et al.*, 1984). These finding stimulated a review of insulin effects upon bovine adipocytes in short term incubations (2h) (Etherton & Evock, 1986). Similar results were obtained and it was found that insulin profoundly affected adipocyte metabolism. The rate of acetate incorporation into lipid was maintained by insulin concentrations ranging from 0-10 ng/ml (approx. 25-225 μU/ml). Maximal

response to insulin was attained at 30 ng/ml (approx. 730 μU/ml). This is very much different to the findings of this study, which found little response to insulin above 10μU/ml. The results of Etherton & Evock (1986) demonstrated that lipogenesis responded to insulin concentrations normally found *in vivo*. Lack of response to insulin above 10 μU/ml in this study may reflect insulin or insulin-like growth factor contamination of the BSA (Etherton & Evock, 1986), which may bind to the insulin receptors on the membrane of the adipocytes, preventing a response to the insulin added to the medium.

Numerous other information (see Prior & Smith, 1982) suggests that insulin plays a primary role in the control of adipose metabolism. Insulin appears to increase the uptake of glucose, availability of glycerol-3-phosphate and to stimulate lipoprotein lipase with the net overall effect of increasing triglyceride deposition.

3.6.2.2 *Effect of Carbohydrate and Protein Level on Acetate Metabolism by Ovine Perirenal Adipose Tissue in vitro.*

Carbohydrate source (barley or sugarbeet pulp) and protein level (11.5 & 20.1% CP) influenced the rate of acetate incorporation into CO_2 and lipid respectively. Two-way analysis of variance indicated that feeding fermentable fibre (sugarbeet pulp) increased the incorporation of acetate into CO_2 ($P<0.05$) compared to fermentable starch (barley). In comparison, increased levels of crude protein increased the incorporation of acetate to lipid ($P<0.05$).

Feeding sugarbeet pulp resulted in a greater demand for acetate oxidation in perirenal adipocytes compared to barley. This effect is most profound for the SPL treatment, 26% of total acetate metabolised by adipocytes was oxidised compared to 16-18% on all other treatments (Table 3.5.1).

This may reflect a low availability of glucose (or glucose precursors), limiting the provision of NADPH and glycerol-3-phosphate which are necessary for the incorporation of acetate into lipid. In these circumstances increased oxidation of acetate in the TCA cycle will provide limited NADPH (via isocitrate

Table 3.5.1 *The Percentage of Total Acetate Metabolised as CO_2 and Lipid.*

TRT	% Acetate CO_2	% Acetate Lipid
SPL	26	74
SPH	17	83
BPL	18	82
BPH	16	84

dehydrogenase) and ATP, for fatty acid synthesis.

Higher crude protein levels increased the amount of acetate incorporated into lipid. This effect was most prominent between treatments SPL & SPH. On treatment SPL 74% of total acetate metabolised by adipocytes was incorporated into lipid, compared to 83% on treatment SPH (Table 3.5.1).

Presumably the effect of protein upon fatty acid synthesis is mediated via an increased supply of glucogenic precursors, (amino acids). The provision of glycogenic precursors, in turn, enhances the incorporation of acetate into lipid via greater supplies of NADPH, glycerol-3-phosphate and ATP. Walsh *et al.* (1990) have reported similar observations in steers receiving silage diets supplemented with fishmeal (150g fresh weight per kg silage DM). Compared to steers fed on silage only, the rates of acetate incorporation into lipid were increased approx. 2.1 fold in steers fed on silage plus fishmeal (determined using the *in vitro* incorporation of [1-14C] acetate into lipid in subcutaneous adipose tissue, sampled from the brisket region). Waghorn *et al.*, (1987) reported increased rates of fatty acid synthesis (1.7 fold) in Romney wether sheep fed on diets high in crude protein (22% CP) compared to ones low in crude protein (12% CP). The rates of fatty acid synthesis were determined by intravenous injection of tritiated water, and after 2h animals were slaughtered and fat samples obtained and the amount of ^3H_2O as fatty acid determined. These results agree with this study, in

that protein has a major effect upon fatty acid synthesis.

3.6.3 Efficiency of Utilisation of ME

The efficiency of utilisation of ME from a fibre based diet is lower compared to an equivalent amount of ME from a starch based diet. This may be related to the inability of the ruminant animal to metabolise excess amounts of acetate commonly associated with fibre diets (Hovell *et al.*, 1976; Blaxter, 1980; MacRae & Lobley, 1982). The results of this study support this hypothesis.

Differences in the molar proportions of rumen VFA existed, higher acetate and lower propionate were observed on sugarbeet pulp when compared to barley treatments. The increased oxidation of acetate on SPL may reflect a limited availability of glucose for lipogenesis and hence the demand for NADPH and ATP may in part be met by the oxidation of acetate. The increased oxidation of acetate may also be the result of an increase in the rate of substrate cycling between acetate and acetyl-CoA. A previous experiment demonstrated that the activities of the enzymes involved in this cycle namely acetyl-CoA hydrolase and acetyl-CoA synthetase tend to be higher on sugarbeet pulp based diets (Chapter 2).

Insulin stimulates fatty acid synthesis and it has been shown that the range of insulin concentrations *in vivo* stimulated acetate incorporation into lipid (Etherton & Evock 1986). The increased efficiency of ME use of barley based diets may simply be the result of increased plasma insulin stimulating the uptake of acetate and glucose and the activity of lipoprotein lipase by adipocytes and consequently enhancing the rates of fatty acid synthesis from acetate.

However it is likely that this is only part of an explanation explaining the efficiency of ME utilisation. MacRae & Lobley (1982) suggested that in those studies which failed to find any differences in the efficiency of utilisation of acetate, the availability of glucose precursors (either propionate or amino acids) was plentiful. The increased rates of lipogenesis on treatments SPH and BPH indicate that the level of crude protein in the diet may influence the utilisation of acetate. This is reflected in the

levels of plasma acetate. Higher plasma acetate concentrations were associated with lower levels of crude protein. It is well known that amino acids may act as precursors for the synthesis of many important metabolites, especially glucose and glycolytic intermediates (Trenkle, 1980; Lindsay, 1979, 1982; MacRae & Lobley, 1986). When the availability of propionate for glucose synthesis is limited, to facilitate the utilisation of acetate, N retention is reduced, reflecting a breakdown of body protein, providing amino acids for gluconeogenesis (Ørskov et al., 1979, Girdler *et al.*, 1986). The concentrations of plasma amino acids, plasma urea and the tyrosine flux and the rate of tyrosine oxidation were higher in lambs fed diets resulting in an acetate rather than an propionate type of fermentation, suggesting that amino acids are used for other purposes (Abdul-Razzaq *et al.*, 1989). In the same study the efficiency of protein retention as expressed by the ratio deposited protein:synthesised protein was higher in animals fed diets resulting in a propionate rather than an acetate fermentation.

If all these observations are integrated it may be concluded that the efficiency of ME utilisation is related to the maintenance of glucose homeostasis. When a ruminant animal is fed a diet which results in a large proportion of acetate and which provides little glucose precursors, the animal will breakdown protein providing amino acids which may be used for glucose synthesis. In turn glucose may facilitate the utilisation of acetate. These effects would be mediated by insulin and glucagon.

Hence this hypothesis would explain the observations of Emmans *et al.*, (1989) who found that the efficiency of ME utilisation was associated with protein rather than lipid synthesis and would also explain why the efficiency of ME utilisation may be related to the utilisation of acetate. This theory represents a dramatic illustration of the interaction between carbohydrate and protein metabolism and would merit further investigation.

CHAPTER FOUR

Separation and Concentration of Organic Acids

and

Calculating the Activity of the Substrate Cycle between Acetate and Acetyl-CoA

Abstract

1. A technique was developed for the separation and concentration of organic acids. This involved the use of open column ion exchange chromatography, freeze drying and high performance liquid chromatography. Organic acid recoveries were 43-69%.

2. The use of tracers to determine the activity of substrate cycling is described, and applied to the substrate cycle between acetate and acetyl-CoA.

Key Words: Organic acids Chromatography Substrate cycling

4.1 CONCENTRATION and SEPARATION of ORGANIC ACIDS

The experiment measuring the rate of substrate cycling between acetate and acetyl-CoA, required a technique to be developed for the concentration and separation of organic acids (primilarly acetate, acetoacetate and 3-hydroxybutyrate) from deproteinised blood. The method used was a modification of that used by Jessop (1984) and Rumsby *et al.* (1987). The separation procedure depended upon the use of two chromatographic procedures, open column ion exchange and High Performance Liquid Chromatography (HPLC).

The method was developed using deproteinised ovine blood, spiked with [^{14}C] acetate, hence permitting the efficiencies of the various stages involved to be assessed.

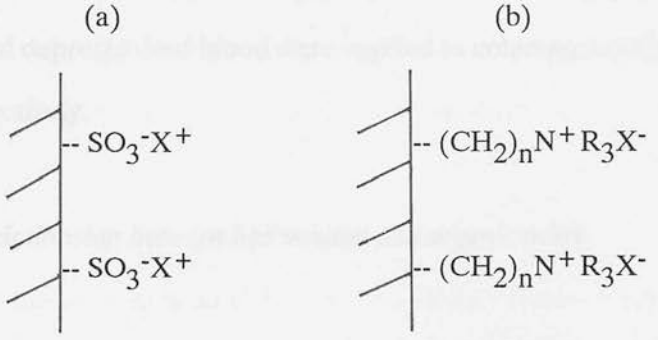
Blood was obtained from sheep by venopuncture. This was kept on ice and transported to the laboratory. The blood was pooled (approx. 100ml) in a 250ml beaker and an equal volume of 6% $HClO_4$ was added. 8ml fractions were centrifuged for 20min at 3000xg (MSE Mistral 4L), at 4^{o}C. Supernatants were pooled in a beaker, kept on ice and the pH was adjusted to 7.0 using 2M potassium hydroxide/0.5M triethanolamine. The deproteinised blood was spiked with [1-^{14}C] sodium acetate and the radioactivity in 3x250µl aliquots was determined. The deproteinised blood was then frozen (-20^{o}C) until required.

4.1.1 *Stage 1 Open Column Ion Exchange Chromatography*

Ion exchange chromatography is frequently conducted as an initial step to "clean-up" the protein free supernatent. This separates compounds such as amino acids which may otherwise elute simultaneously with the organic acids during HPLC analysis, making quantitative analysis difficult (McCord *et al.*, 1984). In columns designed to separate compounds by the mechanism of ion exchange the stationary phase consists of a support matrix, either silica or resin, to which is bonded a charged moeity. Each charged group forms an ionic bond with a counterion such as H^+, Na^+, Ca^{2+} in the case of cation exchange columns or Cl^-, OH^-, acetate$^-$ in the case of anion exchange columns (Fig. 4.1a & b). The mobile phase usually contains this counterion at low

concentrations. Introduction of the cationic species to a cation exchange column or anionic species to an anion exchange column leads to competition between species and the counterion for the oppositively charged sites affixed to the stationary phase. Since the formation of ionic interactions between the column bound charges and the counterion is a dynamic process, the presence of the original counterion in the mobile phase ensures that the species to be separated migrate through the column at a rate inversely proportional to their affinity for the column bound charge. To elute species that are strongly bound it is often necessary to progressively increase the concentration of the original counterion in the mobile phase during the course of the separation.

Fig. 4.1 *The interaction between the column bound charge and the counterion.*



Choice of the original counterion affects the separation of a given mixture of compounds by altering the relative affinities of the compounds to be separated and the counterion for the column bound charge.

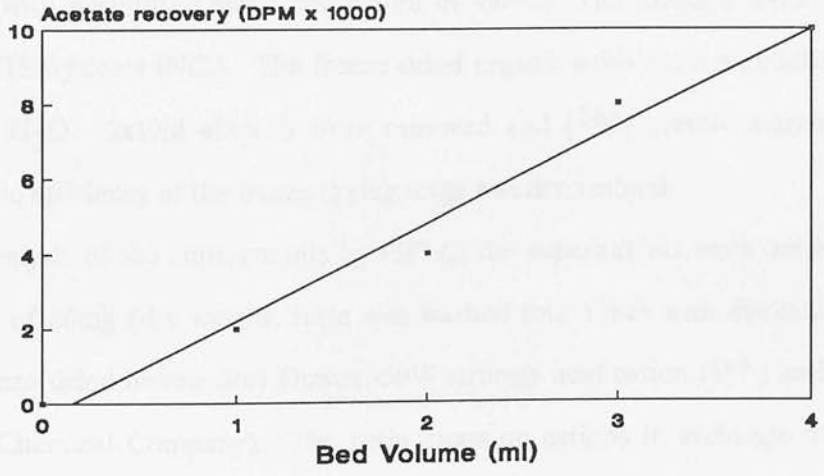
A strongly basic anion exchange resin, Dowex-1 chloride form (100-200 micron mesh), obtained from Sigma Chemical Company was used. The exchange group of the polystrene based resin is ϕ -$CH_2N^+(CH_3)_3Cl^-$.

Resin bed column holders - Econocolumns (BioRad Laboratories) 8mm x 5cm long were used. A frit (TechElut Reservoir Frit - HPLC Technology Ltd, Macclesfield, Cheshire) was placed at the bottom of each column. Each frit was moistened with methanol and rinsed with distilled H_2O to initiate column flow. The resin was first

washed several times in distilled H_2O. Columns were packed to the required volume. The recovery of organic acids is dependent upon the form of resin used. Since $R\text{-}COO^-$ ions will displace OH^- ions more readily than Cl^- ions the resin was converted to the OH^- form from the Cl^- by the columnwise application of 5 volumes of 1M NaOH followed by a thorough washing with distilled H_2O. This step was performed immediately before application of sample. Deproteinised blood spiked with [^{14}C] acetate was applied to the column. Neutral and positively charged compounds were washed from the column with 6ml of distilled water and the organic acids (as represented by ^{14}C acetic acid) were eluted with 15ml 10% $HClO_4$.

It was calculated that the amount of acetate contained in 5ml of blood (10ml of deproteinised blood), assuming a blood acetate concentration of 1.0mM, would be sufficient for detection by HPLC. Hence it was necessary to determine the amount of resin which would bond all the organic acids in 10ml of deproteinised blood. 2, 4, 8 and 10ml of deproteinised blood were applied to columns containing 1, 2, 3. and 4ml of resin respectively.

Fig. 4.2 *Relationship between bed volume and organic acids*



A 4ml bed volume was required to bind all the organic acids in 10ml of protein free supernatant (determined by recovery of [^{14}C]) (Fig 4.2). Addition of 10ml deproteinised blood to a column containing 3ml of resin resulted in a recovery of only 70% of the ^{14}C acetate contained in the 10ml. Fig. 4.3a illustrates the elution pattern of ^{14}C acetate with time from the commencement of application of $HClO_4$ to the column (determined by collecting fractions every 15sec over an eight minute period and assessing the amount of [^{14}C] by liquid scintillation counting). Organic acids began to wash off the column after approximately 2.75min and were completely eluted by the sixth minute. Fig. 4.3b indicates the elution of acetic acid versus the amount of perchloric acid added to the column. The acid eluted between approximately 4.5 and 12ml.

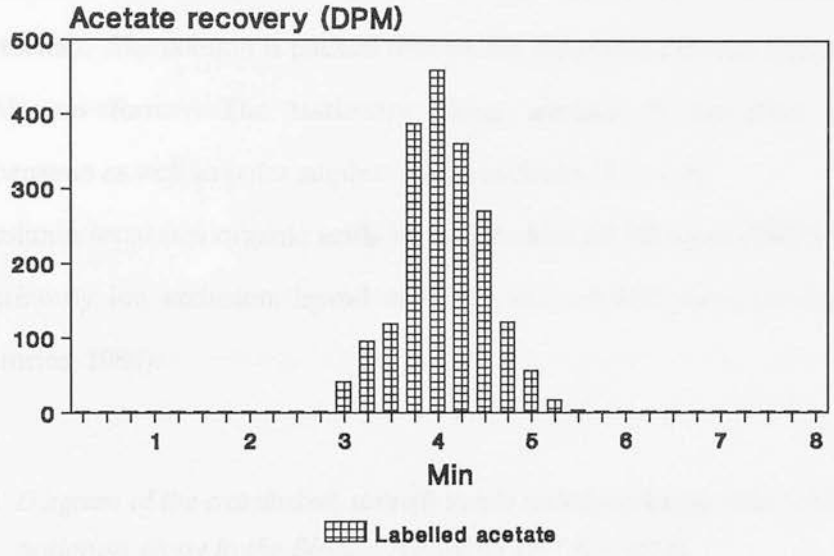
The pH of the organic acid fraction was adjusted to 9.0 with 5M KOH, on ice to precipitate $KClO_4$. The supernatant was decanted and total volume determined (usually about 20ml). Two 250μl fractions were removed and the [1-^{14}C] determined. Hence the efficiency of the first separation procedure (ion exchange) was assessed.

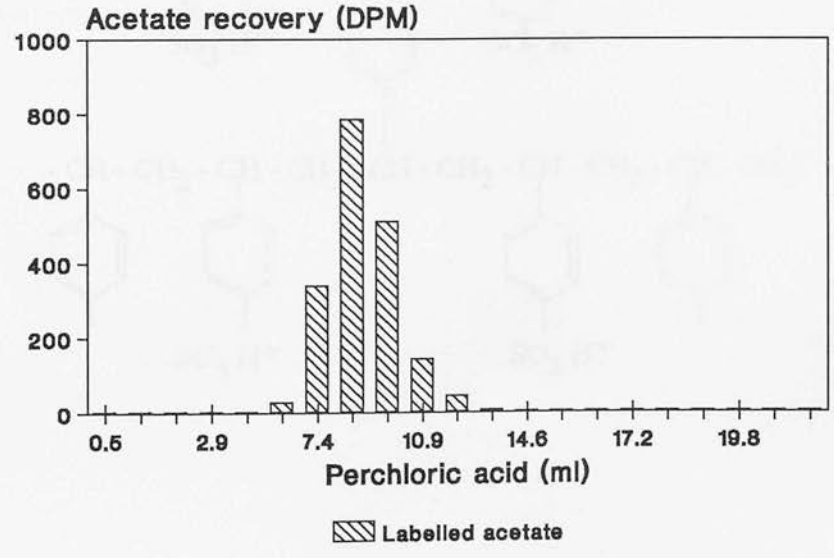
4.1.2 *Stage 2 Freeze Drying*

The neutralised organic acid fraction was further subdivided into 2x20ml cylindrical vessels (with perforated caps) and frozen at -60^{o}C. The samples were then freeze dried (FTS Systems INC.). The freeze dried organic acids were redissolved in 0.5ml distilled H_2O. 2x10μl aliquots were removed and [^{14}C] acetate activity assessed. Hence the efficiency of the freeze drying stage was determined.

For analysis of the supernatants by HPLC, the supernat nts were acidified by the addition of 80mg (dry weight; resin was washed four times with distilled water and then freeze dried before use) Dowex -50W strongly acid cation (H^+) exchange resin (Sigma Chemical Company). The resin mops up cations in exchange for H^+ ions resulting in acidification of the supernatant.

Fig. 4.3 a) *Elution period of the organic acids and b) the influence of volume of perchloric acid upon the elution of the organic acids, determined using [1-^{14}C] acetate.*



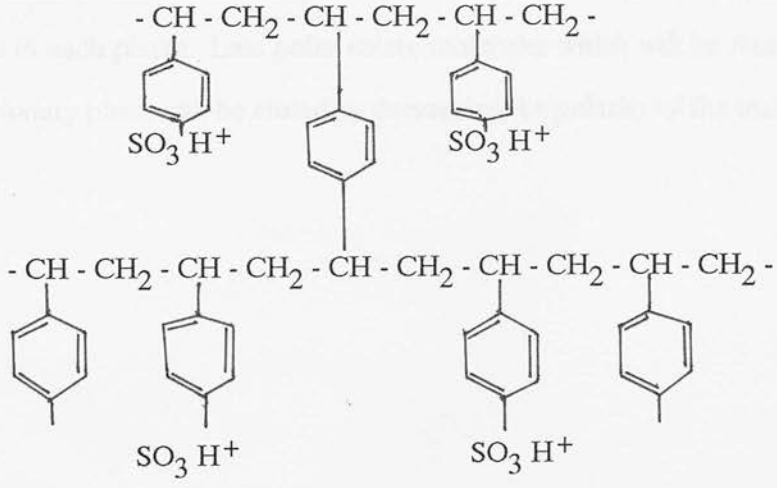


4.1.3 *Stage 3 Separation of Organic Acids by High Performance Liquid Chromatography.*

Separation of organic acids was achieved by isocratic HPLC, using a column specially manufactured for the analysis of organic acids - an Aminex HPX-87H column (BioRad Laboratories). The column is packed with an 8% crosslinked cation exchange resin in the hydrogen form. The stationary phase consists of non-polar polystyrene-divinylbenzene as well as polar sulphonic acid moieties (Fig. 4.4).

The column separates organic acids by Ion Moderated Partition (IMP) mechanisms using primarly ion exclusion, ligand exchange and reverse phase partition (BioRad Laboratories, 1980).

Fig. 4.4 *Diagram of the crosslinked, strongly acidic cation exchange resin used as the stationary phase in the BioRad HPX-87H HPLC column.*



Ion Exclusion - in columns designed for solute separation by ion exclusion, the stationary phase carries either a fixed negative or positive charge. Ions of like charge are excluded from the intra-particle space due to charge repulsion (Fig 4.5). The more highly charged the species is the more rapidly it elutes from the column since it is excluded from a greater proportion of the intra-particular volume. In all cases this form of separation occurs in combination with other mechanisms.

Ligand Exchange - Separation by ligand exchange is based on the interaction of the sample with a counterion (compared with the interaction of the solute with the column bound charge in ion exchange chromatography) ionically bonded to the stationary phase (Fig. 4.6).

Reverse Phase Partition - Separation of solutes by reverse phase chromatography is perhaps the most widely used of HPLC techniques. The stationary phase is less polar than the mobile phase and solutes partition between the two phases in relation to their solubilities in each phase. Less polar solute molecules which will be strongly retained by the stationary phase can be eluted by decreasing the polarity of the mobile phase.

Fig. 4.5 *Ion exclusion. Molecules of like charge to the stationary phase are excluded from the intra-particular volume.*

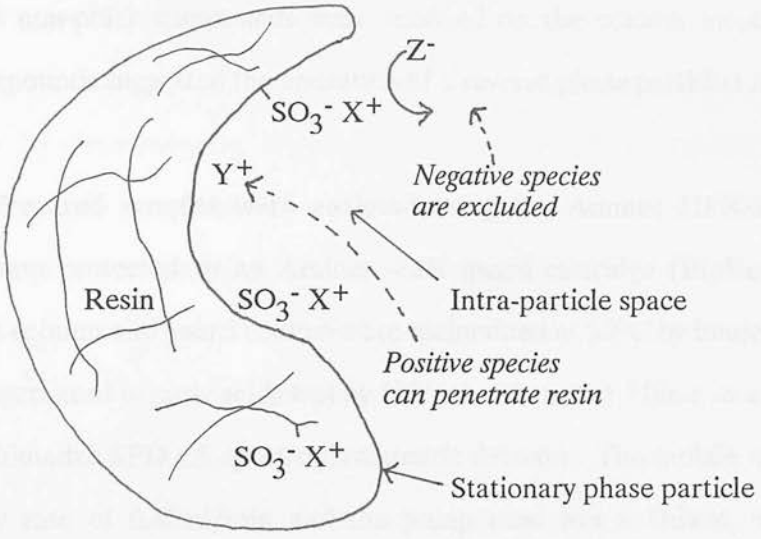
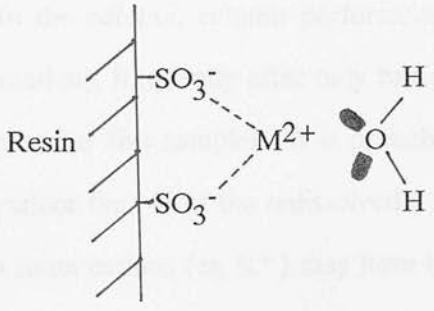


Fig. 4.6 *The interaction between the non-bonding orbitals of an oxygen atom of a solute and a counterion of the stationary phase*



Separation of organic acids on the BioRad HPX-87H indicated that the correlation betwen retention and pka was not as good as would be expected and the observation that non-polar compounds were retained on the column more strongly than polar compounds suggested the operation of a reverse phase partition mechanism.

Prepared samples were analysed using the Aminex HPX-87H organic analysis column protected by an Aminex -85H guard cartridge (BioRad Laboratories Ltd). The column and guard column were maintained at 55^{o}C by immersion in a water bath. Detection of organic acids was by U.V. absorbance at 210nm in a 8μl flow cell fitted to a Shimadzu SPD 6A spectrophotometric detector. The mobile was 5mM H_2SO_4 at a flow rate of 0.45ml/min and the pump used was a Gilson, model 302. Optimal separation of organic acids was achieved using these operating conditions (Jessop, 1984; Rumsby *et al.*, 1987).

The separation of seven organic acid standards is presented in Fig. 4.7. Fig. 4.8 is a typical chromatogram describing the separation of a typical acidified protein free blood supernatant.

Maintaining column resolution was a major problem. Depending upon the sample volume injected onto the column, column performance deteriorated rapidly (peaks begin to split and broaden), frequently after only two samples and the column rarely performed adequately after five samples. It is probable that the Dowex -50W resin, even though it may reduce the pH of the redissolved fraction to 1, may not remove all the cations and thus some cations (eg K^+) may have been injected onto the column. If so these ions may convert the column resin from the H^+ form, to for instance the K^+ form. Bed volume may therefore have been reduced, resulting in the formation of a void at the top of the column and loss of resolution. 100μl sample volumes resulted in the column deteriorating more rapidily than 50μl volumes (compare Fig. 4.8 [50μl] and Fig. 4.9 [100μl]). This was corrected by back-flushing the column with 25mM H_2SO_4 (flow rate 0.1 ml/min), at 65^{o}C for 10-16h. Periodically the column was

cleaned using 40% acetonitrile in 5mM H_2SO_4 (flow rate 0.1 ml/min), at 65°C for 4h.

The experiment (as will be discussed later) was primarily interested in collecting the ketone body (acetoacetate, 3-hydroxybutyrate) and acetate fractions. From the chromatograms presented it is clear that these fractions elute in the order acetoacetate, 3-hydroxybutyrate, acetate. It was not possible to completely separate the two ketone bodies, and so they were collected as one fraction. The distinction between the end of the 3-hydroxybutyrate fraction and the beginning of the acetate fraction was not always clear. Hence the fractions collected were used only to determine the amount of radioactivity present in each and the concentrations of the ketone bodies and acetate were determined enzymatically on the original deproteinised blood samples to minimise errors, as a result of contamination.

Fig. 4.7 *Chromatogram showing the separation of a mixture of seven organic acid standards under the following conditions:*

Column: BioRad Organic Acid Analysis Column HPX-87H

Flow rate: 0.45 ml/min

Mobile Phase: 5mM H_2SO_4

Column temp: 55°C

Detection: UV absorbance at 210nm

Peak identity:

1. Citric acid
2. Malic acid
3. Succinic acid
4. Acetoacetic acid
5. 3-Hydroxybutyric acid
6. Acetic acid
7. Propionic acid

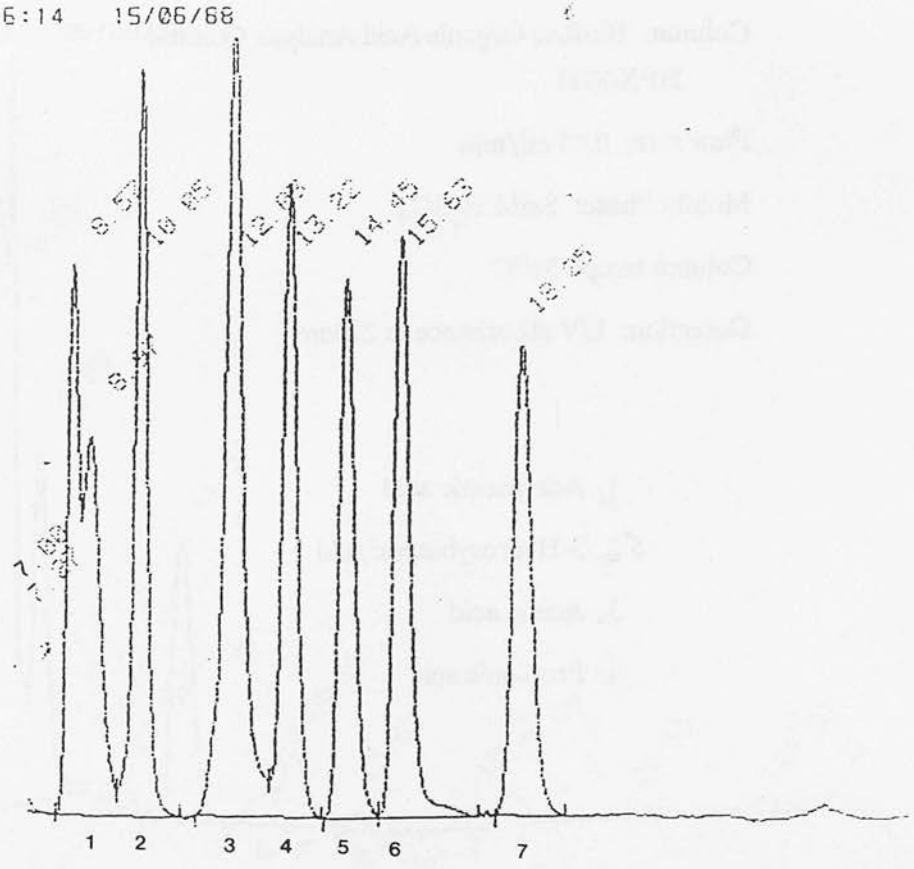

16:14 15/06/88
9.50
10.05
12.03
13.22
14.45
15.63
18.25
1 2 3 4 5 6 7

Fig. 4.8 *Chromatogram showing the separation of the organic acids present in a 50µl sample of a protein free supernatant of blood.*

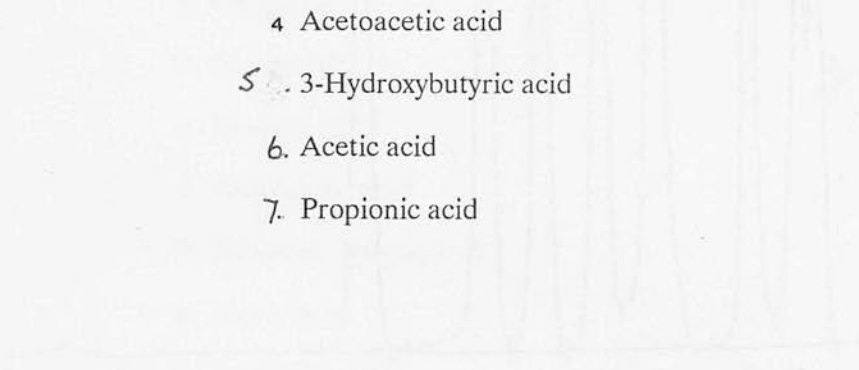
Column: BioRad Organic Acid Analysis Column
HPX-87H

Flow rate: 0.45 ml/min

Mobile Phase: 5mM H_2SO_4

Column temp: 55°C

Detection: UV absorbance at 210nm



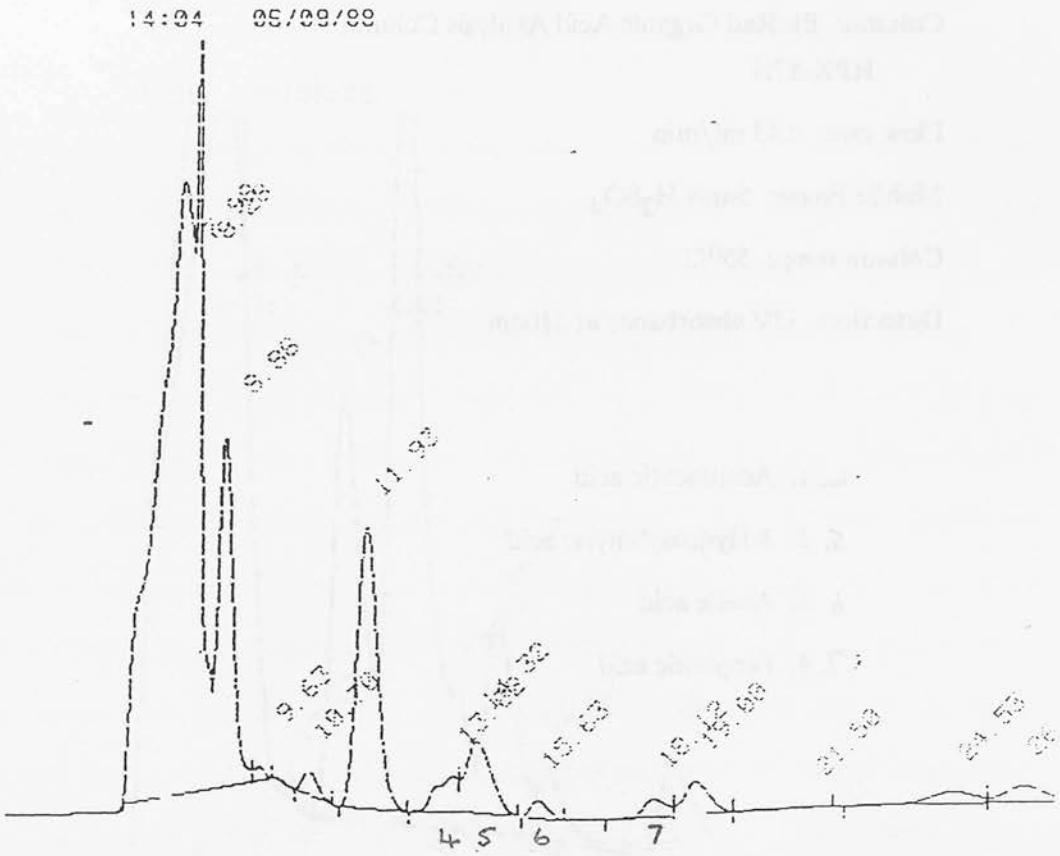

14:04 05/09/88
4 5 6 7

Fig. 4.9 *Chromatogram showing the separation of the organic acids present in a 100µl sample of a protein free supernatant of blood.*

Column: BioRad Organic Acid Analysis Column
HPX-87H

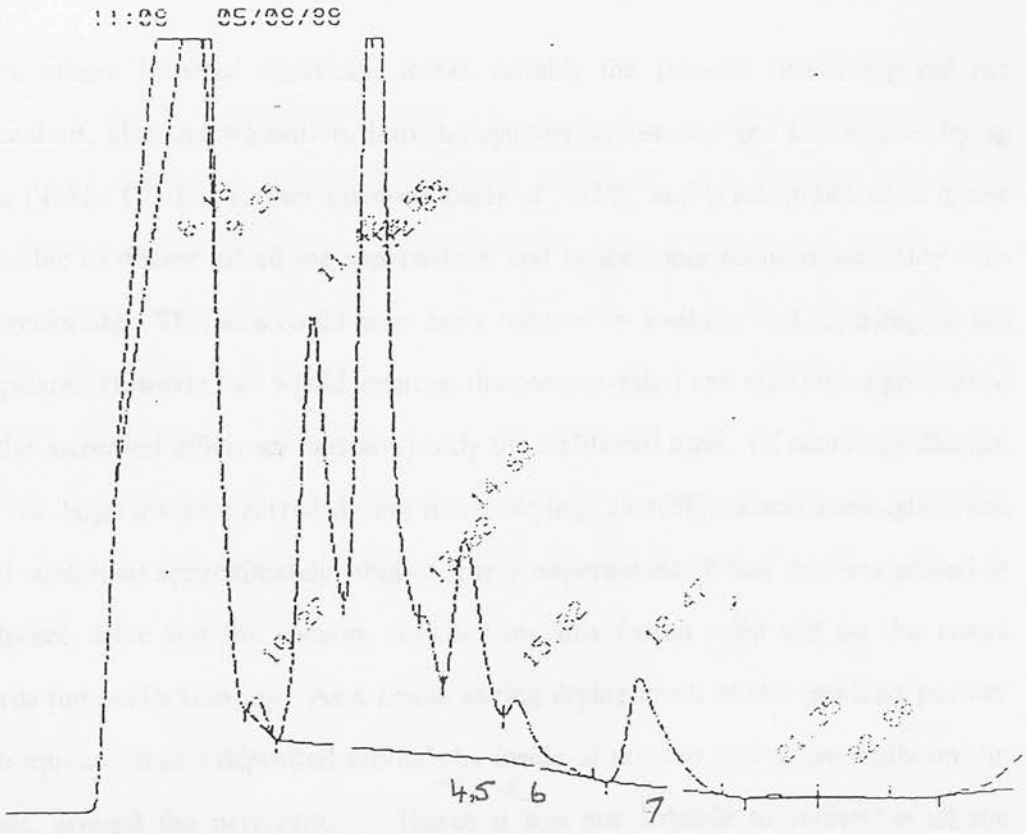
Flow rate: 0.45 ml/min

Mobile Phase: 5mM H_2SO_4

Column temp: 55°C

Detection: UV absorbance at 210nm

4. Acetoacetic acid
5. 3-Hydroxybutyric acid
6. Acetic acid
7. Propionic acid


4.5
6
7

4.1.4 Losses Incurred during the Procedure

Table 1 *Losses associated with the various stages*

Stage	Range (%)	Mean (%)
Ion exchange	1-2	1.5
Transfer stage	5-15	12.0
Freeze drying	25-40	28.0
HPLC	0	0

Two stages involved significant losses, notably the transfer (decanting off the supernatant, after neutralisation, into the cylindricial vessels) and the freeze drying stages (Table 1). The former involved losses of 5-15%, and is inevitable since it was impossible to decant off all the supernatant, and hence some remains asociated with the precipitate. This loss could have been reduced by washing and centrifuging the precipitate. However this would lengthen the concentration and separation procedure and the increased efficiency may not justify the additional time. Of more significance were the large losses incurred during freeze drying (25-40%). Each 20ml cylindrical vessel contained approximately 10ml of frozen supernatant. When this was placed in the freeze drier and the vacuum switched on, this frozen solid slid up the vessel towards the perforated cap. As a result, during drying much of the resulting powder was frequently found deposited around the inside of the cap and occasionally on the outside, around the perforations. Hence it was not possible to redissolve all the powder, increasing the losses. This procedure was improved by subjecting the samples to short periods of vacuum during the initial freeze drying stage, in an attempt to degas the frozen solid, which helped prevent the solid sliding up the vessel. Alternatively this procedure was also improved by freezing the supernatant with the vessel at a slight gradient rather than standing upright. When subject to vacuum this frozen solid did not move.

4.2 Using Tracers to Determine Substrate Cycling

Tracer methods are used to provide quantitative information about metabolic systems in terms of the amounts of substrates present, their rates of supply, consumption and interconversion, and about the structure of such systems in terms of the number and sizes of pools, and their routes and rates of communication (Hodgson, 1987). The computational procedures involved may be complex and frequently the interpretation of data is made difficult by the extensive transformations that may occur between metabolites (Shipley & Clark, 1972; MacRae & Lobley, 1986, Hodgson, 1987).

The use of tracers in metabolic research is a huge field. This discussion is interested in the use of tracer methodology to assess cyclic processes and in particular a substrate cycle between acetate and acetyl-CoA (Jessop *et al.*, 1986; Crabtree *et al.*, 1987).

The substrate cycle between acetate and acetyl-CoA may be determined using the simplest experimental situation, in which tracers are administered continuously (Crabtree *et al.*, 1987). This discussion will focus on the principle of the continuous administration of tracers, the theory behind the use of a single label to determine cycling and how this may be used to evaluate the rate of substrate cycling between acetate and acetyl-CoA, across the liver.

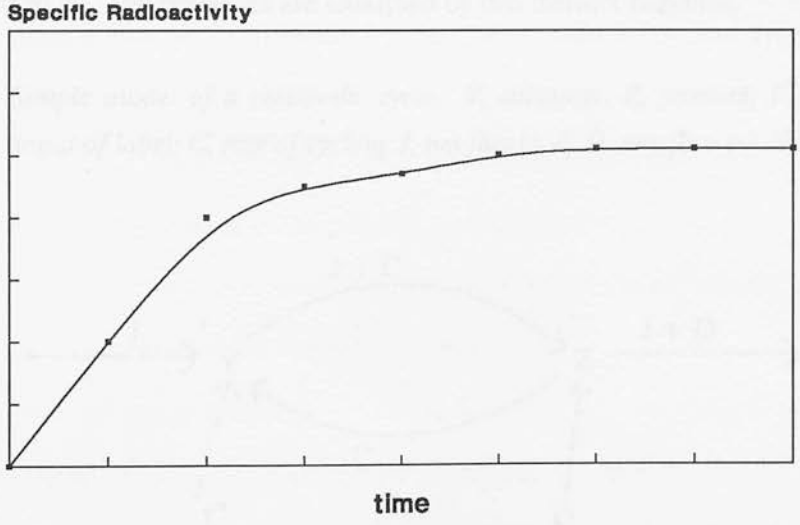
4.2.1 *Continuous infusion*

When a tracer is infused continuously an isotopic steady steady-state in which the specific activities of both the tracer metabolite and many of its metabolite products reach "constant" or "plateau" values. At plateau the rate of loss of tracer from the sampled pool equals the tracer infusion rate (Hodgson, 1987). Fig. 4.10 is a typical curve obtained when administering a tracer continuously. The rate of irreversible loss (ILR) (the rate at which tracee atoms leave the sampled pool never to return) of compound from the sampling pool is:

$$ILR = \frac{\text{rate of infus ion}}{\text{plateau SA}}$$

The time taken to achieve plateau specific radioactivity is influenced by the substrates and systems under study. For example, plateau values in plasma acetate specific radioactivity have been reached within 1-2h of continuous infusion in wethers and ewes (Pethick *et al.*, 1981; Crabtree *et al.*, 1987), but in experiments involving labelled infusions of short-chain fatty acids into the rumen, longer periods (minimum 18h) were required (Wilson *et al.*, 1983).

Fig. 4.10 *Typical plot of specific radioactivity versus time during continuous infusion*



4.2.2 *Modelling cycling using a single label*

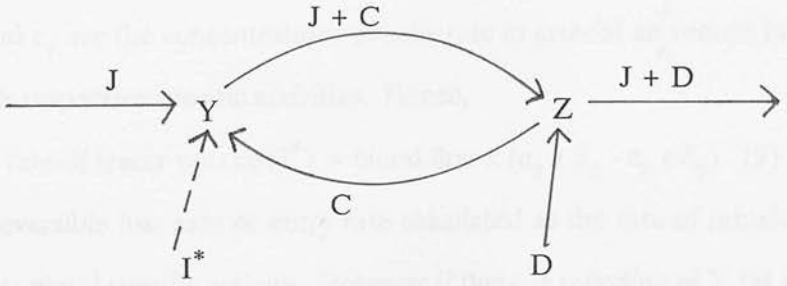
The modelling used in assessing cycling is described in some detail by Crabtree & Lobely (1988). It is based on that developed by Katz and his colleagues (Katz & Grunnet, 1979), in which the isotopic flux is related to its corresponding mass flux (i.e. reaction rate) by the equation:

isotopic flux of substrate Y to product Z = mass flux of Y to Z x specific activity of Y

For radioactive isotopes the "specific activity" is referred to as specific radioactivity and is the amount of tracer (Y^*) divided by the amount of unlabelled tracee (Y). For stable isotopes the specific activity is the molar ratio, tracer:tracee, and is referred to as the isotope enrichment or molar (atom) % excess.

Fig. 4.11 represents a simple metabolic cycle in which Y is converted to Z and Z may be recycled to Y. The reactions are catalysed by two distinct enzymes.

Fig. 4.11 *Simple model of a metabolic cycle. Y, substrate; Z, product; I^*, continuous input of label; C, rate of cycling; J, net flux to Z; D, any flux(es) diluting Z.*



(Crabtree & Lobley, 1988)

Under steady-state conditions the isotopic fluxes which produce labelled **Y** equal those which remove the label, so that no net labelling of **Y** occurs. Hence balanced isotopic equations may be written and then solved to yield the pathway rates in terms

of the specific activities of the labelled intermediates and the rate of supply of labelled precursor. If the label is added in tracer amounts, so that its mass flux is negligible compared with J, then:

$$\text{isotopic flux to Y} = I^* + C \times S_z$$

$$\text{isotopic flux from Y} = (J+C) \times S_y$$

where S_y and S_z are the specific activities of Y and Z respectively.

In a steady state these fluxes are equal, so that,

$$I^* + C \times S_z = (J+C) \times S_y \qquad (1)$$

hence,

$$I^*/S_y = J + C \times (1-S_z/S_y) \qquad (2)$$

or letting $R = I^*/S_y$

$$R = J + C \times (1-S_z/S_y)$$

In Fig. 4.11 the product Z, may be either metabolised further or recycled to substrate Y. If the input of isotope (I^*) represents the rate of isotope infusion into a precursor pool blood, then,

$$\text{amount of tracer per vol. arterial blood} = c_a \times S_a$$

$$\text{amount of tracer per vol. venous blood} = c_v \times S_v$$

where c_a and c_v are the concentrations of substrate in arterial and venous blood and S_a and S_v, their respective specific activities. Hence,

$$\text{rate of tracer uptake } (I^*) = \text{blood flow} \times (c_a \times S_a - c_v \times S_v) \qquad (3)$$

R is the irreversible loss rate or entry rate calculated as the rate of infusion of tracer divided by its blood specific activity. However if there is recycling of Y (at rate C), the value of R does not equal any of the fluxes in the system unless the specific activity of the product (S_z) is either zero or is equal to the specific activity of the substrate (S_y). In the former case Y behaves as an "irreversible tracer" and R measures the actual gross flux, J+C and in the latter case ($S_z = S_y$) R measures the actual net flux (irreversible loss rate), J.

Hence if recycling occurs the values of both S_z and S_y must be known.

For trans-organ studies equation 3 becomes,

$$R = \text{blood flow} \times (c_a \times S_a - c_v \times S_v)/S_y$$

This is often simpled by assuming that the specific activity of the substrate in venous blood, S_v, is the same as that in the tissue, S_y. With this assumption for R, equation 2 becomes,

$$\text{blood flow} \times (c_a \times S_a - c_v \times S_v)/S_v = J + C \times (1-S_z/S_v)$$

Since the net mass flux, J, = blood flow x (c_a - c_v), this equation may be arranged,

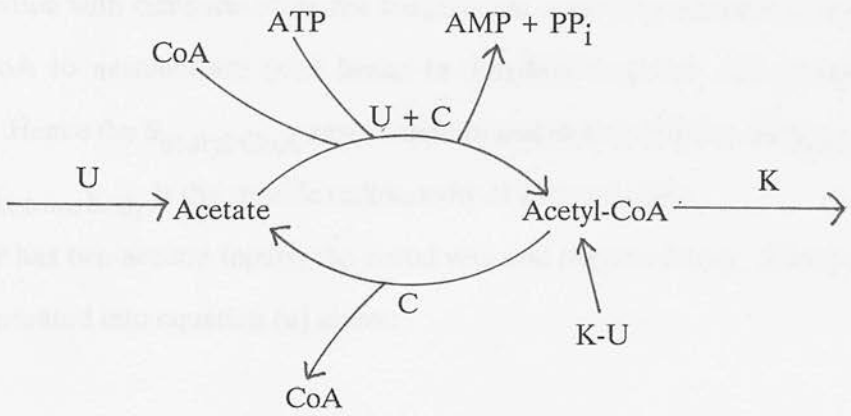
$$C = \text{blood flow} \times c_a \times (S_a - S_v)/(S_v-S_z) \quad (4)$$

Hence the rate of cycling, C, can be calculated by infusing singly labelled or a suitable precursor of Y, provided that S_Z can be measured or calculated indirectly and S_Z is less than S_v, that is the diluting flux, D, in Fig 4.11 must not be zero, hence Z will be diluted relative to that in Y.

4.2.3 *Acetate-acetyl-CoA substrate cycle*

The above principles have been applied in assessing the activity of the substrate cycle between acetate and acetyl-CoA (Fig. 4.12) in sheep muscle (Crabtree *et al.*, 1987).

Fig. 4.12 *The acetate-acetyl-CoA cycle in sheep muscle. C, rate of acetyl-CoA hydrolysis; U, net flux of acetate to acetyl-CoA (J in Fig. 4.11); K, tricarboxylic acid cycle; K - U, flux of acetyl-CoA from unlabelled fuels (D in Fig. 4.11); acetate and acetyl-CoA are Y and Z respectively in Fig. 4.11.*



Isotope input into the muscle was by arterio-venous uptake after infusing [1-^{14}C] acetate into a jugular vein. Dilution of acetyl-CoA (Z in Fig. 4.11) was provided by the oxidation of unlabelled fuels. e.g. glucose, fatty acids and ketone bodies. Acetyl-CoA in this example is mitochondrial and hence the specific radioactivity of acetyl-CoA was assessed indirectly. This was done by assuming that the acetate labels a single pool of mitochondrial acetyl-CoA which participates in only two reactions: condensation with oxaloacetate in the tricarboxylic acid cycle (K) and recycling to acetate (C) (Fig. 4.12). The model assumed that the venous concentration and specific radoactivity of acetate reflect those of the cellular pool (as discussed above) and that there is only one pool of acetyl-CoA. As above, if all pathways and tracers are in a steady-state, the net labellings of acetate and acetyl-CoA are both zero. Hence for acetate,

$$B.c_a.s_a + C.s_{acetyl\text{-}CoA} = s_v.(B.c_v + C+U) \quad (a)$$

where B is blood flow, c_a and s_a are the arterial acetate concentrations and specific radioactivities and c_v and s_v are the venous concentrations and specific radioactivities.

In Chapter 5, an experiment is described, which attempts to assess the activity of the substrate cycle between acetate and acetyl-CoA in calf liver. The equations described above for the cycle in sheep muscle are different for liver, for two main reasons.

1) In liver it is assumed that acetyl-CoA participates in at least three reactions: a) condensation with oxaloacetate in the tricarboxylic acid cycle; b) condensation of two acetyl-CoA to acetoacetate (and hence to 3-hydroxybutyrate) and c) recycling to acetate. Hence the $S_{acetyl\text{-}CoA}$ may be determined indirectly from the $S_{ketone\ body}$, where $S_{ketone\ body}$ is the specific radioactivity of ketone bodies.

2) Liver has two acetate inputs: the portal vein and hepatic artery. Hence this must be incorporated into equation (a) above:

$$\text{Portal acetate supply (PA)} = B_{pv} \times c_{pva} \times s_{pva}$$

and

$$\text{Hepatic acetate supply (HA)} = B_{ha} \times c_{caa} \times s_{caa}$$

where B_{pv} and B_{ha} are the portal and hepatic blood flows respectively; c_{pva} and s_{pva} are the portal acetate concentration and specific radioactivity respectively and c_{haa} and s_{haa} are the carotid acetate concentration and specific radioactivity respectively.

CHAPTER FIVE

Measurement of the Rate of Substrate Cycling between Acetate and Acetyl-CoA in Calf Liver *in vivo*

Abstract

1. Two experiments were conducted, using catheterised animals, to assess the degree of substrate cycling between acetate and acetyl-CoA in calf liver.

2. Expt. 1 involved the infusion of [U-^{13}C] acetate. Unfortunately the amount of ^{13}C required to obtain a 1% atom excess was underestimated and no results were acquired.

3. Expt. 2. Two calves (approx. 160kg) were infused with [2-^{14}C] sodium acetate and PAH. Blood flow data were very erratic and were not suitable for use in the various calculations. Hence mean blood flows for these animals from a previous experiment were used. However this introduced substantial variation into the results which were obtained.
The specific radioactivity of acetate in the carotid artery was greater than that in either the portal or hepatic vein. Since acetate was infused into a mesenteric vein this observation is difficult to reconcile.

4. It was not possible to make any conclusions on the rate of substrate cycling between acetate and acetyl-CoA.

Key Words: Infusion Acetate Substrate cycle Energy

5.1 INTRODUCTION

Basal energy expenditure by an animal has been categorised into costs for service functions essential to the whole animal and costs to support the existence of a particular cell or tissue (Baldwin *et al.*, 1980). Baldwin *et al.* (1980) suggested that significant improvements in animal efficiency by reduction of energy spent on service functions is unlikely through external manipulations and suggested that modification of cell and tissue costs, which may account for 40-50% of basal energy consumption may be the way to improve the efficiency of animal production. Hence it is of considerable significance to investigate ATP-consuming processes (Milligan, 1971; Milligan & Summers, 1986; Reeds *et al.*, 1985). One such process is substrate cycling.

Substrate cycles exist at certain key points in intermediary metabolism, at which the actions of a pair of enzymes, catalysing forward and backward reactions, each greatly displaced from equilibrium, lead to substrate/product cycles resulting in the expenditure of energy (ATP) (Newsholme & Crabtree, 1976).

In the ruminant, blood acetate is derived from rumen fermentation and approximately 30% from endogenous (tissue) metabolism. This "endogenous" acetate production which occurs in many tissues of both ruminants and non-ruminants has been suggested to represent the reverse reaction of a substrate cycle between acetate and acetyl-CoA. This cycle catalysed by the simultaneous activities of acetyl-CoA synthetase (EC 6.2.1.1) and acetyl-CoA hydrolase (EC 3.1.2.1) has been shown to operate in rat hepatocytes (Jessop *et al.*, 1986) and in sheep muscle in vivo (Crabtree *et al.*, 1987). As a result of the high endogenous entry rate and dominance of acetate metabolism in the ruminant the possibility of substantial energy costs associated with this substrate cycle is particularly pertinent.

In rat hepatocytes the rate of the acetate cycle was increased by an increased concentration of acetate (Jessop *et al.*, 1986). If this cycle responds in a similar manner in ruminants it may help to explain why the efficiency of metabolisable energy (ME) use is lowered on feeding fibre based diets which give rise to large quantities of

rumen acetate, when compared to feeding starch based diets (Blaxter, 1980; MacRae & Lobley, 1982). MacRae & Lobley (1982) suggested that this lowered efficiency may result from a restricted rate of fat synthesis from acetate and hence increased acetate oxidation, resulting in ATP production. Excess ATP may fuel the substrate cycle between acetate and acetyl-CoA and this energy would therefore be lost as heat.

Crabtree *et al.*, (1987) measured the activity of the acetate cycle in sheep muscle *in vivo* and investigated the influence of increasing blood acetate upon the cycling rate. The latter had only a small, but positive effect upon cycling. It was concluded that this cycle in muscle is unlikely to contribute significantly to any increased heat production as a direct result of changes in blood acetate concentrations. It was calculated that the cycle in muscle may contribute only approx. 0.5% of the total heat produced by the animal.

Calculations from the rates of the enzymes involved in the acetate cycle in sheep muscle *in vitro*, also suggest that the potential for cycling in this tissue is limited and is unlikely to account for a significant proportion of basal energy expenditure (Chapter 2). Similar calculations from the enzymes rates of both sheep and cattle in liver suggest that in this tissue the cycle may account for up to 2.5% of basal energy expenditure (Jessop *et al.*, 1990).

Hence it would appear that the activity of the acetate cycle in ruminant liver would merit further investigation. Pethick *et al.* (1981) observed a decrease in specific radioactivity of acetate across sheep liver, which most probably reflects acetate cycling. Therefore it was decided to use a similar technique to measure the activity of the substrate cycle between acetate and acetyl-CoA in the liver of calves.

The opportunity to conduct this experiment arose through collaboration with the Institute of Grassland and Animal Production (IGAP), Hurley. The animals were surgically prepared as part of ongoing investigations at this institute.

5.2 METHODS and MATERIALS

Isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; enzymes, cofactors, buffers, heparin and resins were obtained from Sigma Chemical Company, Poole, Dorset. U.K.; and all inorganic reagents were obtained from BDH Ltd., Poole, Dorset, U.K., and were of the highest purity available. 0.9% Sodium chloride intravenous infusion BP, was obtained from Travenol Laboratories Ltd., Caxton Way, Thetford, Norfolk.

5.2.1 *Animals*

1988 - Four Friesian castrates (approx. 160kg) housed in metabolism crates, were allocated to a 2x2 cross-over design and fed hourly on a forage (grass pellets) or forage-concentrate (50:50, grass:flaked maize pellets) diet at levels of 24 g dry matter/kg liveweight (grass) and 19 g DM/kg liveweight (grass/flaked maize) to provide equal metabolisable energy intakes. The forage diet and the forage-concentrate diet contained estimated *in vivo* organic matter digestibilities of 0.62 and 0.78 (DM basis) and 510 and 290 g neutral-detergent fibre/kg respectively.

1989 - Two Friesian castrates (approx. 160kg) were housed in metabolism crates and fed hourly on grass nuts (fed at the same level and was of the same composition as above).

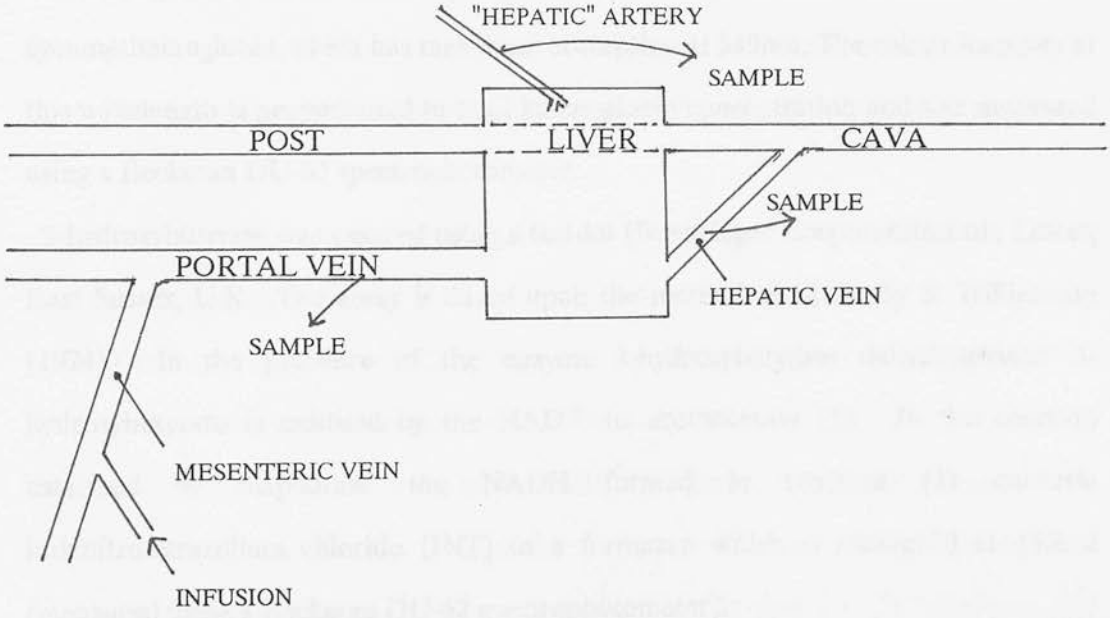
Water and a salt lick were freely available. The mesenteric, portal, hepatic veins and the carotid artery were catheterised (Fig. 5.1).

5.2.2 *Infusion*

A solution containing 0.9% (w/v) sodium chloride, sterilised by filtration (0.2μm pore size), either 0.25mg/ml [U-^{13}C] 99% atom excess (year 1988) or 45kBq/ml [2-^{14}C] acetate (year 1989) and 4% (w/v) para-aminohippuric acid was infused via the mesenteric vein, at a rate of 2ml/min, commencing at 9 am. After 90min 20ml

samples of blood were withdrawn simultaneously from the portal, hepatic and carotid catheters into heparinised syringes (approx. 100 units). Further blood samples (approx. 1ml) were withdrawn into small plastic syringes for the measurement of pH, pO_2 and pCO_2. Blood sampling was repeated at 30min intervals for a further 150min, at which time the infusion was stopped. Hence the total period of infusion was 4h. The infusion was repeated after 3 days.

Fig. 5.1 *Schematic diagram illustrating the position of the sampling and infusion cannulas for the procedure.*



5.2.3 *Metabolite determination*

15ml of blood from each vessel was mixed with an equal volume of ice cold 6% perchloric acid and centrifuged at 3000xg for 20min at 2°C (Sorvall RT6000B Refrigerated Centrifuge). The supernatant was decanted into test-tubes and the pH was adjusted to approx. 7 with 2M KOH/0.5M triethanolamine. Samples were frozen until required for assay. The pH, pO_2 and pCO_2 were determined using Instrumentation Laboratory System 1302 pH/Blood gas analyser.

Haemoglobin was assayed using a test kit (Sigma Chemical Company). The method is based on the fact that in the presence of alkaline potassium ferricyanide, haemoglobin and its derivatives, except sulfhaemoglobin, are oxidised to methaemoglobin. The latter then reacts with potassium cyanide to form cyanmethemoglobin, which has maximum absorption at 540nm. The colour intensity at this wavelength is proportional to total haemoglobin concentration and was measured using a Beckman DU-62 spectrophotometer..

3-hydroxybutyrate was assayed using a test-kit (Boehringer Corporation Ltd., Lewes, East Sussex, U.K. The assay is based upon the method of Mellanby & Williamson (1974). In the presence of the enzyme 3-hydroxybutyrate dehydrogenase, 3-hydroxybutyrate is oxidised by the NAD^+ to acetoacetate (1). In the reaction catalysed by diaphorase the NADH formed in reaction (1) converts iodonitrotetrazolium chloride (INT) to a formazan which is measured at 492nm (measured using a Beckman DU-62 spectrophotometer).

(1) D-3-Hydroxybutyrate + NAD^+ ------> Acetoacetate + NADH + H^+

(2) NADH + INT + H^+ ----------> NAD^+ + formazan

Plasma acetoacetate was calculated from the concentration of 3-hydroxybutyrate by assuming that the plasma acetoacetate is approximately 20% of the 3-hydroxybutyrate concentration (Riis, 1983). Total ketone body concentration was calculated as

acetoacetate plus 3-hydroxybutyrate.

Plasma acetate was assayed according to the method of Guynn & Veech (1974), as described in Chapter 3.

5.2.4 *Acetate concentration and specific radioactivity*

The theory and procedure for the separation and concentration of blood acetate has been discussed in detail in Chapter 4. The practical aspects will be briefly described again in this section.

Eleven ml of neutralised deproteinised blood were spiked with [2-^{14}C] sodium propionate. 2x250μl samples were removed for the determination of radioactivity (Beckman Scintillation Counter LS 5000 CE). Radiolabelled propionate acted as an internal marker and allowed the efficiencies of the various stages associated with the procedure to be determined (similar to adding [1-^{14}C] acetate to samples in Chapter 4).

Ten ml of the above sample were applied to a column (5 x 1 cm, 4ml bed volume) of Dowex-1 OH^- form (100-200 micron mesh). The OH^- form of the resin was freshly prepared from the Cl^- form by the columnwise application of 5 volumes of 1M NaOH followed by a thorough washing with distilled H_20. Neutral and positively charged compounds were washed from the column with 6ml distilled H_20 and the organic acids were eluted with 15ml $HCl0_4$. The organic fraction was adjusted to pH 9.0 (approx.), the supernatant was decanted, volume determined and 2x250μl samples were removed for determination of the radioactivity present. The supernatant was subdivided into 2x20ml cylindrical vessels (with perforated caps) and frozen at -60^{o}C. The samples were freeze dried (FTS Systems INC.). The freeze dried organic acids were redissolved in 0.5ml distilled H_2O, the supernatant decanted and 2x10μl samples were removed for the determination of radioactivity.

The supernatants were acidified by the addition of 80mg (dry weight) Dowex -50W strongly acid cation (H^+) exchange resin. The organic acids (in 50μl samples) were

separated by HPLC using a BioRad HPX-87H Organic Acid Analysis column (BioRad Laboratories), using 5mM H_2SO_4 as the mobile phase at a flow rate of 0.45ml/min. The column was maintained at 55°C by immersion in a water bath. Detection of organic acids was by U.V. absorbance at 210nm in a 8μl flow cell fitted to a Shimadzu SPD 6A spectrophotometric detector.

The ketone body (acetoacetate and 3-hydroxybutyrate) and acetate fractions in 50μl of acidified supernatant were collected by hand, into separate scintillation vials and the radioactivity in each fraction was assessed. Since propionate was used as an internal marker, the overall efficiency of the separation procedure for each sample was known, and hence the specific radioactivity of the ketone body and acetate fractions in blood could be calculated. Organic acid recoveries were 43-69%.

5.2.5 *Blood Flow*

Dilution of intramesenterically infused *p*-aminohippuric acid solution (PAH) was used to measure portal and hepatic vein and hepatic arterial blood flows, as described by Katz & Bergman (1969). The concentration of PAH in the blood should remain constant after a period of continuous infusion in which the PAH is allowed to equilibrate with the extracellular fluid because of the fact that the PAH is rapidly excreted by the kidneys and provided the infusion rate is less than the maximal ability of the kidneys to excrete PAH (Katz & Bergman, 1969). Portal vein - arterial differences will be substantial since a great part of circulating PAH is excreted by the kidneys and since the portal blood flow remains constant (during steady-state conditions) the portal vein - arterial differences will remain constant. The method depends upon a lack of movement of PAH from the portal blood into the gut; the PAH should not be excreted or chemically altered in its passage through the portal bed or liver; and the infused PAH should be well mixed in the portal vein. Katz & Bergman (1969) found that no significant excretion or metabolism of PAH occurs in the portal bed or the liver, but some PAH is acetylated by the liver. However this is

readily overcome by deacetylating the samples as a first step, prior to the determination of PAH.

The amount of PAH entering the portal system equals the amount of PAH leaving by way of the portal blood and therefore:

$$B_{pv} = \frac{I}{C_{pv} - C_a}$$

where B_{pv} is the rate of blood flow (ml/min) in the portal vein; I is the infusion rate of PAH (mg/min); and C_{pv} and C_a are the concentrations of PAH (mg/ml) in the portal and arterial blood, respectively.

The concentration of PAH in the hepatic vein is equal to the concentration in the portal vein less a certain amount due to dilution by the hepatic arterial blood. Therefore:

$$B_{hv} = \frac{I}{C_{hv} - C_a}$$

where B_{hv} and C_{hv} are the blood flow and PAH concentration, respectively, in the hepatic vein. The blood flow in the hepatic artery (B_{ha}) is therefore:

$$B_{ha} = B_{hv} - B_{pv}$$

PAH was determined using the method of Smith et al. (1945), with the modification of Katz & Bergman (1969). 0.5ml whole blood was mixed with 6ml trichloroacetic acid (10%) and centrifuged at 3000xg for 5min. The supernat nt was frozen until required for assay. Supernat nts were thawed and 5ml was boiled for 0.5h in a boiling water bath. A glass marble was placed on the top of each test-tube, which acted as a condenser. Samples were cooled and 1ml of 0.24M hydrochloric acid added and tubes were mixed. 0.5ml sodium nitrite (0.1%) was added, tubes were mixed and left for

4min. 0.5ml ammonium sulphamate (0.5%) was added, tubes were mixed, and left for 4min. 0.5ml Naphthylethylenediamine dihydrochloride (0.1%) was added, tubes were mixed and left for 15min, after which the absorbance at 546nm was determined, using a Beckman DU-62 spectrophotometer.

A standard curve in the range 0.2-2.5 μg/ml PAH was used for the determination of PAH concentration.

5.2.6 *Calculations*

Some of the calculations are similar to those used by Bergman & Wolff (1971) and Pethick *et al.*, (1981).

Acetate entry rate:

$$\text{entry rate (mmol/h)} = \frac{I}{\text{acetate SRA}}$$

where I is the infusion rate of [1-^{14}C] acetate (disintegrations per min per h) and SRA is the mean arterial specific radioactivity (disintegrations per min per mmol).

$$\text{The net hepatic utilisation of acetate (mmol/h)} = [B_{pv}.c_{pva} + B_{ha}.c_{caa} - B_{hv}.c_{hva}]$$

where B_{pv}, B_{ha}, B_{hv}, are the blood flows (l/min) for the portal vein, hepatic artery and hepatic vein respectively; c_{pva}, c_{caa}, c_{hva} are the concentrations of acetate (μmol) in the portal vein, carotid artery and hepatic vein respectively. Gross utilisation of acetate (mmol/h) is

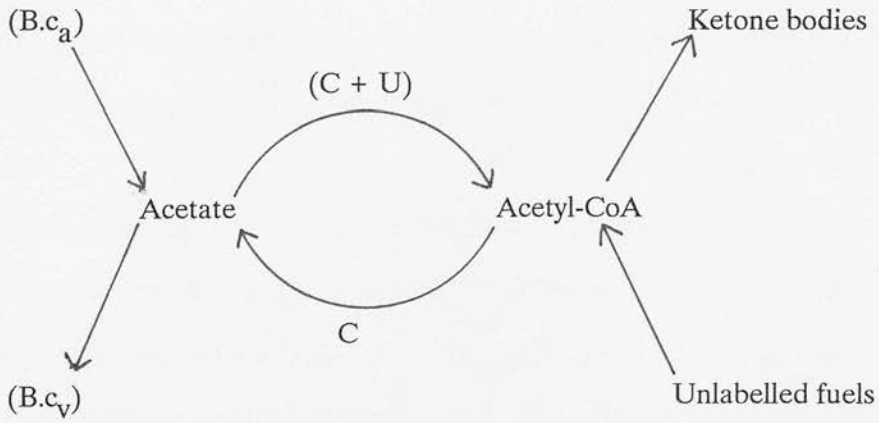
$$\frac{[F.{}^{14}C_{pv} + (1\text{-}F){}^{14}C_{ca} - {}^{14}C_{hv}]}{spva + s_{caa}} \quad x \quad B_{hv}$$

where F is B_{pv}/B_{hv}; $^{14}C_{pv}$, $^{14}C_{ca}$, $^{14}C_{hv}$ are the amounts of radioactive blood acetate (disintegrations/min per ml blood); spva and s_{caa} are the specific radioactivity of acetate (disintegrations per $\frac{*}{\wedge}$) in the, portal vein and carotid artery, respectively. Acetate production (mmol/h) = gross utilisation - net utilisation.

$\frac{*}{\text{min per mmol}}$

Calculation of the rate of acetate cycling

Fig. 5.2 *Calculating the rate of substrate cycling between acetate and acetyl-CoA in calf liver. C, rate of acetyl-CoA hydrolysis; U, net flux of acetate to acetyl-CoA;*



The model assumes that (1) the venous concentration and specific radioactivity of acetate reflect those of the cellular pool and (2) The specific radioactivity of total ketone bodies may be used to calculate the specific radioactivity of acetyl-CoA. If all pathways and tracers are in a steady state, the net labellings of acetate and acetyl-CoA are both zero. Therefore for liver:

$$B_{pv} \cdot c_{pva} \cdot s_{pva} + B_{ha} \cdot c_{caa} \cdot s_{caa} + C \cdot s_{acetyl\text{-}CoA} = s_{hva} (B_{hv} \cdot c_{hva} + C + U)$$

where B_{pv}, B_{ha}, B_{hv}, are the blood flows (l/min) for the portal vein, hepatic artery and hepatic vein respectively; c_{pva}, c_{caa}, c_{hva} are the concentrations of acetate (μM) in the portal vein, carotid artery and hepatic vein respectively; s_{pva}, s_{caa}, s_{hva} are the specific radioactivities of acetate (disintegrations per $\overset{*}{\wedge}$) in the portal vein, carotid artery and hepatic vein respectively.

$\overset{*}{\wedge}$ min per mmol

5.3 RESULTS and DISCUSSION

Expt 1 - 1988. Since the metabolism unit at Hurley was not yet licensed for the use of radioactive isotopes this experiment was restricted to the use of stable isotopes. The infusions were carried out over a 12 week period, April-July. Subsequent analysis of the samples for ^{13}C at the Scottish Universities' Research and Reactor Centre, under the direction of Dr T. Preston, revealed that the amount of ^{13}C in the samples was not above normal background levels. The level of [U-^{13}C] acetate infused into the animals (0.25mg/ml) was calculated necessary to enrich blood acetate by 1% (hence 1% atom excess). On re-examination of this calculation, an error was found. The amount of [U-^{13}C] acetate necessary to achieve approx. 1% atom excess in a 160kg steer ought be 30-35mg/ml. Unfortunately this experiment has not yielded any data.

Expt 2 - 1989. In this experiment the use of radioactive isotopes was permitted. This experiment involved only 2 animals, referred to as X & Y. The animals were infused on a Tuesday and the infusion was repeated on the Friday. Hence TX & TY and FX & FY refer to the infusions conducted on animals X & Y on the Tuesday and Friday respectively.

During infusions on the Tuesday both animals ate and drank normally. On the Friday animal X developed a pyrogenic reaction, 45min after the infusion had commenced. This was characterised by a increase in body temperature and respiration rate (occurred over a 5-10min period). The infusion was terminated and the animal's temperature and respiration rate returned to normal within 1hr. From the subsequent tables it will be observed that not all samples were obtained from the portal vessel of animal Y during each infusion. A sample was only obtained if the animal was lying down. Presumably when the animal stood during sampling the catheter lay close to the vessel wall, so that when suction was applied the catheter adhered to the wall of the vessel and no blood could be withdrawn.

Blood haemoglobin concentrations remained relatively constant throughout each

infusion (Table 5.3.1). The pH, pO_2 and pCO_2 for each animal and vessel are given in Table 5.3.2.

Blood flows are presented in Table 5.3.3. To demonstrate the irregularity of these results, the blood flows determined for individual sampling points during an infusion are given. In many instances the portal vein blood flow was greater than that in the hepatic vein, hence resulting in a negative value for blood flow in the hepatic artery. One can only speculate as to why negative blood flows and such variation were obtained. One possible reason may be that the PAH entering the portal system from the mesenteric was insufficiently mixed, resulting in high concentrations of PAH in the portal vein. However in the liver the PAH infused will be well mixed and the concentrations of PAH in the hepatic vein would be considerably lower than that in the portal vein, resulting in calculated negative blood flows in the hepatic artery. Most of the calculations (as outlined in section 5.2.6) depend upon the blood flows in the portal vein, hepatic vein and hepatic artery. The blood flows in Table 5.3.3 are not suitable for use and in the calculations presented in Table 5.3.7, the blood flows used in the portal vein, hepatic vein and hepatic artery were 8.96, 11.30 and 2.34 l/min respectively.

Table 5.3.1 *Concentrations of haemoglobin in carotid artery, portal vein and hepatic vein for animals TX, TY and FY. Results are expressed as g haemoglobin/100ml blood and represent mean±S.E.M. for the number of sampling points in parenthesis.*

	Carotid	Portal	Hepatic
TX	10.3±0.46 (6)	10.5±0.32 (5)	10.9±0.50 (5)
TY	10.3±0.26 (5)	11.0±0.13 (3)	10.4±0.25 (5)
FY	9.2±0.13 (5)	9.3±0.28 (5)	9.0±0.20 (6)

Table 5.3.2 *pH, pO_2 and pCO_2 for each of the vessels sampled (carotid artery, portal vein, hepatic artery) for animals TX, TY and FY. pO_2 and pCO_2 are expressed in mm Hg and represent mean±S.E.M. for the number of sampling points in parenthesis.*

Carotid Artery

	pH	pO_2	pCO_2
TX	7.40±0.01 (6)	108.0±5.07 (5)	41.8±1.73 (5)
TY	7.40±0.01 (5)	125.2±7.04 (5)	43.0±0.84 (5)
FY	7.40±0.01 (5)	114.0±6.10 (5)	42.4±1.25 (6)

Portal Vein

	pH	pO_2	pCO_2
TX	7.32±0.02 (6)	44.8±2.17 (5)	50.7±2.28 (5)
TY	7.29±0.01 (5)	48.3±1.97 (3)	54.1±1.15 (3)
FY	7.32±0.01 (5)	46.2±2.05 (5)	52.6±1.25 (6)

Hepatic Vein

	pH	pO_2	pCO_2
TX	7.31±0.01 (6)	35.0±0.25 (5)	52.0±0.24 (5)
TY	7.31±0.01 (5)	30.2±0.86 (6)	52.9±0.28 (6)
FY	7.32± 0.01 (5)	32.1±0.75 (5)	51.8±0.26 (6)

Table 5.3.3. *Blood flows in the portal vein, hepatic vein and hepatic artery. The results are expressed in l/min and are given for each sampling point (1-6) during an infusion. N.D. - not determined, or no corresponding value to substract.*

Animal TX

Sampling point	Portal Vein	Hepatic Vein	Hepatic Artery
1	9.40	7.60	-1.80
2	N.D.	7.24	N.D.
3	20.45	6.26	-14.49
4	14.80	8.70	-6.10
5	16.20	8.20	-8.00
6	15.10	6.20	-8.90

Animal TY

Sampling point	Portal Vein	Hepatic Vein	Hepatic Artery
1	N.D.	27.0	N.D.
2	8.11	9.36	1.25
3	3.04	10.0	6.96
4	N.D.	30.4	N.D.
5	N.D.	N.D.	N.D.
6	0.61	8.39	7.78

Animal FY

Sampling point	Portal Vein	Hepatic Vein	Hepatic Artery
1	15.10	10.20	-4.90
2	29.00	18.90	-10.10
3	16.40	12.20	-4.20
4	12.60	16.00	-3.40
5	N.D.	16.80	N.D.
6	21.60	11.40	-10.20

These figures are means for the animals used in this experiment, but obtained from a previous study (Dr N. Fitch, personal communication). It will become clear in the next sections that the use of mean blood flows is very inadequate, and is responsible for a high proportion of the errors obtained in the calculations which will be presented shortly.

Concentrations and specific radioactivity values for acetate and ketone bodies (Tables 5.3.4 & 5.3.5) remained relatively constant throughout the 180min sampling period. The arterial specific radioactivity of acetate varied by less than 14, 10, and 7% of the mean value during the infusion for animals TX, TY and FY respectively. The arterial acetate concentration varied by less than 22, 10 and 9% of the mean value for animals TX, TY and FY respectively. These observations are similar to those reported by Pethick *et al.* (1981) and Crabtree *et al.* (1987). The concentration of acetate in the carotid artery is approx. 60% less than that observed in either the portal or hepatic vein, whereas the specific radioactivity of acetate in this vessel is approx. 180-220% greater. The latter observation is peculiar. The specific radioactivity ought to be lower in the carotid artery than in the portal or hepatic vein. Since the concentration of acetate drops in the carotid artery (utilised by tissues), the specific radioactivity should also drop as the tracer ought to behave in a similar manner to the tracee.

It is difficult to explain these observations. It implies that either the labelled acetate did not behave like cold acetate, or a large amount of labelled acetate is entering the vascular system after the liver. The former is very unlikely and the second conclusion is only reasonable if a large amount of labelled acetate failed to reach the liver after infusion into the mesenteric, and somehow managed to re-enter the vascular system. The mesentery drains only into the portal vein, so it is difficult to comprehend how ^{14}C acetate might escape and then re-enter. Perhaps the label diffused across the mesentery and eventually drained into the lymphatic system, reentering the vascular system close to the

Table 5.3.4a & b *Concentrations of acetate and 3-hydroxybutyrate in the portal vein, hepatic vein and hepatic artery. Values are expressed as μmol/ml of blood and represent means ± S.E.M for the number of sampling points in parenthesis.*

a) Acetate

	Carotid	Portal	Hepatic
TX	0.44±0.04 (6)	0.75±0.02 (6)	0.72±0.04 (5)
TY	0.47±0.02 (6)	0.83±0.04 (3)	0.75±0.01 (6)
FY	0.54±0.02 (6)	0.86±0.04 (5)	0.88±0.02 (6)

b) 3-Hydroxybutyrate

	Carotid	Portal	Hepatic
TX	0.36±0.006 (6)	0.36±0.01 (5)	0.49±0.01 (5)
TY	0.42±0.02 (6)	0.42±0.02 (3)	0.48±0.02 (6)
FY	0.39±0.02 (6)	0.51±0.02 (5)	0.53±0.02 (6)

Table 5.3.5a & b. *Specific radioactivities of acetate and ketone bodies in portal vein, hepatic vein and hepatic artery. Values are expressed as disintegrations per min per μmol and representmean ± S.E.M. for the number of sampling points in parenthesis.*

a) Acetate

	Carotid	Portal	Hepatic
TX	4624±314 (4)	2110±150 (6)	2182±180 (5)
TY	4529±229 (4)	2440±135 (3)	2713±101 (6)
FY	4147±132 (5)	2309±243 (5)	2246±238 (5)

b) Ketone Body

	Carotid	Portal	Hepatic
TX	1575±109 (6)	797±86.0 (6)	814±113 (5)
TY	974±93.3 (6)	1035±83.5 (3)	977±138 (6)
FY	1133±99.8 (6)	685±99.4 (5)	645±86.8 (5)

thoracic region. This would explain an increased specific radioactivity in the carotid artery. Other studies conducted at Hurley have investigated the effect upon the hepatic metabolism of ammonia, of infusing ammonia into a mesenteric vein. These studies have observed that up to 30% of the ammonia infused failed to reach the portal vein (Dr N. Fitch; Dr M. Lomax, personal communication). Accounting for that lost is speculative.

Experiments reported in the literature which have investigated hepatic acetate metabolism infused PAH into the mesenteric and labelled acetate into a jugular vein (Bergman & Wolff, 1971; Pethick *et al.*, 1981). There are no reported experiments which have followed the procedure used in this experiment. Nevertheless as discussed above the variation around the mean specific radioactivity for each vessel is quite acceptable and hence there are no reasons to disbelieve the observed values.

5.3.1 *Whole Body Acetate Turnover, Hepatic Utilisation and Production and Substrate Cycling between Acetate and Acetyl-CoA.*

The data presented in Tables 5.3.6 & 5.3.7 represent means ± S.E.M for 6 data points. In instances where a data point was missing, the mean of the other data points within the infusion was used to represent the missing value. As mentioned in the above section data representing blood flows in the three blood vessels is not satisfactory and in the calculations described in section 5.3.6 and presented in Table 5.3.7, mean blood flows have been used. Unfortunately this is largely responsible for the huge errors which are associated with the fluxes presented and it is difficult to draw conclusions from the results.

Acetate entry rates are presented in Table 5.3.6. These values are approximately 50% less than the entry rates which may be calculated using the equation presented by Pethick & Lindsay (1982) (see Chapter 1, section 1.2.3). If the specific radioactivity of arterial acetate (Table 5.3.5a) were less than that observed in the portal or hepatic vein, then the entry rates would be

Table 5.3.6 *Whole body acetate entry rate for animals TX, TY and FY. Values are expressed in mmol/h and represent mean ± S.E.M. for the number of sampling points in parenthesis.*

	Acetate Entry Rate
TX	70.7±3.14 (6)
TY	71.9±2.19 (6)
FY	81.3±3.56 (6)

Table 5.3.7 *Hepatic net utilisation and production of acetate, and the rate of substrate cycling between acetate and acetyl-CoA. Values are expressed as mmol/h and represent mean ± S.E.M for the number of sampling points in parenthesis.*

	Net Utilisation	Production	Cycling
TX	-20±28.4 (6)	20±28.4 (6)	86±113.6 (6)
TY	7±11.6 (6)	-7±11.6 (6)	-4±26.6 (6)
FY	-60±9.5 (6)	60±9.5 (6)	106±68.9 (6)

similar to that which may be calculated using the equation of Pethick & Lindsay (1982).

Very little may be concluded from Table 5.3.7. This is unfortunate since this is the first experiment involving the infusing of ^{14}C acetate which has been conducted since the studies of Bergman & Wolff (1971) and Pethick et al. (1981). The latter two studies as reviewed in Chapter 1 varied in their conclusions upon the hepatic metabolism of acetate in sheep.

The major objective of this experiment was to assess the rate of substrate cycling between acetate and acetyl-CoA. It is rather disappointing that the results yield very little meaningful information on this substrate cycle. Blood flow during an infusion experiment may vary by at least 30% from the mean value (Dr N. Fitch, personal communication). It may be calculated that a variation of 30% in blood flow would explain approx. 50% of the variation observed in the cycling rates presented in Table 5.3.7.

Nevertheless to demonstrate other calculations which may be done using the data presented, mean values for animal TX have been used to estimate the O_2 and ATP consumption and heat produced by the liver. The proportion of ATP used and heat produced by the liver which may be accounted for by substrate cycling between acetate and acetyl-CoA was determined (Appendix 2).

The O_2 and ATP utilised by the liver was estimated to be 741.6 mmol/h and 3708 mmol/h respectively. The heat associate with liver functioning was calculated to be 21.3% of whole body heat production. This is in good agreement with the values presented by Huntington & McBride (1988) (22-25% of whole body heat production).

It was estimated that substrate cycling between acetate and acetyl-CoA may account for 8.5% of ATP utilisation by liver or 4.6% of the heat produced by the liver.

These calculations have demonstrated the potential data which may be obtained from this type of *in vivo* experiment. Unfortunately this experiment was over shadowed by several problems, which highlight the difficulties associated with these studies.

CHAPTER SIX

DISCUSSION and FUTURE WORK

6.1 *Energy Metabolism*

Research on energy expenditure has a long pedigree and in more recent years one important development has been an examination of the individual processes which consume ATP and thereby lie at the basis of the overall expenditure of energy. As reviewed in Chapter 1 substrate cycles are energetically significant and it was emphasised that the literature is lacking in information regarding substrate cycling in ruminant tissues. Hence this project was interested in a substrate cycle which may be particularly pertinent in ruminant tissues, namely that between acetate and acetyl-CoA. It was estimated from *in vitro* determinations of the enzymes involved in this cycle that in ovine liver this substrate cycle may potentially account for 2.5% of basal metabolic rate, and may therefore be energetically significant *in vivo* (Jessop *et al.*, 1990). Unfortunately the experiment, conducted in collaboration with the Institute of Grassland and Animal Production, Hurley, experienced difficulties and it has not being possible to make any conclusions on the activity of this substrate cycle or its contribution to energy expenditure in bovine liver. This has been a major disappointment in achieving one of the objectives of this thesis.

In 1971 Milligan speculated that "*study of the extent of, and changes in, ancillary energy expenditures could be very helpful in the clarification of observations of particularly low efficiencies of production and differences between young and old, or lactating and non-lactating animals*". Subsequent studies have demonstrated very substantial changes in ancillary energy expenditures, in particular ion transport (notably Na^+,K^+-ATPase or Na^+-pump) and protein turnover, with nutritional and physiological status. (Milligan & McBride, 1985; Reeds & Fuller, 1983; Reeds *et al.*, 1985). Within different tissues the activity of the Na^+ pump and protein turnover varies widely in the maintenance of cellular homeostasis and integrity depending on the metabolic role of that tissue (Kelly & McBride, 1990). For instance tissues of the splanchnic bed (the gastrointestinal tract and liver) make up only 4-6% of whole-body mass, but account for 40% of total ATP utilisation. Skeletal muscle accounts for a much larger proportion of whole-body mass (50%), but accounts for only 20% of whole body ATP use (Huntington &

McBride, 1988; Kelly & McBride, 1990).

Mathematical approaches have been used to aid in achieving an integrated understanding of metabolism (i.e. Gill *et al.*, 1984), but only recently have such models considered the relative costs of individual processes within the background energy costs of growth (Gill *et al.*, 1989a,b). Gill *et al.* (1989a) have mathematically integrated whole-body protein synthesis and degradation based on protein metabolism in 10 individual tissues in growing lambs. Values predicted by the model agreed reasonably well with experimental data and the model was then used to examine the relative contributions of the Na^+,K^+-ATPase, protein turnover and fat turnover and accretion to ATP expenditure in lambs growing at either 90 or 230g/d. Na^+,K^+-ATPase accounted for 39% of the increment in ATP expenditure due to increased nutrient input at the higher rate of growth, while protein turnover and fat turnover and accretion accounted for 17% and 25% respectively. About 17% of the increment is therefore unaccounted and remains to be apportioned appropriately (Gill *et al.*, 1989b).

Mathematical integration of data is a very powerful technique and it highlights areas which require further research. Much of the data used in the model of Gill *et al.* (1989), especially on Na^+,K^+-ATPase were estimates from experimentation *in vitro*. The model indicated the need for information on the accuracy with which *in vitro* estimates of ion transport reflect the situation *in vivo*. It also revealed the need for multiple tissue data on protein turnover and associated energy processes.

In the future current techniques for *in vivo* estimation of biochemical processes will be further enhanced by the use of Electron Spin Resonance (ESR) and Nuclear Magnetic Resonance spectroscopy (NMR) (Blaxter, 198ϥ; Gadian, 1988). These techniques may enable the complete picture of O_2 consumption (ESR) and ATP consumption (NMR) by particular tissues *in vivo*.

The ultimate goal is to increase the efficiency of animal production. Ancillary energy processes are critical to metabolic control but are energetically expensive at the cost of "nutritional efficiency" (Kelly & McBride, 1990). Growth and the rate of growth increase the activity of energy consuming processes. Kelly & McBride (1990)

speculate that one method of increasing energetic efficiency would be to down-regulate the energetically costly events within supportive tissues, yet maintain adequate function to support growth, i.e. a reduction in the rate of cell turnover of Na^+,K^+-ATPase activity within the gastrointestinal tract yet maintain sufficient absorption.

6.2 *Efficiency of ME Utilisation*

In Chapter 3 it was proposed that the efficiency of utilisation of ME and why it is lower on feeding fibre diets, may be related to the maintenance of glucose homeostasis.

Several studies have demonstrated that the proportion of VFA's produced in the rumen influence nitrogen retention (see MacRae & Lobley, 1986). One experiment which demonstrates this observation very well is that of Girdler *et al.* (1986). Four sheep were sustained for six 4-8d periods by the intraruminal infusions of acetate (Ac), propionate (Pr) and butyrate (Bu), buffer and minerals and intraabomasal infusions of casein, emulsified tallow, glucose and vitamins. Isoenergetic infusions provided 1.1xM. In period 1 Ac:Pr:Bu was infused in the ratio 650:250:100 and casein was infused to give zero nitrogen retention. Subsequently propionate was withdrawn (Ac:Bu, 860:140) and replaced by various amounts of glucose. Withdrawal of propionate resulted in negative nitrogen retention, whilst glucose supply restored zero nitrogen retention and further supply increased nitrogen retention. This refects the influence VFA supply may have on the competition for amino acids between protein synthesis and gluconeogenesis.

Since the ruminant absorbs very little glucose from the digestive tract, the need for gluconeogenesis is great. The results in Chapter 3 further emphasise the point that increased supply of protein to the small intestine (on high protein diets, as indicated by the increased proportion of UDP in the diet compared to low protein diets, Table 3.2.5)., influences the utilisation of acetate. Higher levels of crude protein in the diet were associated with lower plasma acetate concentrations and increased rates of

acetate incorporation into lipid.

Carbohydrate source influenced the proportion of acetate oxidation in adipose tissue. Feeding fibre (sugarbeet pulp) resulted in a greater proportion of acetate being oxidised to CO_2 compared to starch (barley). These results support the hypothesis put forward by Blaxter (1962), and subsequently by MacRae & Lobley (1982), that the efficiency of utilisation of ME is related to the molar proportions of VFA produced in the rumen, efficiency of acetate utilisation and is also influenced by the level of crude protein in the diet (i.e. supply of amino acids).

Abzul-Razzaq & Bickerstaffe (1989) have further emphasised the role protein metabolism may have in explaining the efficiency of utilisation of ME. Their results suggested a high rate of protein turnover in lambs fed on a diet resulting in an acetate type of fermentation and a lower rate in lambs fed on a diet resulting in a propionate type of fermentation, which is consistent with a greater supply of amino acids for gluconeogenesis on acetate fermentations.

The influence of protein turnover on the utilisation of ME from fibre/starch diets is further supported by Bryant & Smith (1982). They reported that tyrosine flux was 30% lower in sheep fed on starch when compared to those fed on fibre.

This discussion has attempted to present a much larger view on an explanation for differences in the efficiency of utilisation of ME from different diets, involving VFA metabolism, protein metabolism and glucose homeostasis. This is a very complicated picture and it demonstrates an integration of energy and protein metabolism.

Since the maintenance of glucose homeostasis is central to this theory, glucagon and insulin regulation will also be pertinent. In the ruminant glucagon and insulin stimulate gluconeogenesis and protein deposition respectively (Trenkle, 1980; Lindsay, 1982). Insulin also stimulates lipogenesis by stimulating the uptake of glucose. The lower concentrations of insulin on fibre diets resulting in an acetate type of fermentation (Chapter 3; Abzul-Razzaq *et al.*, 1988) would ensure lower rates of amino acid uptake by peripheral tissues and hence more would be available for gluconeogenesis. Conversely higher insulin concentrations on starch diets resulting in

a propionate fermentation would direct amino acid towards protein synthesis and would also facilitate the utilisation of acetate by promoting glucose uptake in adipose tissue.

No doubt this is only a minor explanation of the hormones involved in regulation of this complex theory. This integrated approach to understanding the efficiency of ME utilisation is worthy of further consideration. Further studies on the role of protein turnover in this hypothesis are required since the studies of Adzul-Razzaq & Bickerstaffe (1989) failed to obtain significant effects of type of fermentation on protein synthesis and turnover. The influence of diet (and consequently type of fermentation) on hormonal regulation of this theory ought to be developed further.

This thesis has concentrated very much on tthe heat of metabolism as discussed in Chapter 1, in attempting to explain the efficiency of ME utilisation. However one must not disregard the importance of the gastrointestinal tract in the utilisation of ME. As mentioned in section 6.1, the portal-drained viscera (PDV) is a highly metabolically active tissue. It is not clear whether or not O_2 or ATP consumption by the PDV is different between fibre/starch based diets (Johnson *et al.*, 1990). Reynolds and Tyrrell (1989) have found a high starch diet to have a significantly lower PDV energy cost than a low fibre starch diet.

Hence an explanation as to why the efficiency of ME utilisation is lower on feeding fibre diets is still open to debate.

CONCLUSIONS

ATP-stimulated acetyl-CoA hydrolase is present in the cytoplasm of ovine and bovine liver, and unlike in certain other species, is not inactivated by cold. This enzyme may be involved in a substrate cycle between acetate and acetyl-CoA. It was calculated that the heat associated with this substrate cycle in ovine liver may account for as much as 2.5% of basal heat production.

The tendency for there to be higher activities of acetyl-CoA hydrolase and synthetase when lambs were fed on sugarbeet pulp when compared to barley diets suggest that the potential for substrate cycling between acetate and acetyl-CoA is greater on sugarbeet pulp diets, in perirenal adipose tissue. This may help to explain part of the increased heat increment associated with feeding fibrous diets. It was interesting to observe the effect of feeding level on these enzymes in perirenal adipose tissue. The higher enzyme activities on the low level of feeding may reflect increased substrate cycling between acetate and acetyl-CoA, regulating metabolism in response to acetate supply.

In Chapter 3, expt. 1, considerable variation was observed in the plasma concentrations of acetate, glucose and insulin in lambs which were fed once daily. This variation was attributed to irregular food intake throughout the day, which was related to the level of feeding. The use of semi-continuous feeding for 3 days before and during the blood sampling period in expt. 2 helped to achieve near-steady-conditions.

The influence of the level of crude protein in the diet on plasma acetate concentrations was intriguing. High (20.1%) and low (11.5%) levels of crude protein were associated with lower and higher concentrations of plasma acetate respectively. Carbohydrate source (fibre or starch) and protein level influenced the incorporation of acetate into CO_2 and lipid in perirenal adipose tissue, respectively. Feeding sugarbeet pulp (fibre) resulted in higher rates of acetate oxidation than barley (starch), whereas higher levels of crude protein increased the rate of acetate incorporation into lipid, compared to lower levels of crude protein. These results highlight the interaction between substrates and support the theory that the efficiency

of utilisation of ME may be related to acetate metabolism, which in turn is influenced by the provision of amino acids.

The technique developed for the separation and concentration of plasma organic acids was time consuming. It would be beneficial to increase the precision of the organic separation, especially between the ketone body and acetate fractions.

Several difficulties were experienced in the *experiment conducted in vivo*. In such experiments blood flow rates are critical. Attaining accurate blood flow data is difficult and problems such as "negative" blood flow rates have been experienced previously (i.e. Pethick *et al.*, 1981; Dr M. Gill, personal communication), using the PAH dye dilution method. The high acetate specific radioactivities in the carotid artery are unusual and not easily explained. It is disappointing that this experiment has yielded little information on the substrate cycle between acetate and acetyl-CoA *in vivo*.

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APPENDIX 1

Converting Enzyme Units from nmoles per min per mg protein into μmoles per min per g wet weight.

Using 2.5 nmoles per min per mg protein as typical enzyme activity.

Assuming that liver is approximately 35% dry weight and of the dry matter approx. 50% is protein, then:

1g wet weight = 0.35g dry weight

2.86g wet weight = 1g dry weight

Enzyme rate = 2.5 nmoles per min per mg protein

= 2.5/2 nmoles per min per mg dry weight

= 1.25/2.86 nmoles per min per mg wet weight

= 0.44 nmoles per min per mg wet weight

= 0.44 μmoles per min per g wet weight

For simplicity 0.5 μmoles per min g wet weight is used in the text.

APPENDIX 2

O_2 consumption, ATP utilisation and heat production by the liver.

O_2 Consumption

pO_2 was adjusted to that at pH 7.4 by using the equation:

$$pO_2\ (7.4) = pO_2\ (\text{measured}) \,.\, 10^{s}$$

where s = -0.48(7.4-pH), and the percentage saturation of haemoglobin calculated from a dissociation curve for bovine blood at pH 7.4 (Bartels & Harms, 1959). The concentration of O_2 was obtained as the sum of that dissolved in the plasma and that bound to the haemoglobin assuming 1g of haemoglobin binds 1.34ml of O_2 when fully saturated (Adams & Hahn, 1982)

hence, Content = combined O_2 + dissolved O_2

$$= (S/100 \times [Hb] \times 1.34) + \alpha pO_2 \text{ ml } O_2/\ 100\text{ml blood}$$

where,

S = per cent oxygen saturation of haemoglobin

[Hb] = haemoglobin concentration in g Hb/ 100ml blood

pO_2 = partial pressure of oxygen, in mm Hg

α = solubility coefficient of oxygen, = 0.000031 ml O_2/ ml blood/ mm Hg oxygen pressure

Using this equation and the data in Tables 5.3.1 & 5.3.2, the concentration of O_2 (μmol/ l blood),

Carotid artery = 6030

Portal vein = 4080

Hepatic vein = 3390

Assumed blood flows 2.34, 8.96 and 11.3 l/min for the carotid artery, portal vein and hepatic vein respectively.

Using the equation given in section 5.2.6 for calculating net flux, the net utilisation of oxygen by the liver is

$$= 741.6 \text{ mmol/h}$$

Assuming 5 mol of ATP synthesised from ADP + P_i per mole of O_2 consumed (see Gill *et al.*, 1989a), then ATP utilised by liver is

$$5 \times 741.6 = 3708 \text{ mmol ATP/h}$$

Since 1 mmol ATP = 77 Joules (see Gill et al., 1984), heat produced by the liver is,

= 3708 x 77 x 24 = 6852.4 kJ/day

= 6.85 MJ/day

Estimated Heat Produced by The Whole Animal

Assume M/D diet = 11.9 MJ/kg DM

DM intake = 24g/kg liveweight
= 3.84 kg DM
= 42.2 MJ/day

$$\text{Maintenance Energy} = \frac{0.53\,[W/1.08]^{0.67} + 0.0043W}{k_m}$$

= 22.2 MJ/day for 160kg steer assuming k_m = 0.71 (ARC, 1980)

Heat production = Maintenance + Production x $(1-k_f)$

= 22.2 + 20x0.55

= 32.2 MJ/day

(k_f = 0.45 ARC, 1980)

The heat produced as a proportion of total heat production of the animal is,

= 6.85/32.2

= 0.213 or 21.3%

ATP Utilisation and Heat Produced as a result of Substrate Cycling between Acetate and Acetyl-CoA.

Mean rate of cycling (Table 5.3.7) = 85.7 mmol/h

since 2 ATP are hydrolysed by the cycle then ATP utilised by cycle is,

85.7 x 2 = 171.4 mmol ATP/h

and since 1 mmol ATP = 77 Joules, heat produced

= 85.7 x 2 x 77 x 24 = 316.8 kJ/day

Total ATP utilised by liver = 3708 mmol/h, hence proportion utilised by substrate cycling between acetate and acetyl-CoA is,

= 316.8/3708 = 0.085

or 8.5%

Total heat produced by liver = 6.85 MJ, hence proportion produced as a result of substrate cycling between acetate and acetyl-CoA is,

= 0.317/6.85 = 0.046

or 4.6%