University of Glasgow

INTRAGASTRIC INFUSION AND ITS USE IN THE INVESTIGATION OF NITROGEN AND GLUCOSE METABOLISM IN RUMINANTS

By

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being

a thesis submitted in part fulfilment of the requirements for Doctor of Philosophy and comprising a report of studies undertaken at the Hannah Research Institute, Ayr, in the Faculty of Science of the University of Glasgow.

April 1986

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To Anne and Zoë

ACKNOWLEDGEMENTS

I thank the Director and General Council of the Hannah Research Institute for permission to undertake this work. I am equally indebted to the Department of Agriculture and Fisheries for Scotland for financial assistance.

I also acknowledge the contribution made by the following people :-

Dr P.C. Thomas for his guidance throughout the work and preparation of the thesis;

Dr E.R. Ørskov, N.A. MacCleod and D. Kyle at the Rowett Research Institute for their invaluable help at the outset of this work;

Mr J. McCann for his hard work and dedication as my assistant in what proved to be particularly demanding experimental work;

Miss E. Brechany for analysis of blood;

Dr D.G. Chamberlain for helpful comments and analysis of plasma amino acids;

Dr D. Reid for advice and help with statistical procedures;

My wife, Anne, for help in the preparation of the thesis and finally, I give many thanks to Mrs E. Barbour, my long-suffering typist.

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Ac	acetate
Acetyl CoA	acetyl co-enzyme A
ACS	acetyl CoA synthetase
ACTH	adrenocorticotrophic hormone
ADF	acid detergent fibre
ADL	acid detergent lignin
ADS	acid detergent solubles
ANOVA	analysis of variance
ANS	8-anilino-napthalene sulphonic acid
ATP	adenosine-5-triphosphate
AV	ateriovenous
BCG	bromocresol green
BSA	bovine serum albumin
Bu	butyrate
CF	crude fibre
CoA	co-enzyme A
cpm	cycles per minute
CPM	counts per minute
CrETDA	chromium II ethylenediamine tetracetic acid
CS	citrate synthetase
d	day
DCP	digestible crude protein
DM	dry matter
DMD	dry matter digestibility
EAA	essential amino acids
ED	external diameter
EE	ether extract
FAD	flavine adenine dinucleotide
FCM	fat corrected milk
FMN	flavine mononucleotide
g	gravity
GCN	L-γ-gluataryl-3-carboxy-4-nitroanilide
GDR	German Democratic Republic
GE	gross energy
GG	glycylglycine
GOD	glucose oxidase
GOT	glutamic oxaloacetic transaminase
GPT	glutamate-pyruvate transaminase
γGT	L-γ-glutaryltransferase
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
HRI	Hannah Research Institute
HSA	human serum albumin
HVN	hypothalamic ventromedial nucleus
ID	internal diameter
INT	2-(p-iodophenyl)-3-nitrophenyl-5-phenyl tetrazolium chloride
$kgW^{0.75}$	metabolic liveweight
LEM	leukocytic endogenous mediator
LHN	lateral hypothalamic nucleus
L-LDH	L-lactate dehydrogenase
LW	liveweight

MADF	modified acid detergent fibre
MDH	malate dehydrogenase
ME	metabolizable energy
mol	Mole
N	nitrogen
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NDF	neutral detergent fibre
NDI	neutral detergent insolubles
NDS	neutral detergent solubles
NEAA	non-essential amino acids
NEFA	non-esterified fatty acids
NFE	nitrogen free extract
NPN	non protein nitrogen
3-OH butyrate	D-(-)-3-hydroxybutyrate
OM	organic matter
OMD	organic matter digestibility
OP	osmotic pressure
PAH	p-aminohippiric acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
Pr	propionate
q	metabolizability, ME/GE
RIA	radioimmunoassay
RPM	revolutions per minute
RSD	residual standard deviation
SDH	sorbitol dehydrogenase
STH	somatotrophic hormone
TCA	tricarboxylic acid cycle (Krebs')
TCAA	trichloroacetic acid
UDP	undegradable protein
VFA	volatile fatty acids
VFI	voluntary food intake
VIC	Veterinary Investigation Centre
v/v	volume per volume
WOSAC	West of Scotland Agricultural College
w/v	weight per volume
w/w	weight per weight

SUMMARY

1. Current techniques used for measurements of the composition of feeds given to ruminants and of the subsequent utilization of nutrients by these animals were reviewed. Intragastric infusion, an alternative technique for the precise control of nutrient supply under experimental conditions, was introduced and its use for the investigation of nutrient utilization was discussed.

2. Experiments were undertaken to develop the intragastric infusion technique for use at the HRI for investigations into the effects of propionate supply on N utilization in ruminants.

3. In the first experiment propionate was isoenergetically withdrawn from 'control' infusions given to a cow which were designed to provide an above-maintenance supply of energy together with adequate protein to sustain zero N retention (420mg $N/kgW^{0.75}$ per d). The quantity of N excreted by the cow was increased from 47 to 54g/d (15%) in response to propionate withdrawal. The animal maintained its homeostasis, as judged by its plasma glucose concentration, and was able to derive 2.37g glucose/$kgW^{0.75}$ per d from the protein that was catabolised. In view of the types of glucose-precursor supplied to the animal, this quantity of glucose was equivalent to the animal's minimum requirement.

4. A more extensive study of the effects of propionate supply on N utilization was conducted with a larger group of animals and this was most conveniently achieved using sheep. Propionate was isoenergetically withdrawn from 'control' infusions and N excretion was increased by 2.64g/d (27%). From this a minimum glucose requirement of 2.44g/kgW$^{0.75}$ per d was calculated and proportions of this requirement were used to supplement isoenergetic propionate-free infusions in subsequent treatments. When glucose was reintroduced in quantities equivalent to the minimum requirement, a positive retention of N of 0.06g/kgW$^{0.75}$ per d was measured, despite there being no change in the 480mg casein N/kgW$^{0.75}$ per d provided by the infusions. When glucose was again isoenergetically withdrawn from the infusions the quantity of N excreted by the animals was 13% lower than in the identical treatment initially imposed.

It appeared that restriction of glucose-precursor supply was associated with increased efficiency of utilization of N and/or a reduction in glucose utilization. The effects of the treatments on the plasma concentrations of certain metabolites and amino acids suggested glucose sparing could have occurred and that non-essential amino acids were major contributors to gluconeogenesis. This despite the provision of ME in quantities calculated to allow positive energy retention.

5. In a third experiment, after an initial 'control' period (P1), propionate was withdrawn from the infusions (P2) and then reintroduced at 50% of the quantities given in (P1) for a prolonged period (P3, 32d). Finally propionate was fully reintroduced (P4). Blood analyses were made to provide information on the time scale and

nature of the metabolic response.

In P2 there was an increase in N excretion from 0.41 to 0.59g/kgW$^{0.75}$ per d (43%) despite a constant supply of 413mg Casein N/kgW$^{0.75}$ per d throughout the experiment. In P3 and P4, 0.38 and 0.36g N/kgW$^{0.75}$ per d were excreted respectively. P3 was intended to perpetuate the adaptive response initiated in P2 but, judging by the quantities of N excreted and the changes in the blood plasma constituents that were measured, the adaptive response had not been perpetuated and there was little difference in the effects of restricting propionate by 50% (P3) or not restricting propionate supply at all (P1 and P4).

6. A more severe perturbation of glucose-precursor supply was imposed on the animals. Following an initial 'control' period (P1), propionate was isoenergetically withdrawn from the infusions (P2) for as long as was practicable for the animals to avoid clinical hypoglycaemia. Propionate was then reintroduced to the infusions (P3) in the same quantities as given in P1.

The N excreted by the animals during the 3 treatments was 0.47, 0.64 and 0.46g/kgW$^{0.75}$ per d respectively despite the provision of 473mg casein N/kgW$^{0.75}$ per d throughout the experiment. Analysis of the N data from P2 showed there to be a tendency for N excretion to fall after the first 5 days of the treatment. This change in N excretion was not, however, associated with any changes in the constitutents of blood plasma that were measured.

7. The acute and chronic effects of intragasric infusion on ruminants were discussed.

8. The effects of the treatments imposed on the animals during the experiments were discussed collectively. when 'control' infusions were administered, the animals maintained homeostasis and the quantity of N required to maintain the tissues was 370mg $N/kgW^{0.75}$ per d for both cows and sheep.

The initial isoenergetic withdrawal of propionate from the infusions resulted in changes in the animal's utilization of substrates which were similar to those observed in fasting animals. This occurred despite the provision of energy in quantities calculated to allow positive energy retention. In these circumstances glucose was utilized at a rate of $2.52g/kgW^{0.75}$ per d which was less than equivalent values measured by other methods. As judged by the concentrations and proportions of amino acids in the blood plasma the major contributors to gluconeogenesis were the non-essential amino acids with a concomitant sparing of the essential amino acids. There were exceptions to this rule of which methionine was of particular interest.

Analysis of all the N data from the first period of experiments 2, 3 and 4 in which propionate was isoenergetically withdrawn from the infusions revealed a relationship between N excretion (g/d) and time (d) described by the expression:

$$y = 0.4427 + 0.131\ x - 0.02383x^2 + 0.00122\ x^3$$

$(r = 0.70,\ P < 0.05)$.

where y = N excretion ($g/kgW^{0.75}$ per d)

x = The time (d) following propionate withdrawal from the infusions.

Reintroduction of glucose-precursor supply resulted in reduced N excretion and indications that the efficiency of utilization of N had increased. The notion that this was associated with an improved husbandry of glucose was introduced together with the idea that the effects of glucose sparing were residual. The idea that an integral portion of the animal's requirement for protein is normally available for gluconeogenesis was discussed as an explanation for the reduced requirement for N maintenance which was associated with periods in which glucose-precursor supply was restricted. The resultant reduction in glucose requirement through the instigation of metabolic mechanisms designed to spare glucose would result in a reduction in the gluconeogenic demand on protein, which would then be made available for protein synthesis.

9. The wider implications of the intimate association between protein and energy metabolism indicated by the experimental findings were discussed. Particular reference was made to their effects on current ideas regarding the physiological mechanisms of compensatory growth as well as the accuracy of modern protein rationing systems.

SECTION I

INTRODUCTION

SECTION I

INTRODUCTION

The ultimate aim of research into ruminant nutrition is to accurately relate the nutrient content of animal feeds to the quantity and composition of animal products. To achieve this it is necessary to acquire a quantitative understanding of nutrient supply to the animal's tissues and of nutrient utilization for various metabolic purposes.

Nutrient supply is dependent upon factors which show considerable natural variation viz the quantity and composition of the consumed feed and its fate during digestion. Nutrient utilization is dependent on the interaction between nutrient supply and the animal's dynamic physiological state. Accordingly, attempts by researchers to quantify nutrient supply and utilization are fraught with difficulties.

This introduction discusses the factors that affect nutrient supply and utilization in the ruminant and considers the limitations of the methods that are currently used in quantitative studies of digestion and metabolism. A recently developed technique for the precise control of nutrient supply under experimental conditions is introduced and its use in the investigation of nutrient utilization is discussed.

NUTRIENT SUPPLY IN RUMINANTS

Nutrient supply to the rumen

The quantity and composition of nutrients entering the rumen for digestion are dependent on the composition of the food given to the animal and the quantity consumed by the animal.

Composition of foods. Compositional variation between and within the foods given to ruminants is considerable as can be illustrated by reference to the major categories of food given to ruminants in the UK. The single most important food is grazed or conserved grass. Legumes (lucerne, clover, peas, beans, vetches) and brassicas (kale, rape, cabbage, fodder beet, swede) are also used but in comparatively small amounts. The seasonality of crop production necessitates forage conservation for housed and outwintered stock and because forages are normally bulky and of a low nutrient concentration there is often a requirement for supplementary concentrates to meet the nutrient demands of productive stock.

The nutrient composition of grass is predominantly dependent on the species and strain grown. Growing conditions, fertilizer policy, growth stage, duration between defoliations and dry matter (DM) content also contribute to compositional variation (see Reid, 1982). As plants mature there is a reduction in soluble carbohydrate, protein, lipid and moisture content with a concomitant increase in the structural carbohydrates - cellulose, hemicellulose and lignin (Table I(1)) which imposes important limitations on nutrient availability. Regrowth following defoliation also affects grass composition by reducing the soluble carbohydrate and increasing

Table I(1): The effect of maturity on the chemical composition (g/kg DM) of herbage[1].

Chemical component	Stage of Maturity			
	Young leafy	Late leafy	Head emerged	Seed setting
Dry matter (DM)	150	250	300	350[2]
Crude protein	185	152	138	96
Soluble sugars, hexose, fructose + sucrose	138	118	113	106
Lipid	91	76	65	47
Cellulose	213	221	239	267
Hemicellulose	158	189	194	257
Lignin	27	36	43	73

1. Dried ryegrass S.23 (Waite et al., 1964).
2. McDonald et al., 1981.

the protein content (Thomas & Morrison, 1982), an effect also produced by high nitrogen (N) fertilization (Castle, 1982).

Conservation aims to facilitate the storage of forage for finite periods with minimal nutrient loss, but during conservation grass undergoes various changes in composition. When conservation is by drying, as in the case of hay, there is a reduction in soluble carbohydrate content due to continued plant respiration and microbial fermentation taking place in the early stages of storage. This has the effect of increasing the proportion of protein and fibre in the food. However, because of heating, which occurs in all hays below 90% DM, there is a concomitant reduction in digestibility (Watson & Nash, 1960).

With ensilage, fresh or wilted forage is stored in clamps or silos from which air is excluded by compaction and sealing (see McCullough, 1977; McDonald, 1976). Detrimental microbial activity is minimised either by previous acidification, with, for example, formic or sulphuric acids, by inhibition with formalin or by the addition of soluble carbohydrate to encourage anaerobic fermentation. The production of lactate and other acidic end-products of fermentation reduce the silage pH to around 3.8-4.2 at which point fermentation is inhibited.

During ensilage changes in nutrient content occur through the loss of effluent and associated leaching of soluble sugars, nitrogenous compounds, organic acids and minerals. The nutrient types lost depend on the stage and type of fermentation and the quantities depend on the initial crop moisture content (Castle, 1982; Ellern & Vic-Mo, 1979). Nutrient loss may also occur with exposure to air or dilution with extraneous water which leads to secondary,

aerobic fermentation by clostridial bacteria or yeasts. Secondary fermentation invariably reduces the feeding value of silage by producing 'butyric' characteristics (Table I(2)). Similarly basic compounds formed as by-products of clostridial proteolysis and gaseous ammonia increase pH and perpetuate nutrient loss (Thomas & Morrison, 1982). Clearly the composition of conserved food varies considerably from that of the same foods when fresh, the degree of variation being dependent on the degree of nutrient loss or conversion during the storage period.

Compositional variation is also apparent in concentrate foods but to a smaller degree. Concentrate foods for ruminants are normally used to supplement forage diets. The range of concentrate foods is large but within each class the compositional variation is small. For example, an energy concentrate such as barley varies little from 12-13 MJ metabolizable energy (ME), 82g digestible crude protein (DCP) and 53g crude fibre (CF) per kg DM (ARC, 1984). DM content remains relatively constant at 86% with variations ($\pm$ 2%) depending on processing. Concentrate foods are often compounded to give rations of specified nutrient content which results in the overall compositional stability found within particular compound feeds.

Determination of food composition. The most widely used method of determining the nutrient content of food is the proximate analysis, or Weende system. This system relies on standardized methods which were originally defined by the Association of Official Analytical Chemists (see Lassiter & Edwards, 1982).

Table I(2): The chemical composition of silage

Parameter	Silage type			
	Lactic	Butyric	Wilted	Restricted[1]
pH	3.9	5.9	4.2	5.1
DM (g/kg)	190	170	308	212
Protein N (g/kg total N)	235	353	289	740
Ammonia N (g/kg total N)	78	246	83	30
Lactic acid (g/kg DM)	102	1	59	26
Acetic acid (g/kg DM)	36	24	24	10
Butyric acid (g/kg DM)	1	35	1	1
Water soluble CHO. (g/kg DM)	10	6	48	133
Mannitol (g/kg DM)	41	-	36	-
Ethanol (g/kg DM)	12	-	6	4

1. Restricted by the addition of 75:25 W/W formalin: formic acid. (Adapted from McDonald and Edwards (1976).

In the scheme of analysis dry matter (DM) content is determined by drying in an oven at 135^{o} or 95^{o}C for 2h or 5h respectively. With silages this procedure can underestimate the true DM content of a feed due to the loss on heating of volatile residues (e.g. volatile fatty acids) which may account for up to 20% of DM (Wilson, et al., 1964). The use of a toluene distillation technique (Dewar & McDonald, 1961) takes account of volatile losses within its assessment of DM and is the preferred technique for silage analysis.

Heating in a muffle furnace for 30 mins at 600^{o}C removes the organic matter contained in a sample. The ash residue which remains after heating is a measure of the inorganic matter content of the sample. However, due to the high temperatures involved in the analysis, volatilization of certain inorganic minerals may occur which may result in losses of iodine, selenium, sodium, chlorine, potassium, phosphorous or sulphur,. Assessments of the inorganic matter content of foods by this method are thus prone to underestimation. The lipid components in the food are determined by boiling a dry, powdered food sample with ether. Fats, fat soluble vitamins (A, D, E, and K), oils, waxes, sterols, organic acids and pigments are extracted and determined gravimetrically when the ether is subsequently evaporated. These fractions are termed the 'ether extractives' or 'extract' (EE). Phospholipids and protein bound fats are unaccounted for in this fraction which effectively describes the crude fat content of foods.

Insoluble structural carbohydrates largely undigested in the monogastric are approximately defined by the crude fibre (CF) fraction. This is determined on the ether extracted residues which are successively boiled for 30 min with 1.25% w/v sulphuric acid

(H_2SO_4) and 1.25% w/v sodium hydroxide (NaOH) to remove all but cellulose, hemicellulose, lignin and minerals. Subsequent ashing at 600°C for 30 minutes removes organic matter and this represents the CF fraction.

Protein content is described in terms of crude protein (CP). N content is determined by the Kjeldahl method (see Davidson et al., 1970) in which a catalysed digestion with sulphuric acid (H_2SO_4) converts organically bound N to ammonium sulphate ($(NH_4)_2SO_4$). The resulting solution is rendered alkaline to facilitate ammonia (NH_3) release and distillation prior to absorption in standard acid and back titration with standard alkali. The N content is then used to calculate crude protein content on the assumption that crude protein contains 16% N.

The remaining food fractions are grouped and quantified by mathematical difference into the nitrogen-free extract or extractives (NFE). Some cellulose, hemicellulose and lignin as well as sugars, fructans, starch, pectins, organic acids, resins, tannins, pigments and water soluble vitamins are included in this fraction which is said to contain the more digestible of the structural carbohydrates (McDonald, et al., 1981).

Proximate analysis provides a means of classification of individual foods in terms of their component fractions and this potentially provides a method for the assessment of the nutrient content of foods. However, the adequacy of this assessment is dependent on the distinctive nature of the analytical fractions chosen and in this respect the Weende system has recognised shortfalls. In particular the CF fraction, assumed to represent the indigestible portion of the diet, was found to be an unreliable

measurement when it was shown that that up to 90% of straw and grass hemicellulose and lignin could be included in the NFE fraction (see Lassiter & Edwards, 1982).

Further subdivision of feed fibre fractions was patently necessary. Johnson et al. (1961) devised a crude fractionation technique to determine lignin content because of its strong association with indigestibility. Lignin was dissolved in acetyl bromide prior to spectrophotometric analysis. As the importance of lignin and its effect on nutrient availability became more fully appreciated (Morrison, 1976; Jarridge, 1980) the technique was improved (Morrison, 1972 and 1972a, 1973).

Van Soest (1967) isolated plant cell walls with detergents prior to fractionation into constituent lignin, cellulose and hemicellulose leaving a 90% digestible cell content fraction. This method represents a major improvement in forage analysis and is illustrated schematically in Fig. I(1).

Food analysis provides a means of quantifying food composition and its variability. However, the extent to which this is accomplished depends on the parameters measured and the accuracy and reproducibility of the techniques employed.

In practice, the problems of food analysis are compounded by the difficulty of obtaining samples representative of the bulk of the food consumed. Concentrate foods present fewer problems due to their relative uniformity but forage foods, because of their variability require regular and repeated sub-sampling (Stranks et al., 1983).

Food intake. Accepting the above difficulties of food sampling and analysis it is therefore possible to quantify the composition of

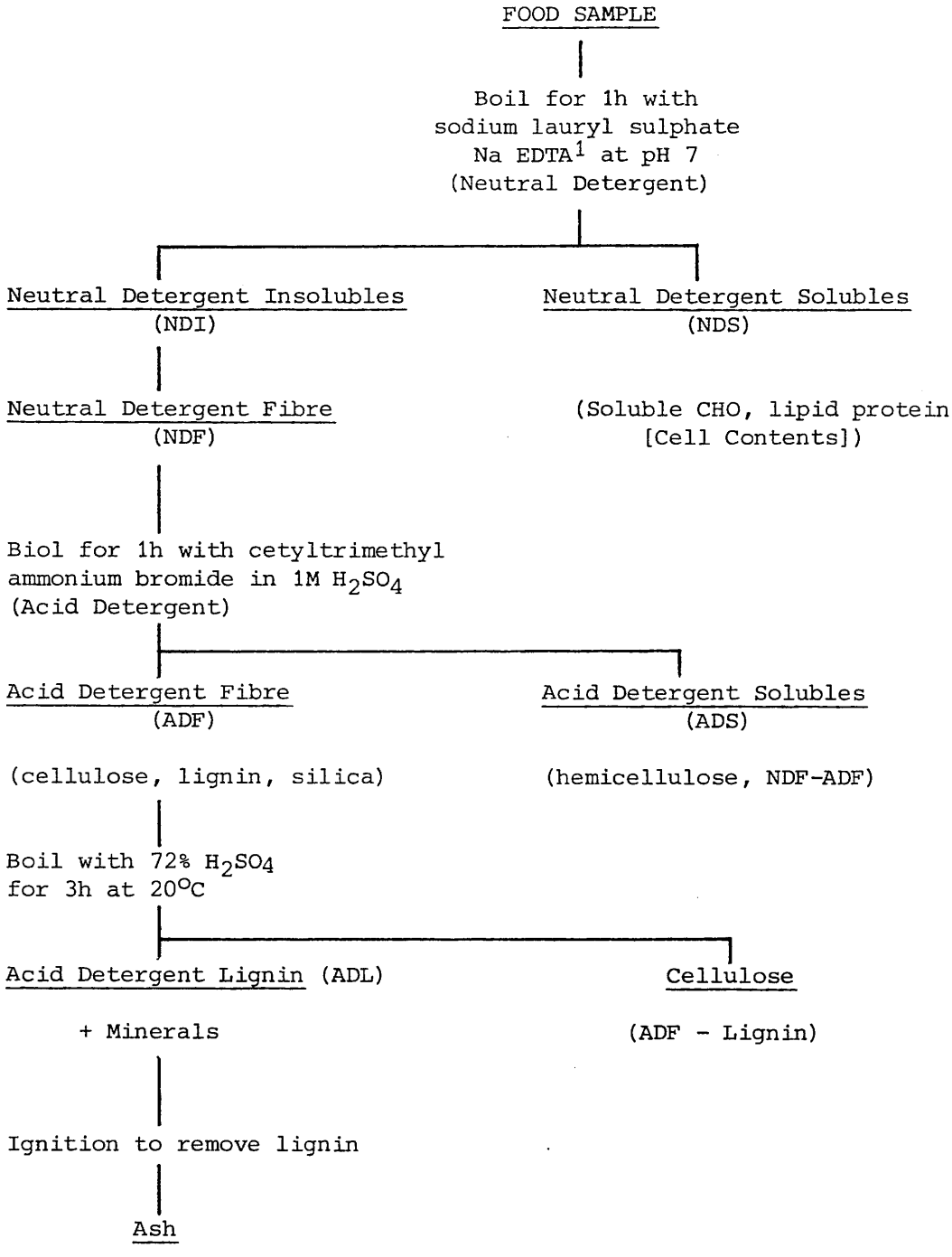


Figure I(1): Procedural flow chart for the fractionation of plant fibre using detergents (after Van Soest, 1967).
1. Sodium ethylenediamine tetracetic acid.

foods. When this information is combined with measurements of food consumption, an estimation of nutrient supply to the animal can be made. The quantity and type of food offered for consumption must, however, fall within the limitations imposed by the animal's voluntary food intake (VFI). Thus the limits of VFI represent a constraint over the range of dietary treatments that the experimenter can impose on the animals.

Physiologically, VFI is determined by the combined effects of the satiety (hypothalamic ventromedial nucleus (HVN)) and hunger (lateral hypothalamic nucleus (LHN)) centres of the brain (see Baile & Della-Fera, 1981). Peripheral receptors in the rumen wall, intestine, liver and brain itself monitor nutritional status by detection of key metabolic substrates and influence the animal's desire to consume food by their action on the hypothalamic nucleii (Forbes, 1983). Interruption and adjustment of this feedback system is possible by the higher centres of the brain in response to visual or olfactory cues and past experience. Accordingly VFI is the result of interaction between various food, animal and environment related factors (see Forbes, 1970).

The ultimate constraint on VFI is gut distension (Campling, 1970; Balch & Campling, 1962; Leek & Harding, 1975). Reticulorumen wall mechano-receptors induce satiety by stimulation of the HVN in response to the quantity of food residues held within the gut. Animals with large bodysize tend therefore to demonstrate high VFI by way of gut capacity.

Measures to reduce the bulk of foods, by removing water and air by dehydration and compaction, increase VFI (Campling & Lean, 1983). Reductions of particulate size (Marsh, 1978) and structural

disruption of plant tissue by physical and chemical means, to optimise the rate of digestion and consequent passage of feed (Van Soest, 1975) have a similar effect. Optimum digestion can generally be achieved provided adequate nutrients and a favourable environment are available for the rumen microflora (see following section on carbohydrate digestion).

Within the limitations of bulk, ruminants attempt to maintain energy balance (Baumgardt, 1974) regardless of dietary energy concentration. High demands for energy as a consequence of physiological changes associated with periods of rapid growth (Wilson & Osbourn, 1960), pregnancy and lactation stimulate increased VFI (Bines, 1979). Conversely a reduction in energy demand as a result of the deceleration of specific metabolic pathways through inhibition by toxins, disease or nutrient deficiency leads to reduced intake (Forbes, 1983). Growth hormone:insulin ratio is thought to mediate the response; insulin predominating during periods of nutrient abundance and growth hormone in periods of scarcity. Shortfalls in energy requirement are met through hormone-mediated, endogenous lipolysis but long-term energy equilibrium can only be achieved by varying feed and energy intake to allow either a predetermined (Hervey, 1979) or variable (Wirtshafter & Davis, 1977) degree of body condition.

Modification of the basic intake response to bulk and energy requirement occurs continuously. Rumen-wall chemoreceptors respond to acetate and propionate concentrations as well as pH (Martin & Baile, 1972) by reducing VFI whilst duodenal receptors respond to lactic acid (Forbes, 1983) and hepatic receptors respond to propionate (Baile & Forbes, 1974). The brain may respond directly to

glucose deficiency by increasing VFI if an increase has not previously been initiated by the reduction in circulating insulin and increase in growth hormone levels.

Potentially over-ruling all these agents is the neural activity of in the higher centres of the brain. Greater dry matter intakes are achieved with palatable foods (Greenhalgh & Reid, 1967) than with similarly digestible unpalatable foods. Accordingly dry matter intakes of poorly ensiled forage containing large quantities of protein breakdown products are low (Waldo, 1977; Wilkins et al., 1971) even when the effects on VFI of the associated amino acid deficiencies and metabolic inhibitors (histamine, tegramine, seratonin) are taken into account. Behavioural changes associated with social eating, order of dominance and reproduction also modify ruminant intake as agonistic priorities change with circumstance. These and other variables with smaller influence on VFI make its prediction extremely difficult.

Prediction of VFI. Currently various predictive relationships are proposed for ruminant VFI ranging from simple expressions of DM intake per unit liveweight for sheep and growing beef animals (ARC, 1976; ARC, 1984) to more complex partial regressions based on liveweight and yield for lactating ruminants (ARC, 1984). All are estimates for a particular set of circumstances and a reliance on the 'experience and judgement of the feeder' is deemed essential for correct estimation (ARC, 1976). The technical review of ruminant nutrient requirements carried out by the ARC Working Party (1980)

provides estimates of maximum DM intakes for lactating cows. Their starting point is the value of 135g DM/kg of metabolic liveweight ($kgW^{0.75}$) per d which is based on various experimental sources. It is a mean value for the complete lactation of cows yielding an average of 5000kg fat corrected milk (FCM) per 305 day (d) lactation. Adjustments for above or below average daily FCM production and stage of lactation are made and the values given are relevant to diets in the range of metabolizability (q or the ratio of ME to gross energy (GE), ME/GE) of 0.55 to 0.65.

VFI and its prediction impose a major limitation on the design and execution of animal feeding experiments. Manipulation of the components of experimental diets must remain within the constraints of VFI for individual animals which precludes the use of extremes of nutrient type and quantity

Conclusions

In summary, nutrient supply to the animal is seen to depend on the composition and quantity of the foods consumed. Food composition is variable and the accuracy with which it can be determined depends on the fractions chosen for measurement and the accuracy of the analysis employed. Furthermore, if manipulation of nutrient supply is necessary for experimental purposes it is largely limited by practical constraints on diet formulation, by the range of food that are acceptable to the animal as sole dietary ingredients and by the need to maintain food intake. These together with the constraint imposed by the upper limit of voluntary food intake reduce the scope for achieving extreme ranges of nutrient supply.

Digestion

Digestion of dietary constituents. Nutrients consumed by ruminants are largely digested in the rumen, abomasum and duodenum. Significant digestion also occurs in the caecum and colon. The end products of digestion are absorbed, either on site or further down the alimentary tract.

On entering the rumen, ingested food undergoes considerable microbial modification. As a consequence the quantity and type of nutrients made available for absorption bear little resemblance to those ingested. For convenience the microbial degradation of food can be regarded as occuring in three concurrent parts; carbohydrate, protein and lipid digestion.

Carbohydrate digestion. Plant tissue, which comprises the major part of the diet may contain up to 75% carbohydrate depending on species, age and previous crop management. Carbohydrates may be divided into three categories: cellulose and hemicellulose; pectins; and starches, dextrans and soluble carbohydrates (Leng, 1970). In the rumen all undergo a degree of microbial degradation to initially yield pyruvate (Fig. I(2)) prior to the formation of volatile fatty acids (VFA), carbon dioxide and methane (Fig. I(3)). Gaseous products are removed from the rumen by eructation whilst VFA's of which approximately 90% are found as acetic, propionic and n-butyric acid (Annison, 1983), with smaller quantities of iso-butyric, n-valeric (McDonald et al., 1981), are absorbed across the rumen wall.

The VFA's produced in the rumen are found to be energetically equivalent to between 53 and 62% of the digestible energy of the food

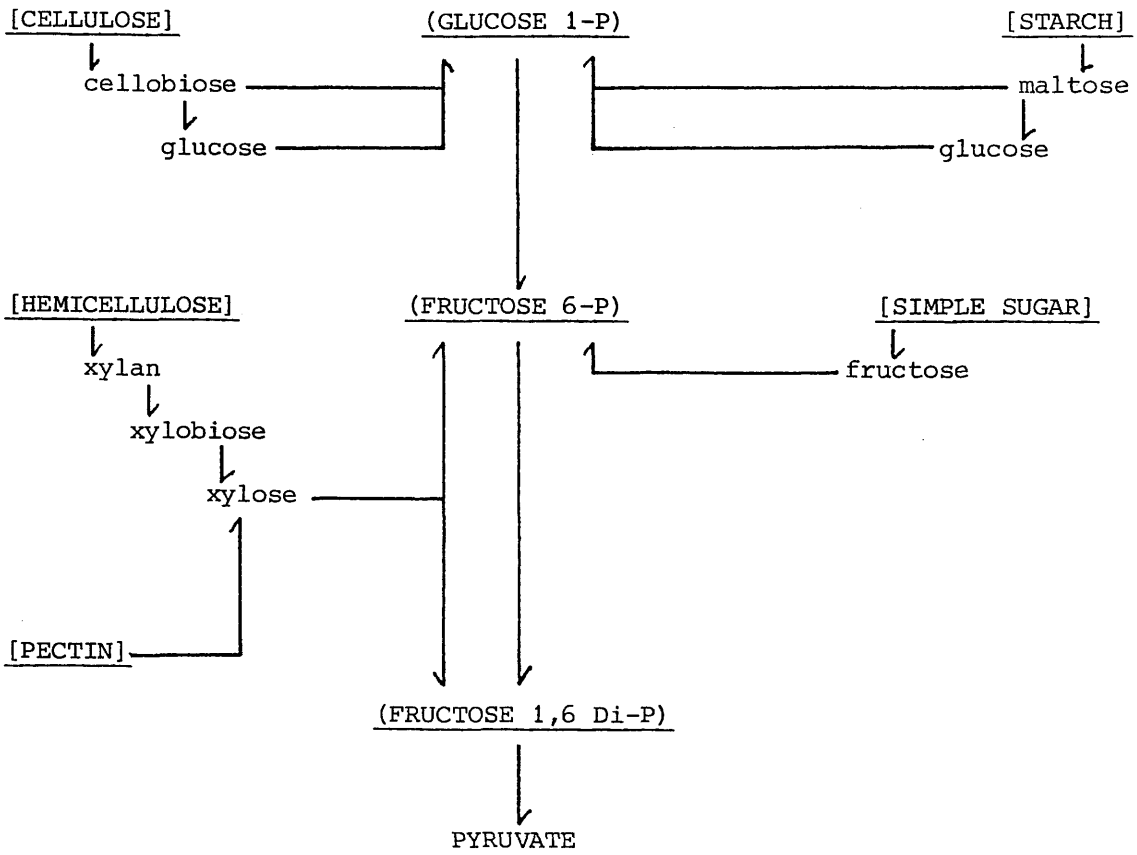


Figure I(2): Initial stages in the fermentation of the major plant carbohydrates by the rumen microbes (From Lewis & Hill, 1983). [SUBSTRATES] and (MAJOR INTERMEDIATES).

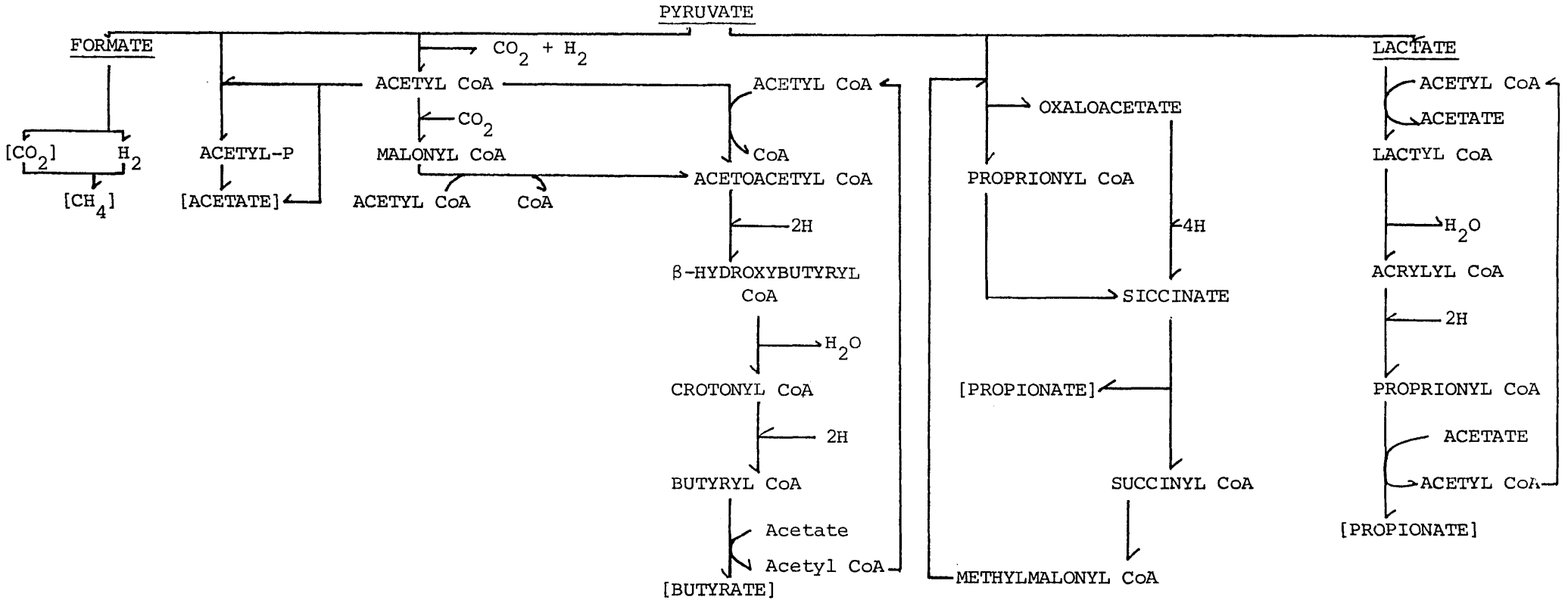


Figure I(3): Pyruvate degradation in the rumen during the fermentation of plant carbohydrates. (After Leng, 1970).

(Whitelaw et al., 1970). Variation in the relative production rates of VFA is normal and largely depends on the amount of pyruvate available in the rumen which itself reflects the rate of polysaccharide degradation and the soluble sugar content of ingested food.

Acetate provides the major electron sink for pyruvate oxidation during fermentation, the hydrogen released being made available for methane and propionate formation. A proportion of the acetate undergoes condensation and reduction to form butyrate. When pyruvate is present in abundance those reactions lead to increased hydrogen ion concentration in the rumen. The lowering of pH associated with this inhibits methanogenesis and the consequent removal of hydrogen from the rumen. This in turn leads to an increase in the reduction of pyruvate to propionate and a reduction in the oxidation of pyruvate to acetate.

Accordingly, forage diets with low soluble sugar content and the requirement for a large degree of physical and chemical action to facilitate polysaccharide degradation result in acetate biased fermentations. Typically 65-74% acetate, 15-20% propionate and 8-16% butryate (Thomas & Rook, 1977; McDonald et al., 1981). Increasing the rate of forage degradation by grinding increases propionate and or butyrate production at the expense of acetate (Osbourn et al., 1976). Similar effects are also demonstrated with moderate levels of concentrate supplementation. Diets composed of a high proportion of concentrates can lead to extreme propionate concentrations (30% of total VFA on a molar basis) at the expense of acetate and to a lesser degree, butyrate. Generally associated with this there are

reductions in rumen pH and cellulolytic activity (Rook, 1975; Annison, 1983).

Level of feeding influences VFA production and is dependent on other, associated effects; high levels tend to produce a propionate biased fermentation whilst restricted meal feeding may produce propionate peaks and an overall reduction in digestive efficiency. The rate of propionate production is roughly proportional to rumen digestible organic matter levels (Sutton, 1980). Large dry matter intakes resulting in significant dilution of digesta may also facilitate a shift in emphasis from propionate to acetate production as a result of an associated reduction in digestibility and rate of pyruvate formation.

The relationship between the composition of ingested carbohydrate and the quantity and relative proportions of the VFA produced is difficult to quantify. VFA production reflects both the substrates fermented and the metabolic activity of rumen microbes (Sutton, 1969) which is itself dependent on the composition of the microbial population and dietary and non-dietary factors effecting the rumen environment. Furthermore, the accuracy with which rumen VFA molar concentrations represent VFA production is questionable despite its widespread use. The relationship is said to hold for high forage diets (Annison, 1983; Thomas & Rook, 1977) but as a consequence of increased rates of absorption at low pH (Daneilli et al., 1945), and the possibility of differential absorption (Stevens, 1970) it is not generally applicable to mixed diets (see Table I(3)). Hence changes in rumen liquor concentrations of VFA can only safely be attributed to qualitative rather than quantitative shifts in VFA production (see also Sutton & Morant, 1978).

Table I(3): Total VFA production (mol/d), concentration (mmol/l) and VFA proportions (mmol/mol) produced and present in the rumen of sheep given various diets. (After Sutton and Morant, 1978).

Diet type	Total VFA produced (mol/d)	Concentration of VFA in the rumen (mmol/l)	VFA proportions produced and present in the rumen (mmol/mol total VFA)			
				Acetate	Propionate	Butyrate
100% hay	3.56	59.60	Molar proportions produced	730	200	70
			Molar proportions present	740	190	70
			Difference[1]	20	10	10
40% hay	5.13	75.30	Molar proportions produced	600	220	180
60% concentrate			Molar proportions present	660	190	150
			Difference	70	30	60
10% hay	6.15	97.40	Molar proportions produced	610	300	100
90% concentrate			Molar proportions present	570	310	120
			Difference	80	50	30

1. The mean difference between the molar proportions produced and the molar proportions present regardless of sign.

A substantial quantity of structural β-linked carbohydrate leaves the rumen undegraded and this passes through the small intestine to the caecum and colon where a further degree of microbial fermentation ensues and VFA is absorbed . The more soluble, α-linked carbohydrates undergo extensive rumen degradation (Leng, 1970) with little reaching the small intestine (see Armstrong & Beever, 1969; Lewis & Hill, 1983) depending on food source and degree and type of processing. Of that reaching the small intestine most is digested and absorbed as glucose.

Protein digestion. Protein digestion in the ruminant leads to a complex cycling of nitrogenous compounds (Fig. I(4)).

In the rumen, soluble proteins are degraded through peptides and amino acids to ammonia and ketoacids. Non protein N (NPN) consumption facilitates microbial amino acid and ammonia production and is supplemented by recycled urea from the saliva (Nolan et al., 1973) and by diffusion across the rumen wall (Thorlacius et al., 1971), although the quantitative importance of the latter route is subject to some controversy (Hecker & Nolan, 1971). Incorporation of peptides, amino acids and ammonia into microbial protein occurs continuously provided adequate energy and micro-nutrients (e.g. sulphur) are available (see Harrison & McAllan, 1980). Studies of the dynamics of rumen ammonia metabolism indicated that more than half of microbial N was derived from ammonia (Pilgrim et al., 1970; Nolan and Leng, 1972). Inadequate rumen ammonia concentration may lead to uncoupled fermentation of organic matter (Satter & Slyter, 1974; Beever et al., 1974) whilst excess is recycled, wasted through excretion in urine or causes toxicity. The optimum ammonia level is

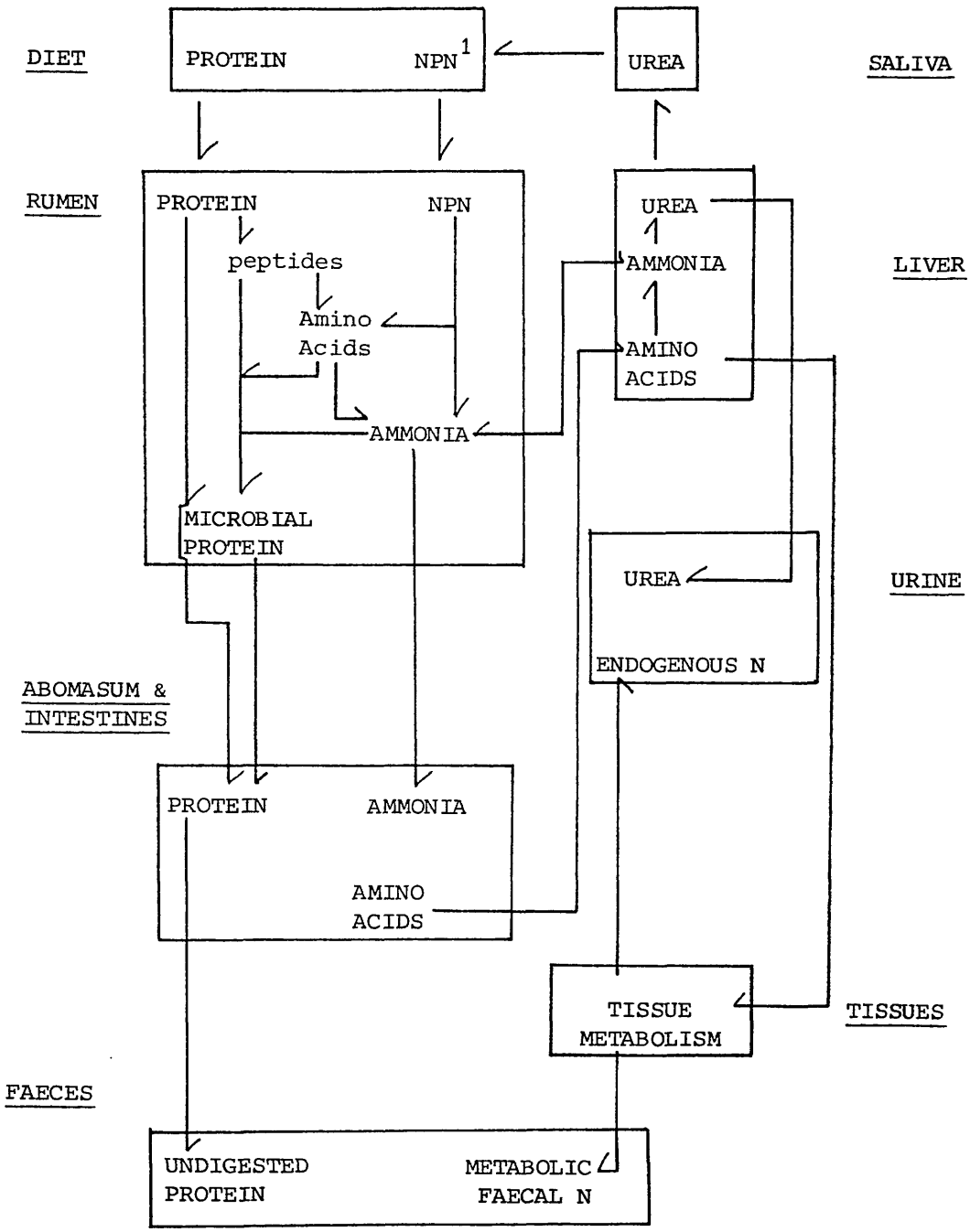


Figure1(4): Nitrogen (N) digestion and associated metabolism in the ruminant (After Lewis & Hill (1983)). 1. Non-protein nitrogen.

subject to much controversy (see Miller, 1973; Okorie et al., 1977) and depends on the availability of energy in the rumen.

Undegraded protein (UDP) together with microbial protein flows into the abomasum and small intestine. There the action of pancreatic and intestinal proteases cause protein hydrolysis prior to absorption of the constituent amino acids. Nitrogenous products remaining unabsorbed undergo further microbial degradation in the caecum and colon.

The quantity and composition of protein absorbed from the small intestine depends on the relative proportions of microbial and undegraded dietary protein flowing from the rumen (see Hagmeister et al., 1976).

Microbial protein production is influenced by the quantity of adenosine-5-triphosphate (ATP) made available from the fermentation of dietary carbohydrate. It has been estimated that 10.5g bacterial dry matter can be produced per mol of ATP (Bauchop & Elsden, 1960) and that approximately 75% of microbial energy requirement is associated with protein synthesis (McMeniman et al., 1976). Unsuccessful attempts have been made to use this relationship for the prediction of microbial protein yield from hexose fermentation and VFA and methane production (Hungate, 1966). This failure has been attributed to variability in microbial yield (Thomas, 1973; Mercer & Annison, 1976) and to the presence of both bacteria and protozoa in the rumen population. Despite this, it is generally accepted that approximately 32g of microbial N is produced per kg of organic matter (OM) apparently digested in the rumen, although this value does vary with the type of diet given (ARC, 1984).

Changes in the rate of microbial synthesis are associated with propionate concentration (Jackson et al., 1971), dietary sulphur content (Hume & Bird, 1970) rumen protozoal population (Eadie et al., 1970) and liquid and solid phase dilution rates (Harrison & McAllan, 1980).

Dietary protein undegraded by the microbial population (UDP) flows into the small intestine. Protein degradability increases with solubility and is variable with food type and previous processing. Ørskov and Mehrez (1977) and Mehrez and Ørskov (1977) quote values of 80%, 38% and 82% respectively for barley, white fishmeal and silage. Manipulation of protein supply through physical and chemical 'protection' is used widely (Chalupa, 1975) but the degree of protection achieved is subject to variation. High levels of food intake also increase the amount of undegraded dietary protein leaving the rumen (Mercer & Annison, 1976) and this is associated with an overall reduction in dry matter digestibility.

Protein flowing from the rumen is further modified through the inclusion of mucosal secretions and desquammated epithelial tissue associated with the digestive process. The precise magnitude of this endogenous fraction is however, difficult to determine due to the difficulty of separating it from other proteins. Ingested protein is, hence, subject to considerable modification in the rumen and in consequence the quantity and amino acid composition of protein entering the abomasum, and available for digestion and absorption, bears little resemblance to the quantity and/or quality of protein in the food.

Lipid Digestion. Lipids constitute 5-10% of forage and 4-5% of cereal DM content (Annison, 1983). Linolenic acid (C18:3) and other mono and digalacto-1,2-diglycerides predominate the forage lipids whilst linoleic (C18:2) and triglycerides predominate the cereal lipids. Both di- and triglyceride fractions show a high level of unsaturation (Table I(4)). Phospholipids, glycolipids, sterols and waxes also occur but in smaller amounts.

In the rumen, bacterial lipases bring about the lipolysis of complex lipids with the liberation of largely unsaturated free fatty acids (FFA) together with glycerol and galactose for fermentation (Garton, 1977). Extensive biohydrogenation occurs with the production of trans-fatty acids, 80-90% of linolenic and linoleic acids being hydrogenated to stearic acid (C18:0) (Bickerstaff et al., 1972; Sutton et al., 1970),(see also Table I(5)) Lipid synthesis de novo from glucose and acetate also occurs in the rumen; its contribution to total lipid supply being small but significant (Sutton, 1980).

The degree of microbial modification of dietary fats in the rumen depends on the composition of the microbial population. Ciliate protozoa, for example, which are important in the hydrogenation of unsaturated fats, are particularly sensitive to dietary factors which cause low pH (Storry, 1970). This coupled with the variable type and accessibility of lipid in the diet and with substrate dependent synthesis de novo of fatty acids by the rumen microbes (Sutton et al., 1970) explains the diverse relationship between the lipid passing to the duodenum and that present in the diet (see Table I(6)).

Table I(4): Fatty acid composition of mature ryegrass (g/kg fatty acid).

Fatty acid		Content g/kg fatty acid
Lauric	12:0	5
Myristic	14:0	10
Palmitic	16:0	169
Palmitoleic	16:1	19
Stearic	18:0	10
Oleic	18:1	27
Linoleic	18:2	127
Linolenic	18:3	632

Table I(5): Comparison of the fatty acid composition of conserved and fresh forage with rumen digesta (weight % of major constitutent fatty acid groups).

Fatty acid		Ryegrass[1]	Hay	Rumen[2] digesta	Rumen[3] digesta
Palmitic	C16:0	16.9	33.9	25.3	26.6
Stearic	C18:0	1.0	3.8	37.0	46.0
Oleic	C18:1	2.7	3.0	6.5	7.0
Linoleic	C18:2	12.7	24.0	3.2	2.8
Linolenic	C18:3	63.2	31.0	15.8[4]	5.3

1. Adapted from Thomas & Morrison (1982). 2. Adapted from Moore et al. (1969). 3. Adapted from Keeney (1970). 4. Estimate from Bickerstaffe et al. (1972).

Table I(6). Difference between duodenal flow and dietary intakes of fatty acids in cows given hay together with barley or maize in two proportions (g of fatty acid/kg DM of food consumed per day).

Fatty acid	Diet[1]				
	1	2	3	4	SEM
C14	0.27	0.49	0.75	0.57	0.06
C16	0.44	1.21	1.26	0.69	0.24
C18	3.69	11.70	3.52	11.18	1.37
Total fatty acid	5.45	13.82	7.00	13.05	1.31

1. Diet types (DM basis):
   1. Hay:barley 40:60; 2. Hay:maize 40:60;
   3. Hay:barley 10:90; 4. Hay:maize 10:90

From Brumpy et al. (1979).

Lipids leave the rumen either bound to food particles, incorporated into microbial cells or as free fatty acids in the liquid phase (Leat & Harrison, 1975). In the hay-fed cow these routes accounted for 80.3%, 4.1% and 15.6% of total outflow respectively (Keeney, 1970). In the small intestine fatty acids of chain length C10-C12 are absorbed directly into the portal circulation whilst longer-chain acids are incorporated into chylomicra and pass into the lymphatic system (Garton, 1969).

Techniques used for the investigation of digestion in ruminants. The microbial and animal metabolism of ingested nutrients means that the products of digestion available for absorption bear few compositional or quantitative similarities to the original food. This problem is exacerbated by the dynamic nature of the microbial population and its response to variations in the amount and relative proportions of nutrients supplied to it and the prevailing conditions in the reticulorumen. Attempts to quantify the relationship between food constituents and nutrients made available for absorption have led to the development of various experimental techniques.

Measurements of digestibility. Traditionally, quantitative estimates of the digestibility of foods were used as a measure of nutrient supply, the digestible nutrient content of the food being assumed to provide a measure of nutrient uptake during the digestive process. The adequacy of such 'black box' procedures is limited, however, through an inability to account for nutrient removal by means other than absorption (e.g. the loss of energy by microbial heat of fermentation or by the eructation of methane); endogenous

nutrient addition (e.g. proteinaceous, secretions and gut desquamations); and extensive nutrient modification (e.g. biohydrogenation of unsaturated lipids and lipogenesis de novo) (Schneider & Flatt, 1975).

In recognition of the inadequate description of nutrient uptake derived from digestibility trials, surgical and isotopic procedures were developed to directly measure the disappearance of nutrients from the gut.

Surgical techniques. Initial investigations into the digestion of nutrients involved the dissection of specific areas of the digestive tract of slaughtered animals that had previously been acclimatized to specific experimental diets. These procedures yielded much qualitative data regarding nutrient disappearance and formation along the digestive tract. However, it was shown that shedding of intestinal mucosa occurred when animals were subject to the shock of humane slaughter (Badaway, 1957, 1958). This endogenous addition of sloughed epithelial tissue resulted in data of questionable validity. Data of a more quantitative and accurate nature than that derived from digestion and slaughter experiments can only be obtained with the use of surgically cannulated animals.

Cannulation procedures. Continuous access to the digesta contained in various sites along the digestive tract can be achieved by cannulation in the rumen, omasum, abomasum, duodenum, ileum or caecum or in combinations of these areas (Hecker, 1974). The sampling of digesta and measurement of the concentration of individual nutrients allowed by such techniques coupled with

measurements of digesta flow yield quantitative estimates of the net disappearance of nutrients from the gut.

Various types of cannula have been used including simple T-piece designs (Phillipson, 1952) from which 'spot' samples can be taken; re-entrant designs (Markowitz et al., 1964) in which localized areas of the digestive tract are effectively exteriorized to allow total collection of digesta; and, more recently, the 'Komarek' design (Komarek, 1981) which combines the simplicity of surgical preparation of the T-piece cannula with the sampling characteristics of the re-entrant.

Controversy exists, however, over the effects of cannulation. MacRae (1975) and MacRae & Wilson (1977) suggest certain techniques may cause 'chronic stress' and observed small changes in digestibility and voluntary food intake associated with reduced digesta flow. These findings were in agreement with Nicholson and Sutton (1969), Topps et al. (1968), Wenham (1979), and Hutton et al. (1964) but conflicted with the observations of Buttle et al. (1982).

The procedures used to establish digesta flow rate varies with the sampling technique employed. With T-piece cannulae intermittent, spot sampling is used whilst with Komarek or re-entrant cannulae continuous sampling is possible, where donor digesta is used to replace the sample digesta removed. The latter cannulae also allow the use of continuous automated digesta collection (see MacRae, 1975, Axford et al., 1971; Vera, 1985). The use of indigestible marker or reference substances to establish digesta flow is essential with T-piece cannulated animals whilst being favoured for Komarek and re-entrant sampling and unnecessary for automated digesta collection where total collection and sub-sampling occur. The use of automated

collection procedures is, however, still quite limited which explains the widespread use of marker substances.

Markers. The measurement of digesta flow involves the addition of undigested, unabsorbed markers or reference substances to the diet. Changes in the relative proportions and absolute quantities of marker and nutrient in the food and digesta can, as well as being used to calculate digesta flow and extent of passage, be used to determine food intake and digestibility and correct 'spot' sample data.

Markers may be elements, compounds or particulates (see Kotb & Luckey, 1972) and may associate with the digesta liquid phase (e.g. polyethylene glycol (PEG) or chromium II ethylenediamine tetraacetic acid (Cr.EDTA)), solid phase (e.g. lignin and complexes of ruthenium (Ru) and ceasium (Ce)) or be intermediate between the two (e.g. chromium sesquoxide (Cr_2O_3)).

Selection criteria for nutritional markers are numerous. They must be inert with no toxic, or physiological effects; be neither absorbed nor metabolised, to allow complete recovery from raw or digested food; have no appreciable bulk and mix intimately with the feed whilst remaining uniformly distributed throughout the digesta; have no influence on alimentary secretion, digestion, absorption, motility or excretion and have no influence on gut microflora.

Accordingly the choice and performance of markers leads to considerable discussion. Of the liquid phase markers Cr.EDTA is preferred to PEG which may be poorly recovered due to precipitation and solid phase binding (Thomas, 1978; Pickard & Stevens, 1972). Lignin used for solid phase marking undergoes variable degrees of

digestion whilst the rare earths are difficult to measure (Evans, et al., 1977) and their radioactive complexes may impose a health hazard. Cr_2O_3 is used widely in digestive studies but suffers variable recovery rates (see Offer et al., 1971; Sutton et al., 1976).

Markers pass through the gut at rates approximately equal to particles with similar physical characteristics (Hyden, 1961). The limited choice of markers usually results in a compromise selection with associated inaccuracy. The use of a dual-marker technique to simultaneously mark liquid and solid phases improves accuracy (Faichney, 1975). Within the limits of the techniques available it is possible to derive quantitative estimates of the disappearance of nutrients at various sites along the digestive tract. Investigations into the rates of production of specific metabolites as opposed to their removal necessitates the use of radio-isotope dilution techniques.

Isotopic procedures. The entry or disappearance rate of a specific metabolite from a defined pool is mathematically related to its effect on the dilution of infusions of its isotopically labelled form. Labelled material may be introduced in small quantities over a short finite period (i.e. single injection or pulse labelling) or at a continuous, precisely controlled rate (continuous infusion) which may be preceded by a single injection (primed infusion) to ensure the rapid onset of steady-state conditions (Leng, 1970a).

Isotope dilution procedures are widely used in digestive studies, particularly in the determination of VFA production rates.

These procedures rely on an estimation of the dilution of infused ^{14}C and ^{3}H labelled VFA by the corresponding VFA produced in the rumen. Measurements of protein metabolism are also undertaken with isotopes. Of particular importance is work involving the use of ^{15}N marker techniques (Nolan, 1975). The main areas of inaccuracy found with the techniques described are associated with slow or variable mixing of label within the rumen pool; variable relative production rates in the case of VFA depending on frequency of feeding; sampling error due to proximity of infusion and sampling sites; and re-cycling and transfer of label (Leng, 1970a). Choice of infusion system and subsequent analysis of results also affect accuracy, with single injection and first order kinetics calculations having a lower accuracy than continuous infusion and multicompartmental techniques of mathematical analysis (see Leng, 1970a; Kronfeld et al., 1971).

Arteriovenous difference techniques. An alternative, indirect approach to the measurement of the end products of digestion is to determine nutrient uptake by the hepatic portal vein or whole body by arteriovenous difference (AV) techniques.

Differences in the concentrations of nutrients in portal, hepatic and arterial blood are measured together with simultaneous estimates (Harrison et al., 1971) or assumptions (Cook & Miller, 1965) of the rate of blood flow. The product of blood flow and AV difference in the concentration of nutrients flowing through a blood vessel represents the net nutrient flux.

Several techniques are used for the measurement of blood flow including thermo-dilution (Annison, 1965), radio-telemetry (Hume et al., 1972) and the dilution of dyes or p-aminohippiric acid (PAH) (Katz & Bergman, 1969), as well as electromagnetic flow metering,

ultrasonic devices utilizing the Doppler-shift principle and methods utilizing the Fick-principle (Linzell & Annison, 1975). All give comparable estimates but PAH, through the simplicity of its measurement, its complete removal by the kidneys and low absorption by tissues is most widely used.

The mathematical product of portal blood flow rate and difference in nutrient concentrations between portal blood and the blood supplying the gut is an indication of the quantity of nutrients absorbed by the blood (Bergman, 1975). However, the degree to which nutrients absorbed into the blood represents digestive end-product formation depends on the degree of utilization or modification caused by the portal drained viscera. This may be considerable (Dobson & Philipson, 1968) particularly in the case of VFA absorption, during which up to 30, 50 and 90% of acetate, propionate and butyrate production respectively may fail to reach the portal blood (Bergman, 1975) due to mucosal metabolism. This inaccuracy is further compounded by difficulties encountered with the measurement of high blood flow rates and small AV differences which can lead to further inaccuracies in the order of 10-25% depending on the metabolite being measured (see Bergman, 1975).

The use of isotope dilution procedures to relate whole-body metabolite entry rate to absorption is also possible (Annison & Armstrong, 1970). However, recycling of labelled breakdown-products leads to underestimation of entry rate (Bergman, 1973) whilst the converse is true with endogenous metabolite production (e.g. acetate) (Annison, 1965; Annison & Armstrong, 1970).

A combination of arteriovenous difference and radio isotope dilution measurements, is said to provide a more accurate measure of

nutrient flow through a blood vessel than either method alone and is widely used in preference. However, the problem of visceral metabolism of digestive end products still limits the application of these indirect methods for the investigation of nutrient utilization. They also take no account of nutrient uptake by the lymphatic system, which may reach 20g/d (Garton, 1969a) in hay fed sheep and 200-400g/d in the lactating cow (Hartman & Lascelles, 1966).

The techniques described in this section have provided much data about digestion and have allowed a more quantitative appreciation of the digestive process. The most widely used of these are the procedures involving direct measurements. Recently, however, methods involving the indirect determination of nutrient supply are receiving more attention (see Huntington et al., 1985).

Quantitative aspects of digestion. Use of the techniques discussed in the previous section has resulted in a wealth of data about the digestive process in ruminants. In order to review these data, the process of digestion may be considered in its three, concomitant phases.

Carbohydrate digestion. Carbohydrates consumed in the diet are fermented in the rumen, caecum and colon. The energy digested in the rumen can account for 23-87% of total energy digested with 17 to 51% and 4 to 26% digested in the small intestine, caecum and colon respectively (Thomas & Clapperton, 1972). The overall digestibility of a diet and the relative degrees of digestion occurring in the rumen, caecum and colon depend on the chemical composition of the diet, the type and degree of processing and the level of feeding.

The effect of these on apparent digestibility and the proportion of digestion occurring in the rumen are illustrated in Table I(7).

Ninety per cent of carbohydrate digestion is by fermentation to volatile fatty acids (VFA) (Sutton, 1971). Diets composed solely of forage lead to the production of VFA mixtures comprising 650-740 mmol/mol acetate 150-200 mmol/mol propionate and 80-160 mmol/mol butyrate (Thomas & Rook, 1977). With such rations it is estimated that between 0.7-0.9 mol of VFA is produced in the rumen per 100g of digestible organic matter. When mixed rations are given it is estimated that VFA production is slightly increased to 0.8-0.9 mol per 100g of digestible organic matter (Sutton, 1971). This is often accompanied by an increase in the concentration of propionate to about 300 mmol/mol and an overall reduction in the proportion of digestion occurring in rumen. Other estimates of VFA production are higher, with values of 1.3 mol/100g OM fermented in the rumen proposed by Nicholson & Sutton (1969) and 1.5 mol/100g OM proposed by Hogan et al. (1969). The latter measurement was, however, by an isotopic procedure, the data from which are thought to be overestimations (Sutton, 1971).

The net production of VFA in the rumen of sheep given various rations is illustrated above (Table I(3)).

Estimates of VFA production in lactating cows have yielded widely divergent results. Estimates of acetate production in animals given normal rations range from 25 mmol/d (Davis, 1967) to 77 mol/d (Satter & Willtrout, 1970) depending on the isotopic procedures used. If, as is common, values of acetate production are used to predict the production of the other VFA's from measurements of their relative concentrations in the rumen fluid, considerable inaccuracy results

Table I(7): The effect of diet composition on the site of digestion of energy.

Diet	Apparent digestibility of energy (%)	% of apparent digestion of energy occuring in the rumen
Medium mature grass, chopped	73.4	60.3[1]
Immature grass, chopped	83.3	64.4
Immature grass, chopped, high level of feeding	83.3	61.9
Immature grass, chopped, ground and pelleted	81.0	56.2
Hay (100% of DM)	59.3	67.1[2]
Hay:barley (33:66) DM[3]	71.8	62.3
Hay:concentrate (33:66) DM	74.0	59.0[4]
Hay:concentrate (33:66) DM + coconut oil	71.0	38.0
Dried grass, flaked maize	100[5]	95.7[5,6]
Dried grass, ground maize	100[5]	78.0[5,6]

1. Beever et al. (1971). 2. MacRae & Armstrong, (1969). 3. 200g hay/d + 400g concentrate/d. 4. Sutton et al. (1983). 5. Values refer to the digestibility of starch. 6. Beever et al. (1970).

(Sutton, 1980). Consequently, labelling of all 3 acids is recommended to obtain more accurate quantitative data. This is in accord with the views of Leng & Brett (1966).

As a result of microbial action in the rumen, the quantity of soluble carbohydrate reaching the duodenum from forage diets is generally small (Leng, 1970a), with between 3g and 10g available for hydrolysis to glucose in sheep (Sutton, 1971). This range of values is in agreement with more recent work in which that 100g to 200g per kg of dietary starch escapes fermentation when normal rations are given to cattle (see Rook & Thomas, 1983). The quantity of starch escaping fermentation in the rumen may, however, reach 300g/kg if a high-starch ration containing ground maize is given (Armstrong & Beever, 1969).

Protein digestion. The relationship between N intake and protein flow into the small intestine is extremely variable. When high N diets are given there is usually a net loss of N between mouth and duodenum whereas the feeding of low N diets often results in a net gain (Hogan & Weston, 1970; Beever, 1980). Protein supply to the small intestine for digestion and absorption is the summation of endogenous, undegraded dietary and microbial protein flowing out of the forestomachs.

The quantity of endogenous protein leaving the forestomachs is difficult to differentiate from the total protein flow and as a result only limited quantitative data are available (see Egan, 1980; Demeyer & Van Nevel, 1980; and Lindsay & Armstrong, 1982). It is known, however, that the endogenous protein fraction comprises sloughed epithelial cells and proteinaceous secretions. The sloughed

epithelial cells may be incorporated, to varying degrees, into microbial cells. Despite this, a value of approximately 1g N/d was measured by Wallace et al. (1979) in sheep. The quantity of proteinaceous secretions entering the small intestine of sheep is said to contribute a further 1-2g N/d to the endogenous fraction (Phillipson, 1964). Measurements of the complete endogenous fraction have been attempted. Ørskov et al. (1980) using an intragastric infusion procedure measured endogenous protein flows of 1.4g N/d and 7.88g N/d in sheep and cattle respectively. It must be noted, however, that such procedures can not accurately account for the contribution of desquammated epithelial cells under normal dietary regimes. Siddons et al. (1985) showed that with sheep, a higher value of 4-6g N entered the small intestine as non-urea N of endogenous origin, depending on the degradability of the N in the diet given. The total endogenous N contributed to the protein flowing into the small intestine was said to be in the order of 5.5 to 9.4g N/d.

The quantity of undegraded dietary N reaching the small intestine depends on the quantity degraded and lost as NH_3 or incorporated into microbial protein. Microbial protein, which constitutes a larger proportion of the protein reaching the duodenum (see Ling, 1976; Ling & Buttery, 1978) is dependent on the availability of energy and the matched supply of nitrogen in the rumen. The form in which energy is made available in the rumen affects microbial synthesis; acetate-based fermentations result in less efficient microbial protein synthesis than propionate based fermentations (Harrison et al., 1975; Ishaque et al., 1971). Despite

this a value of 10.5g of microbial DM per mol of ATP formed from fermented substances is generally accepted (see Thomas & Rook, 1977). This corresponds to approximately 23g of bacterial crude protein per 100g of organic matter fermented.

The ammonia concentration required to optimize microbial protein synthesis in the rumen varies, with the microbial population and prevailing conditions. Values from 4-5 mmol/l (Roffler et al., 1974; Pizulewski et al., 1981) through 6.3-9.5 mmol/l (Hume, 1970) up to 17.0 mmol/l (Mehrez et al., 1977) and 20 mmol/l (Miller, 1973) have been proposed despite the fact that Ortega et al. (1979) showed there was no response in microbial protein synthesis between 4.5 and 19.6 mmol/l. At concentrations below 3.1 mmol/l, Satter and Slyter (1974) demonstrated an uncoupling of protein synthesis and VFA production, (see also Beever et al., 1974), whilst levels above 60 mmol/l were associated with acute ammonia toxicity.

The discrepancies between optimum values are thought to reflect the characteristics of the fermentation and the method of measurement of protein flow into the small intestine (see Buttery & Lewis, 1982). The gross composition of the protein leaving the rumen can vary considerably; the N content of bacteria ranges from 50 to 120g N/kg DM (Harrison & McAllan, 1980) whilst values for protozoa range from 45-83g N/kg DM (Lindsay & Armstrong, 1982). There is however a tendency towards uniformity in the amino acid composition of duodenal digesta (Armstrong, 1976) which is attributed to the relative constancy of the amino acid composition of microbial protein (see Table I(8)) and the fact that this source of protein constitutes such a large part of the total protein entering the small intestine when conventional rations are given: estimates range from

Table I(8): The amino acid composition of bacterial and protozoal protein isolated from the rumen of sheep. (% W/W of microbial protein) (After Ling, 1976).

Amino acid	Bacteria	Protozoa
Threonine	[1] 5.4 ± 0.4	5.1 ± 0.5
Valine	5.5 ± 0.3	5.2 ± 0.6
Isoleucine	4.7 ± 0.2	5.8 ± 0.5
Leucine	6.5 ± 0.3	7.2 ± 0.7
Phenylalanine	4.0 ± 0.2	5.3 ± 0.5
Histidine	1.5 ± 0.1	1.8 ± 0.1
Lysine	7.0 ± 0.4	10.1 ± 0.6
Methionine	1.8 ± 0.1	1.7 ± 0.2
Tyrosine	3.9 ± 0.2	4.5 ± 0.5

1. Means ± S.E.

approximately 50% (Siddons et al., 1985) to 85% (Lindsay & Armstrong, 1982). The contribution of undegraded N to the flow of N into the small intestine depends on the degradability of the dietary protein source. Quantitatively, it is the difference between total protein flowing into the small intestine and the sum of microbial and endogenous protein within that protein (ARC, 1984).

Lipid digestion. Dietary lipid is extensively modified in the rumen but there is no degradation of long-chain fatty acids. This, together with the amount of microbial synthesis of lipid and endogenous secretion means that the quantity of lipid passing to the small intestine often exceeds that consumed in the diet (Sutton et al., 1970; Sutton, 1980). The degree to which this occurs depends on the type of diet given as illustrated in Table I(6). Sutton et al. (1970) estimated that in sheep given a hay/concentrate ration (75:25), 11.5g/d of fatty acid or 28.5g total lipid entered the duodenum. These values were increased to 37.8g/d and 64.5g/d respectively when the proportions of forage to concentrate in the ration was changed to 20:80 and 850g/d flaked maize was included in the diet. Knight et al. (1979) calculated that in sheep given a mixed diet of hay and concentrate, 75% of the lipid reaching the small intestine was of microbial origin and 9% resulted from fatty acid synthesis de novo from acetate.

Synthesis de novo was estimated to account for 33% of the quantitative difference between dietary lipids and lipids reaching the duodenum of sheep. The remaining 66% was estimated to originate from endogenous sources (Murray et al., 1978). The effect of dietary factors on the supply of fatty acids to the duodenum is difficult to

predict accurately because the analytical procedures used, are themselves, a source of variation (see Knight et al., 1979). There is clearly a need to differentiate between dietary, endogenous, microbial and de novo-synthesized lipids in the duodenum before the quantitative factors affecting duodenal lipid flows can be resolved.

Predictive techniques for the estimation of nutrient supply.

Techniques discussed thus far have involved direct or indirect measurement of metabolite production in vivo. Alternative procedures exist which utilize this information and enable the prediction of nutrient supply from measurements of less complex or variable parameters. These techniques were designed to facilitate a more rapid assessment of the nutrient value of food and to have a universal application with all food types.

Relationships exist between in vivo measurements of nutrient disappearance during complete or partial digestion and the composition or laboratory assessment of foods (Alderman et al., 1971). Prediction of nutrient supply from these relationships is an important part of the methods of food evaluation currently used (see Osbourn & Terry, 1977).

The ability of plant material to supply nutrients for absorption directly relates to the susceptibility of its cell walls to cellulolytic degradation. This can be measured or predicted by various in vitro and in vivo techniques and related to in vivo measurements of digestibility.

In vivo techniques

In sacco nutrient disappearance. In order to investigate the effect of diet on cellulose digestion, Quin (1938) suspended silk bags filled with known quantities of food inside rumen fistulated sheep for defined periods of time in order to measure nutrient disappearance. Except for the substitution of silk with dacron or nylon this technique remains unchanged today (see Johnson, 1966; Hopson et al., 1963). This despite considerable discussion regarding inconsistent results as a consequence of variations in incubation period, washing technique, location of bags in the rumen, sample homogeneity and level of feeding as well as bag and pore size (see Hopson et al., 1963; Lowrey, 1970; Mehrez & Ørskov, 1977). Lowrey (1970), however, compared 72h in sacco measurements of digestibility with those determined in vivo and demonstrated a consistent 8-12% difference when grasses of variable maturity were tested. He concluded that in sacco and in vivo nutrient disappearance can therefore be related together accurately.

Recently, in recognition of the above problems there have been attempts to standardise the dacron bag procedure. An ARC technical review body (Interdepartmental Protein Working Party, 1985) has circulated proposals for a standard method for the in situ measurement of nitrogen disappearance from dacron bags suspended in the rumen. Bag size, pore size, stitching, quantity and preparation of samples, previous feeding of the animals used and bag washing procedure have all been specified in order to achieve standardisation of the procedure.

In vitro techniques. Classification of the in vitro techniques is best achieved on the basis of whether or not they utilise rumen liquor as the digestive medium.

Techniques based on the digestion of samples with rumen liquor. As a result of the low mathematical correlations observed between one-stage rumen liquor digestions and in vivo digestibility, Tilley et al. (1960) and Tilley and Terry (1963) introduced and standardized a two-stage in vitro digestion procedure; dried grass samples were incubated with rumen liquor for 48h prior to acidification of the digesta to stop fermentation, centrifugation and decantation of dissolved materials. A 48h pepsin incubation followed and the residual material, which representing the undigested food components, was measured and related to in vivo digestibility (Tilley & Terry, 1963). Results from such tests carried out on 148 forage samples yielded the regression equation:

$$\text{In vivo digestibility} = 0.99 \times \text{in vitro digestibility} - 1.01 \quad (SE = 2.31)$$

The above method is thought to provide accurate predictive estimates of the digestibility of grass and hay but its use is not advised in the evaluation of grass silage (Osbourn & Siddons, 1980). Despite the accuracy of the method considerable dispute has arisen regarding the use of one regression equation for all forages (Witt & Pedersen, 1975). This is due to the variation in cellulolytic activity observed between and within individual rumen liquor samples. The use

of standardized feed and selected rumen liquor donors is recommended to maintain the uniformity of the rumen liquor microflora.

Rumen simulation techniques. Further development of *in vitro* digestion techniques has led to rumen simulation. Czerkawski and Breckenridge (1977) developed a device to simulate rumen digestion called 'Rusitec'. Rusitec comprises a perspex, rumen liquor filled reaction vessel immersed in a thermostatically controlled water bath. Food samples contained within dacron bags are placed inside a perforated piston within the reaction vessel moving at 8 cycles per minute (CPM). Artificial saliva is infused into the vessel base and is collected again, together with dissolved metabolites at the top of the vessel for analysis, as are gaseous emissions. Temperature and pH are precisely regulated and feed samples are removed every 2d for the evaluation of nutrient loss.

Rusitec permits precise control of nutrient input and residence time as well as simplifying digesta and end-product collection procedures. Comparative studies have shown that when identical feeds are used there are similar, reproducible VFA patterns in the rumen of sheep and the Rusitec (Czerkawski, 1978; Stanier & Davies, 1981). However, the substrate concentrations in Rusitec and the density of the microbial populations are normally much lower than found *in vivo* and, in the absence of supporting experimental data it is difficult to know whether the Rusitec simulates rumen fermentation and microbial growth in quantitative terms.

Enzymatic and chemical methods. In vitro digestions based on rumen liquor all suffer from the problem of natural variation in cellulolytic activity between liquors. This can be minimized by the use of standardised cellulolytic enzyme preparations or completely obviated by the prediction of digestibility from parameters measured using chemical means.

Enzymatic methods. Jones and Hayward (1973) and Jones and Bailey (1974) replaced the rumen liquor digestion phase commonly used with a digestion with crude cellulase, extracted from Trichoderma viride. Whilst reducing natural variation, use of the enzyme preparation proved to be less accurate than the in vitro method of Tilley and Terry (1963). Current work involved in the isolation and extraction of a range of cellulases is hoped to improve the technique (see Morrison, 1976).

Predictive techniques based on food composition. Attempts to relate the chemical composition of food to subsequent digestibility and nutrient supply are extensively documented (see Minson, 1982; Demarquilly et al., 1980). Digestibility may be derived from summation of the digestibility of individual feed fractions or more frequently by prediction from one chemical fraction. Equations are available which relate dry matter and organic matter digestibility to crude protein (Phillips & Laughlin, 1949; Sullivan, 1964; McLeod & Minson, 1976), cellulose (Sullivan, 1964), lignin (Joshi, 1972; McLeod & Minson, 1976; Morrison, 1972a and 1973; Edwards, 1973), the methoxyl groups of lignin (Shearer, 1961), neutral detergent fibre (NDF) (Van Soest, 1965a), acid detergent fibre (ADF) (Van Soest,

1963), modified acid detergent fibre (MADF) (Clancy & Wilson, 1966) and crude fibre (CF) content (McLeod & Minson, 1976; Bosman, 1967; Jarrige, 1980) with variable success. Multicompartment relationships are also employed in an attempt to increase accuracy. Gaillard (1966) related organic matter digestibility (OMD) to lignin, cellulose, hemicellulose and anhydrouronic acid content and demonstrated a reduction in residual standard deviation (RSD) when the results were compared with unifactorial methods. Also, Van Soest (1965a) and Van Soest & Wine (1968), related dry matter digestibility (DMD) to NDS, NDF, lignin and ADF (see above section on food analysis) which, when applied to 19 legumes and grasses, yielded a correlation of 0.96 with a RSD of $\pm$2.7 digestibility units.

In a recent review (Minson, 1982) the lowest RSD was associated with regressions based on lignin or its methoxyl group. This may be explained by the profound effect of lignin on the digestibility of the largest single fraction of forage dry matter - NDF. The accuracy of predictions based on lignin depend on the analytical technique employed for its measurement, being least accurate with the acid detergent lignin technique (Van Soest, 1963). In general the accuracy of predictions based on chemical analyses falls short of that from procedures employing *in vitro* digestions (Morrison, 1973).

The prediction of OMD and DMD unfortunately provides little information on the availability of individual nutrients, which limits its use for the study of nutrient utilization. Current rationing systems (ARC, 1980; ARC, 1984) also suffer the same inaccuracy when they describe foodstuffs on the basis of their metabolizable energy content (ME). According to the Oscar Kellner Institute, GDR, ME, a

measure of the actual energy available to the animal, can be calculated from the digestible energy content of a food or the digestibility of individual food components (e.g. CP, EE, CF and NFE). The reliance of the system on certain, broad assumptions regarding the loss of energy in methane and urine (see ARC, 1984), together with the inevitable inaccuracies found with the analytical procedures on which the ME system relies, limits its usefulness for precise, experimental studies.

The prediction of protein supply from food composition has also been attempted, despite the complex protein/energy interactions occurring in the rumen. Initial estimates were based on digestible crude protein (DCP) which effectively measured the apparent disappearance of N along the digestive tract. More recent evaluation procedures (ARC, 1980, 1984) have been improved and take into account the partition of microbial and animal requirements. They also attempt to predict nitrogen and energy supply to the microbial population from food characteristics. By necessity the system incorporates several assumptions; that the availability of nitrogen to rumen microbes can be measured (Tamminga, 1979; Jarrige, 1980; Waldo, 1978) and that the classification of foods on the basis of this availability of N is possible; that the energy from 65% of the organic matter apparently digested in the whole gut is available for microbial synthesis in the rumen; that digested organic matter yields 19MJ/kg DM; that degraded N is incorporated into microbial protein with 100% efficiency (Weston & Hogan, 1973; Roffler & Satter, 1975); that approximately 30g microbial N is produced per kg DOM apparently digested in the rumen; and that 80% of microbial nitrogen is available as amino acid with a true absorbability of 85%. Despite

the problems, this method of protein evaluation represents a considerable improvement over previous methods; it attempts to describe the types and quantities of protein reaching the duodenum. Accordingly it is currently the most advanced of the commonly used predictive techniques. Despite this, however, the underlying assumptions on which the system depend are largely based on mean values of data pertaining to various diet and livestock classes (see ARC, 1980, 1984) and their predictive ability, whilst possibly adequate for general ration formulation lacks the precision necessary for detailed experimental purposes.

The increased availability and use of computers together with flexibility of computer language has led to further developments in predictive techniques. Simulations of digestion have now been undertaken in which the diet, described in closely defined chemical terms, can be related to the end products of digestion (see Black et al., 1982; France et al., 1982). More specialized models have also been constructed which simulate N digestion in the rumen (Baldwin & Denham, 1979) and the energetics of rumen fermentation (Reichl & Baldwin, 1974).

Such models are based on data generated from digestion studies of the type described above. However, the predictive ability of such models may extend outwith the nutritional circumstances in which measurements for the database were made (see Baldwin et al., 1981).

Predictive models regarding the metabolism of absorbed nutrients have also been constructed (see Gill et al., 1984; Smith et al., 1980) as well as more complex models which attempt to relate food inputs to animal production (Bywater & Dent, 1976; Bywater, 1976; Oldham & Parker, 1980).

The construction and use of models is still largely in the experimental stage with progress limited by the fact that attempt to describe complex and dynamic biological systems in mathematical terms are extremely difficult. That computer modelling will replace current methods of investigation is doubtful but the technique does provide a means to evaluate current nutritional concepts and identify areas in need of further investigation as well as aiding the interpretation or extension of existing information (Baldwin et al., 1981). Accordingly, computer simulation is playing an increasingly important role in association with the more conventional research techniques.

Conclusions

With the exception of isotopic and arteriovenous procedures the investigative techniques described above are limited in that they can only provide estimates of the availability of broad, nutrient groups (e.g. dry matter, organic matter, energy and microbial or undegradable protein) or of the apparent disappearance of individual nutrients along the alimentary tract. The type and accuracy of data from such procedures obviously limit the potential scope of investigations in which they are used. The ability to perturb nutrient supply, which is essential if nutrient utilization is to be studied, is also limited using existing techniques because of the constraints imposed by voluntary food intake. Manipulation of nutrient supply is further complicated by the fact that the quantities and types of nutrients supplied to the rumen have an influence on the microbial population therein and consequently the

patterns of digestion occurring in the rumen. This occurs in a largely unpredictable manner.

NUTRIENT UTILIZATION

The integrity of an organism is dependent upon an adequate and continuous supply of nutrients needed to meet the specific requirements of the tissues. The usually discontinuous nature of nutrient input into the digestive tract and, as a consequence, nutrient supply to the tissues necessitates considerable, hormone mediated, interconversion and transfer of nutrients between the tissues. This makes good temporal imbalances between nutrient supply and tissue requirements.

The complex nature of nutrient utilization is well recognized and the following section will briefly describe nutrient supply to the tissues as well as general features of metabolism and will dwell specifically on glucose and N metabolism in ruminants to illustrate the integration of pathways in metabolism.

Nutrient supply to the tissues

The digestion of dietary carbohydrate provides an abundant supply of VFA (Warner, 1964; Sutton, 1979; Russell & Hespell, 1981) together with variable quantities of lactate (Leng, 1970a) and unfermented dietary α-glucose polymer (Armstrong, 1974). Prior to absorption into portal blood the major short-chain fatty acids undergo varying degrees of metabolism by the gut mucosa (Masson & Phillipson, 1951; Pennington, 1952; Stevens, 1970). Butyrate and propionate undergo considerable metabolism with the formation of β-hydroxybutyrate and lactate respectively whilst the majority of the acetate produced passes into the blood stream unchanged (Bergman & Wolff, 1971; Annison, 1983).

The quantity of unfermented soluble carbohydrate absorbed from the small intestine is usually small (Baird et al., 1983; Bergman, 1968) but varies with dietary α-glucose polymer content, and rate of passage of digesta (Armstrong, 1974; Armstrong & Smithard, 1979; MacRae & Armstrong, 1966). Lactate is freely absorbed across the rumen wall into the portal circulation.

Lipids entering the small intestine bound to feed particles, incorporated into microbial cells or as free saturated fatty acids are subject to a digestion similar to that found in simple-stomached animals (Lough, 1970; Harfoot, 1978). Free fatty acids of chain length between C10 and C12 are then taken up by portal blood whilst longer-chain acids are incorporated into chylomicra and transported by the lymphatic system (Garton, 1969).

Protein, peptides and amino acids of dietary origin which escape ruminal degradation, together with microbial and endogenous protein are subject to hydrolysis by proteolytic enzymes of the gastric, pancreatic and intestinal mucosae. The amino acids released are then absorbed into the portal blood flow (see Dawson & Porter, 1962) by an 'active' mechanism (see Wiseman, 1968; Munck, 1976). During absorption, however, up to 40% of amino acids may undergo modification (see Bergman & Heitman, 1978) by the intestinal mucosa.

The utilization of nutrients by tissues

The fundamental chemical energy requirements of living cells are derived from the oxidation of suitable substrates and the subsequent transfer of released electrons via electron acceptors (NAD, NADP, FAD, FMN) and transmitters (cytochromes and ubiquinones) to lower energy states. Consequent energy release during this

'electron cascade' is largely harnessed in a tissue-usable form, indirectly associated with the phosphate bond of adenosine-5-triphosphate (ATP) (see Villee & Dethier, 1976). Electron removal from the substrate for transfer is facilitated by mitochondrial decarboxylation and dehydrogenation reactions of Krebs' citric or tricarboxylic acid cycle (TCA) (Fig. I(5)).

The primary fuel for the TCA cycle is acetyl coenzyme A (acetyl CoA) which, condensed with oxaloacetate, forms an isomer of citric acid for subsequent metabolism through intermediates to oxaloacetate once more. One revolution of the TCA cycle results in the release of 8 hydrogen atoms for electron transfer and ATP formation and the formation of one high energy phopshate bond (GTP) at the substrate level.

In order to enter the TCA cycle substrates must undergo catabolism to acetyl CoA or TCA intermediates. Accordingly acetate, β-hydroxybutyrate and other ketones, ketogenic amino acid fragments as well as lysine and leucine (Leng, 1970a) and C2 residues of fatty acid from the β-oxidation of lipids (Bell, 1980) contribute to the acetyl CoA pool. Correspondingly glucose, hepatic glycogen, and glycerol from lipolysis together with lactate from muscle glycogenolysis and absorption from the gut contribute via the glycolytic pathway either to the acetyl CoA pool or via pyruvate carboxylase to TCA intermediates whilst propionate and the majority of deaminated amino acids provide intermediates alone (see Fig. I(5)).

Many of the reactions of the TCA cycle are directly or indirectly reversible and this facilitates nutrient interconversion. An exception, however, is the irreversible oxidative carboxylation of

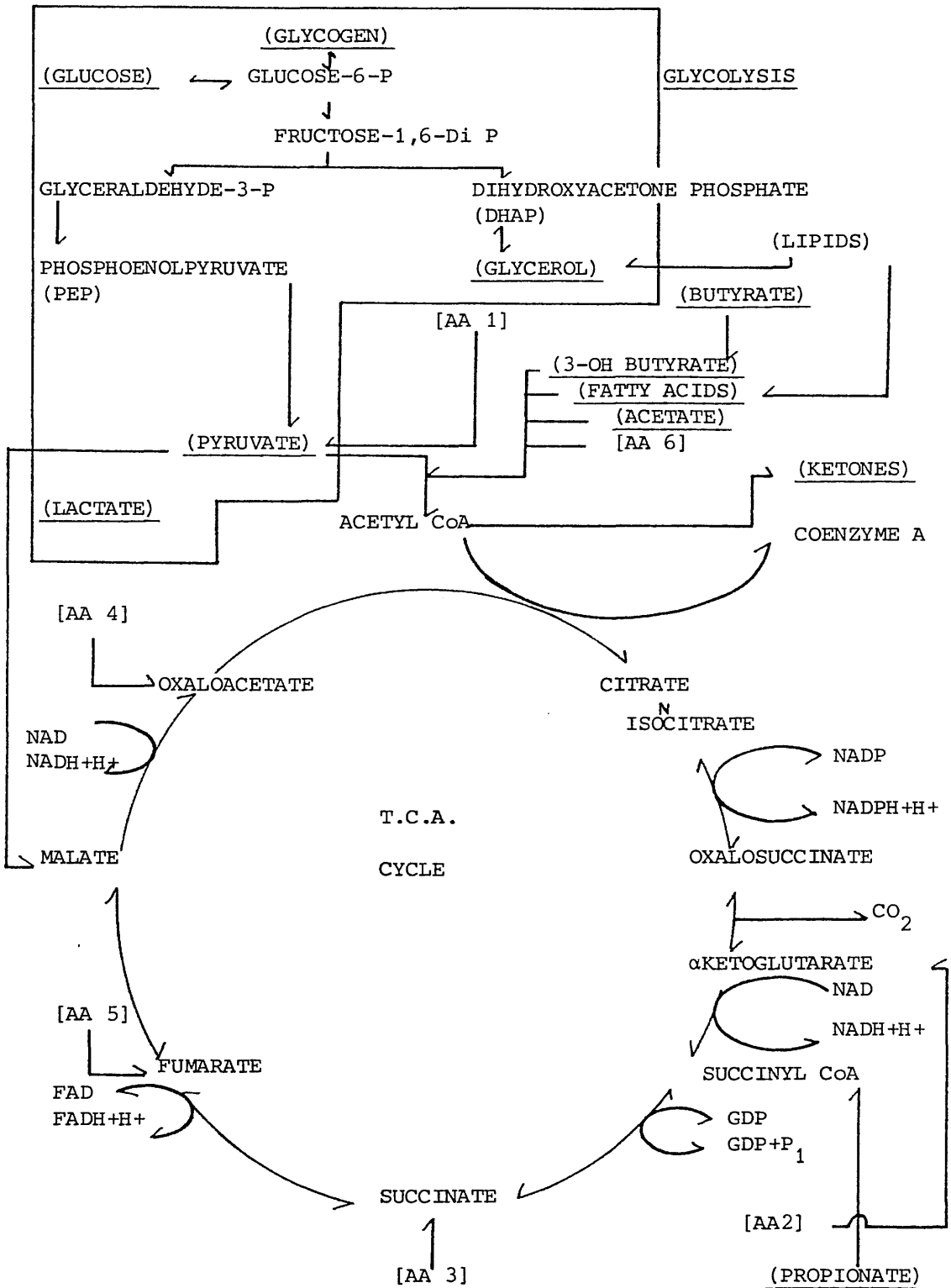


Figure I(5): Glycolysis and the TCA Cycle showing (KEY METABOLITES). [AA1] - Alanine, threonine, glycine, serine, cystine. [AA2] - Arginine, histidine, glutamate, proline. [AA3] - Isoleucine, methionine, valine. [AA4] - Aspartate. [AA5] - Phenylalanine, tyrosine. [AA6] - Phenylalanine, tyrosine, lysine, leucine, tryptophan.

pyruvate to acetyl CoA. Whilst cycle intermediates, pyruvate and their precursors demonstrate considerable interconversion and are potentially glucogenic, acetyl CoA may undergo condensation with oxaloacetate for oxidative purposes, condensation with CO_2 to form long-chain fatty acids for lipogenesis or partial oxidation to ketone bodies to allow the release of coenzyme A, but is not glucogenic.

The interconversion of nutrients is of particular significance to glucose metabolism. Considerable utilization of glucose has been demonstrated in the mammary gland for lactose, glycerol and NADPH synthesis (Lindsay, 1979; Linzell & Peaker, 1971), in the testicles during rete fluid production (Linzell & Setchell, 1969) and in the pregnant uterus (Christienson & Prior, 1978), nervous system (Linzell & Setchell, 1976) and cellular elements of the blood (Leng & Annison, 1962). The formation of reducing equivalents (NADPH) via the pentose phosphate pathway (Bell, 1980) and glycerol (Bergman, 1973) for lipogenesis also require glucose and there is circumstantial evidence of specific glucose requirements in ovine hind limb (Lindsay, 1979) and heart muscle (Lindsay & Setchell, 1976). Consideration of these demands for glucose, of the level of post-ruminal absorption and of the high proportion of dietary energy absorbed as acetate as opposed to glucose has led to the suggestion that the glucose economy of ruminants is in a precarious state (Lindsay, 1959). Accordingly, ruminants must rely heavily on gluconeogenesis together with metabolic adaptations to 'spare' glucose in order that requirements can be met.

Gluconeogenesis. In ruminants the formation of glucose from suitable precursors occurs predominantly in the liver (approximately 85%) and

to a smaller extent in the kidneys (approximately 15%) (Vernon & Peaker, 1983; Bergman & Heitmann, 1978). Four main groups of metabolites are known to contribute significantly to the glucose pool of ruminants; propionate , lactate and pyruvate, amino acids and glycerol (Bergman, 1973; Leng, 1970a; Armstrong, 1965). Other precursors do exist e.g. valerate, ribose, citrate, butyrate via the propanediol pathway (Leng & White, 1964) but all are of minimal quantitative significance. By necessity all are net contributors to oxaloacetate which may be withdrawn from the TCA and converted to glucose via phosphoenolpyruvate (PEP) (Fig. I(5)).

Propionate is known to be the major glucose precursor providing TCA intermediates in the form of succinyl CoA. Measurements of the propor tion of glucose synthesized from propionate in the whole animal using radio-isotope dilution procedures in fed ruminants gave a range of values; 27% (Bergman et al., 1966), 54% (Leng et al., 1967); 50-60% (Leng, 1970a) and 42% (Lindsay, 1978), the general consensus being 30-50% (Bergman et al., 1966, Judson & Leng, 1968; Leng et al., 1967), depending on diet, extent of epithelial metabolism to lactate (Pennington & Sutherland, 1956) (which can reach 50% of total rumen propionate production (Bergman & Wolff, 1971)) and physiological state (Steele & Leng, 1973 and 1973a). In most cells the anaerobic metabolism of glucose also leads to the formation of lactate and small quantities of pyruvate which may be released and converted to glucose in hepatic and renal tissue (Cori, 1931). Although this does not lead to a net synthesis of glucose it does provide the means to use muscle glycogen as a general, instead of specific, glucose source. The total contribution of lactate to glucose synthesis, as determined by renal and hepatic

uptake is approximately 19-28% (Lindsay, 1978). The relative contributions to this from ruminal absorption, epithelial metabolism of propionate and endogenous inputs are difficult to differentiate (Bergman, 1973).

All amino acids except lysine, leucine and taurine (Krebs, 1964) are potential precursors for glucose synthesis because of their ability to provide TCA intermediates (Fig. I(5)). Dietary and muscle bound amino acids comprise the major sources which may be metabolised by hepatic or renal tissue. Estimates for the potential contribution of amino acids to glucose supply vary between 30 and 50% (Black et al., 1968; Bergman & Hietmann, 1978) and the general consensus is that alanine and glutamine are the most glucogenic of the amino acids, each representing 6-8% of glucose turnover (Black et al., 1968; Lindsay, 1978) and 40-60% of all amino acid gluconeogenesis (Bergman & Heitmann, 1978). The complex metabolic cycling of the major gluconeogenic amino acids is best demonstrated diagramatically (Fig. I(6)).

In the fed animal the majority of glycerol found in ruminant tissue exists in combination with adipose tissue triacylglycerides. Low levels of circulating glycerol ensure hepatic and renal gluconeogenesis rarely exceeds 5% of total production (Lindsay, 1978; Bergman, 1973). Periods of undernutrition and adipose tissue mobilization, however, lead to the release of large quantities of glycerol and unesterified fatty acids (Marco et al., 1981). Bergman (1973) suggested that in fasted and ketotic sheep glycerol turnover or rate of release into the circulation could account for between 28 and 40% of the animals glucose production. The net contribution of adipose glycerol to gluconeogenesis would, however, be considerably

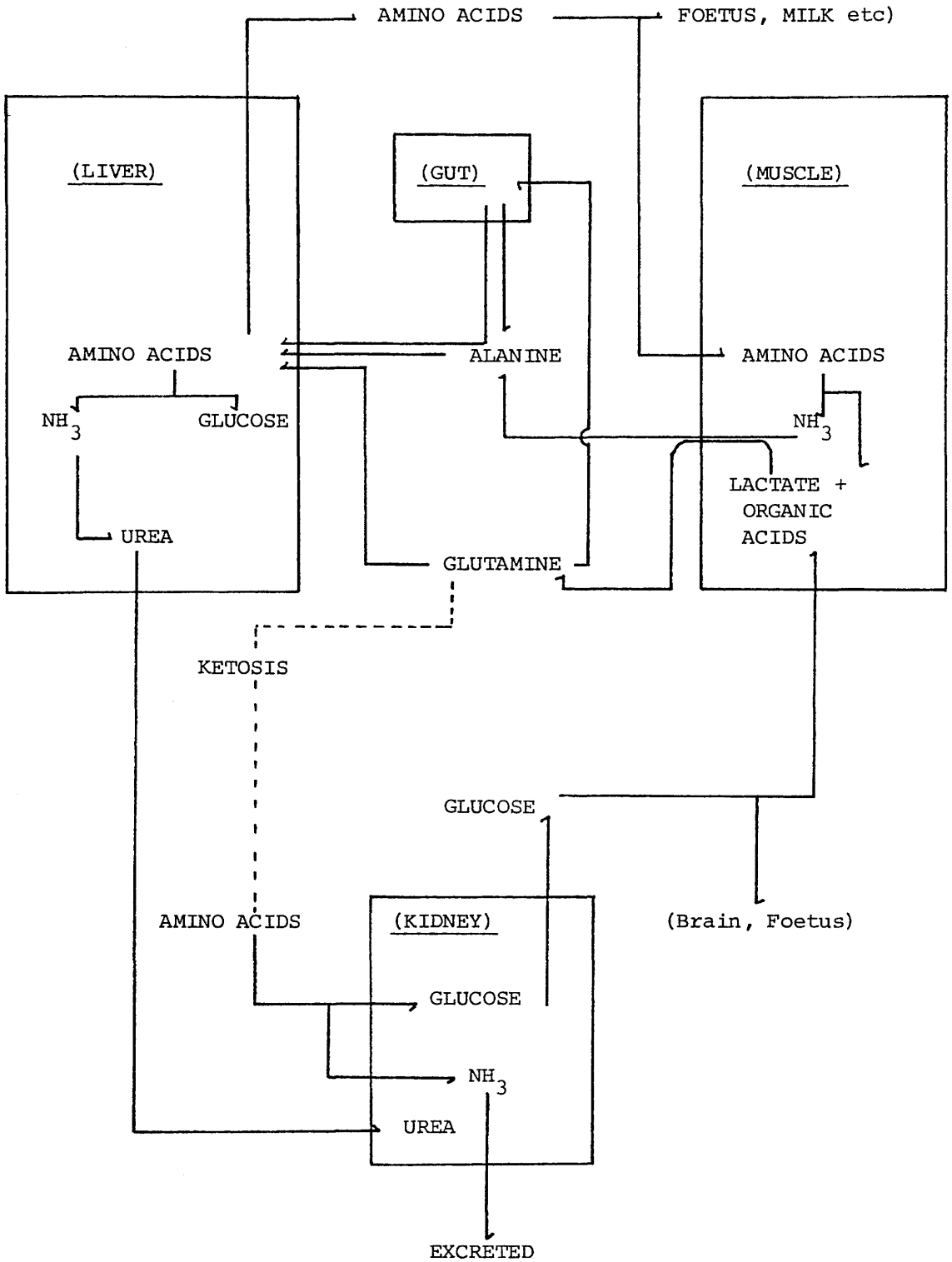


Figure I(6): Amino acid metabolism and gluconeogenesis in ruminants (After Bergman, 1973).

smaller. This is because of the requirement for gluconeogenic substances to facilitate the oxidation of the acetyl CoA released during lipolysis.

Lindsay (1979) concludes that in nearly all circumstances, 90-100% of circulating glucose is derived from gluconeogenesis. The relative contribution of the major gluconeogenic precursors to glucose production is variable in order that glucose supply is maintained and is dependent on precursor supply. Ruminants on high planes of nutrition predominantly utilize dietary amino acids, propionate and absorbed lactate whilst those on lower planes demonstrate the increased utilization of endogenous amino acids, lactate and glycerol. Gluconeogenesis during prolonged starvation, having exhausted liver glycogen, relies solely on endogenous amino acid and triglyceride glycerol which are often inadequate during periods of high nutrient demand (Baird et al., 1972).

Regulation of gluconeogenesis. The complex regulation of gluconeogenesos is achieved in the first instance by a control of peripheral precursor supply which is effected by the regulation of voluntary food intake. At the tissue level precursor supply and flux is hormone mediated. Reduced propionate absorption inhibits pancreatic insulin secretion (Manns et al., 1967; Baird et al., 1972; Horino et al., 1968). Hypothalamic glucoreceptors then permit increased epinephrin secretion from the adrenal cortex which together with reduced insulin secretion stimulates increased hepatic glycogenolysis and mobilization of free fatty acid and glycerol from adipose tissue (Bergman, 1970). Increased amino acid release from muscle also occurs (Cahill et al., 1970), possibly in response to

increased levels of circulating glucagon (Aguilar-Parada et al., 1969).

Prolonged deprivation of glucogenic precursors leads to continuation and exaggeration of this response through the adrenal secretion of glucocorticoids in response to hypothalamic somatotrophic (STH) and adrenocorticotrophic (ACTH) hormone secretion (Bergman, 1970). Hormone mediated control of gluconeogenesis relies on alteration of the activity and synthesis of 'pacemaker' enzymes responsible for overcoming the four major thermodynamic barriers involved (see Ballard et al., 1969).

The economic use of glucose by ruminants is patently necessary and in order to achieve this glucose can be 'spared' by substitution with other metabolites until a situation is reached where glucose utilization is equal to the animal's absolute or minimal glucose requirement.

Glucose may be spared by one of three mechanisms: (1) The substitution of glucose used for non-specific energy provision by acetyl CoA. (2) Gluconeogenesis from non-glucose precursors, and (3) Reduction in glucose utilization at the tissue level. Gluconeogenesis and the use of acetyl CoA for oxidative purposes have been discussed above. Consideration of tissues utilizing glucose (i.e. mammary gland, testis, pregnant uterus, brain, blood, muscle and adipose tissue) by Lindsay (1979) led to the conclusion that alternative metabolites could, in fact, replace glucose for almost all purposes excepting mammary lactose formation. Prior to this it was thought that the brain also had an absolute requirement for glucose because of the small molecular size of glucose and the ease with which it could pass across the blood-brain barrier. Recent indications,

however, of the use of branched-chain amino acids by neural tissue suggest that glucose sparing can also occur in the brain (Leng, 1970a). The permanent neurological damage occasionally resulting from hypoglycaemia in sheep suggests, however, that the level of glucose sparing is small. Glucose sparing through the utilization of ketones is thought not to occur in ruminants (Owen et al., 1967).

Quantitative aspects of glucose metabolism. Glucose utilization is largely dependent on physiological and nutritional state (Bergman, 1964; Bergman & Hogue, 1967; De Bodo et al., 1963). Lactation imposes the greatest demand, mammary uptake accounting for 50-90% of glucose entering the circulation which is equivalent to 560-1509 mg/minute in the lactating cow (Bickerstaffe et al., 1974; Annison et al., 1974). Inter-tissue competition for glucose is considerable with priority shown to neural tissue followed by the gravid uterus and mammary gland with adipose tissue subject to least priority.

Much information relating to glucose utilization has been generated by the use of radioisotope dilution techniques where utilization is equated with the irreversible loss of glucose from the circulation or the entry of glucose into the circulation. Of these alternatives, irreversible loss is a more accurate assessment of utilization because measurements of entry rate will inevitably include some recycled glucose. Both measurements are subject to considerable variation, even when carried out in similar circumstances (see Lindsay, 1979 and Table I(9)). This variation may be partly attributable to the methods used for tracer administration together with the mathematical analysis of results (White et al., 1969, Young, 1976), to differences in initial plasma glucose

Table I(9): Glucose utilization ($g/kgW^{0.75}$ per d) measured using continuous infusions of isotopically labelled glucose with non-pregnant, non-lactating ruminants.

	Nutritional status		
Species	Fed	Fasted	Reference
C[1]	-	5.04	Baird et al., 1983
S[2]	-	2.95-8.02	Lindsay, 1970
S	-	2.95-4.44	Armstrong, 1965
C	-	8.88	Armstrong, 1965
S	-	2.73-3.60	Bergman et al., 1974
S	-	4.8	Annison and White, 1961
S	-	3.6	Bergman, 1973
S	5.76	-	Bergman, 1973
S	4.6-9.8	-	Leng, 1970a
S	6.0	-	White et al., 1969
S	5.48	-	Steel and Leng, 1973a
S	4.85	-	Bergman et al., 1966
S	5.55	-	Wilson et al., 1982
S	5.76-6.1	-	Bergman, 1973
C	5.77	-	Harmon et al., 1983

1. Cow. 2. Sheep. Values refer to the irreversible loss of glucose.

concentration (Bergman, 1964), the degree of C cycling through the TCA cycle (Leng, 1970a; Krebs et al., 1966; Young, 1976) or more importantly to differences in the composition and quality of the diet (Leng, 1970a) and physiological state (Steel and Leng, 1973 and 1973a).

Investigations of glucose kinetics in fed, non-pregnant, non lactating sheep have given results for irreversible loss (IL) of between 2.02 and 6.19 $g/kgW^{0.75}$ per d, (Steel & Leng, 1973). This variation has been related to the effects of plane of nutrition (Ford, 1965) and more specifically, the digestible energy (Judson & Leng, 1968) and crude protein intake (Reilly & Ford, 1971).

Glucose requirement is considerably increased during pregnancy (Langlands & Sutherland, 1968; Steel & Leng, 1973 and 1973a) in part through the specific foetal requirement (Kronfeld, 1958; Bergman, 1963). Increases of 30-40% were measured by Steel & Leng (1973a) on three different planes of nutrition (5.5 to 6.2, 6.5 to 7.5 and 8.5 to 10.5 $g/kgW^{0.75}$ per d for sheep on low, medium and high planes of nutrition respectively). During lactation glucose is required for lactose, glycerol and NADPH synthesis sometimes results in inordinate glucose demands. To demonstrate this, Young (1976) quotes a specific case where a lactating cow yielded 89 kg milk daily with a lactose concentration of 4.9% w/w. Assuming 60% of glucose entry rate could be utilized for lactose synthesis (Bickerstaffe et al., 1974; Bergman & Hogue, 1967) this represents a glucose requirement of 7.4 kg (i.e. $84g/kgW^{0.75}$ per d) 90% of which must be derived from gluconeogenesis.

As discussed earlier, it is clear that ruminants have a minimum glucose requirement and that the rate at which they utilize glucose

depends on the availability of suitable glucose precursors. It may be argued that because of glucose 'sparing' the minimum glucose requirement is apparent when the exogenous glucose precursor supply is zero. To measure this minimum glucose requirement Steel and Leng (1973) withheld food from non-pregnant sheep for 4 days in order to ensure cessation of the exogenous glucose precursor supply. A minimal glucose requirement of 3.2 $g/kgW^{0.75}$ per d was measured which remained stable once the 6th day of starvation had been reached. Measurements of glucose utilization in underfed sheep were made by radioisotope dilution by Annison and White (1961) and the values varied from 10.1$g/kgW^{0.75}$ per d to 5.6$g/kgW^{0.75}$ per d depending on whether the single injection or continuous infusion technique was used. Variation between these and the results of Steel and Leng may partly be attributed to the fact that, the sheep of Annison and White were given small quantities of poor quality feed (lucerne chaff). Measurement of minimal utilization should only occur in animals starved for 4d or more, shorter periods, where exogenous absorption of precursors may still occur, lead to overestimation (e.g. Armstrong, 1965). Since quantitative aspects of glucose utilization depend largely on nutrient supply to the tissues and, as discussed previously, the quantification and perturbation of nutrient supply is difficult by ordinary means, it follows that more control over nutrient supply would be of considerable value for investigative purposes.

Intragastric infusion. Procedures which, to varying degress, facilitate control and manipulation of nutrient supply to the absorptive regions of the ruminant gut have been described. The most

familiar example of this work was the work of Armstrong and Blaxter (1957 and 1957a) and Armstrong et al. (1957) who supplemented sheep fed basal diets of dried grass with exogenous infusions and were also able to sustain fasting sheep for short periods (12d) (Armstrong & Blaxter, 1957a) with intraruminal infusions of VFA. Later Martin and Blaxter (1963) were able to sustain fasting sheep with intraabomasal infusions of egg albumin for 14d.

Such techniques formed the basis of more recent procedures which allowed the total nutrition of an animal by the infusion of nutrients, specifically, Tao and Asplund (1975) in an adaptation of a method of parenteral hyperalimentation (see Dudrick & Rhoads, 1971; Way et al. 1973) sustained animals with intravenous infusions of glucose, amino acids, vitamins and antibiotics supplemented by intraruminal infusions of VFA, antibiotics, McDougall buffer and 12N sodium and potassium hydroxide. However, the incidence of electrolyte imbalance and intravenous infection limited its use with sheep to a period of 3 weeks.

Later, Ørskov et al. (1979) recognised the inadequacies of previous techniques and developed a system for the complete intragastric nutrition of sheep and Macleod et al. (1982) reported a similar technique for the sustenance of cattle. Both systems were able to sustain animals for longer periods than was before possible.

Intraruminal infusions of VFA's (acetate, propionate and butyrate) comprised the major energy components of the infusions. The incidence of reticulorumen acidosis and hyperosmolarity associated with these infusates was minimised by the simultaneous addition of an artificial saliva buffer (sodium chloride, sodium and

potassium bicarbonate) and water. Regular monitoring of rumen liquor osmolarity and pH together with adjustments to the buffer and water infusions helped maintain rumen homeostasis within physiological limits.

Protein nutrition was based on the intraabomasal infusion of a lactic-casein solution to which was added linoleic acid and a multivitamin mixture. Major minerals were infused intraruminally whilst trace minerals were administered in a daily intraabomasal injection (Fig. I(7)).

Intragastric alimentation allows complete qualitative and quantitative control over nutrient supply to chosen areas of absorption in the ruminant gut. The nutrients supplied pertain to known end products of digestion and as simple, uniform compounds lend themselves to accurate, uncomplicated sampling and analysis. These factors together with the effective removal of the gut microflora which ensures little or no modification of the infused nutrients prior to absorption shows the technique to be a potent research tool, information from which, can be used to supplement existing knowledge of nutrient utilization. The complexity of the technique has, however, limited its use to a small number of laboratories of which one has used the infusion procedure in various investigations, including the determination of N secretion in steers and dairy cows fed protein free diets (Ørskov & McLeod, 1982); the measurement of basal urinary N excretion in sheep given protein free infusions (Hovell et al., 1983). Nitrogen balance in cows (Ørskov et al., 1983) and sheep (Hovell et al., 1983a) given various levels of energy; and the investigation of the effects of non-protein energy on fasting N losses (Asplund et al., 1985).

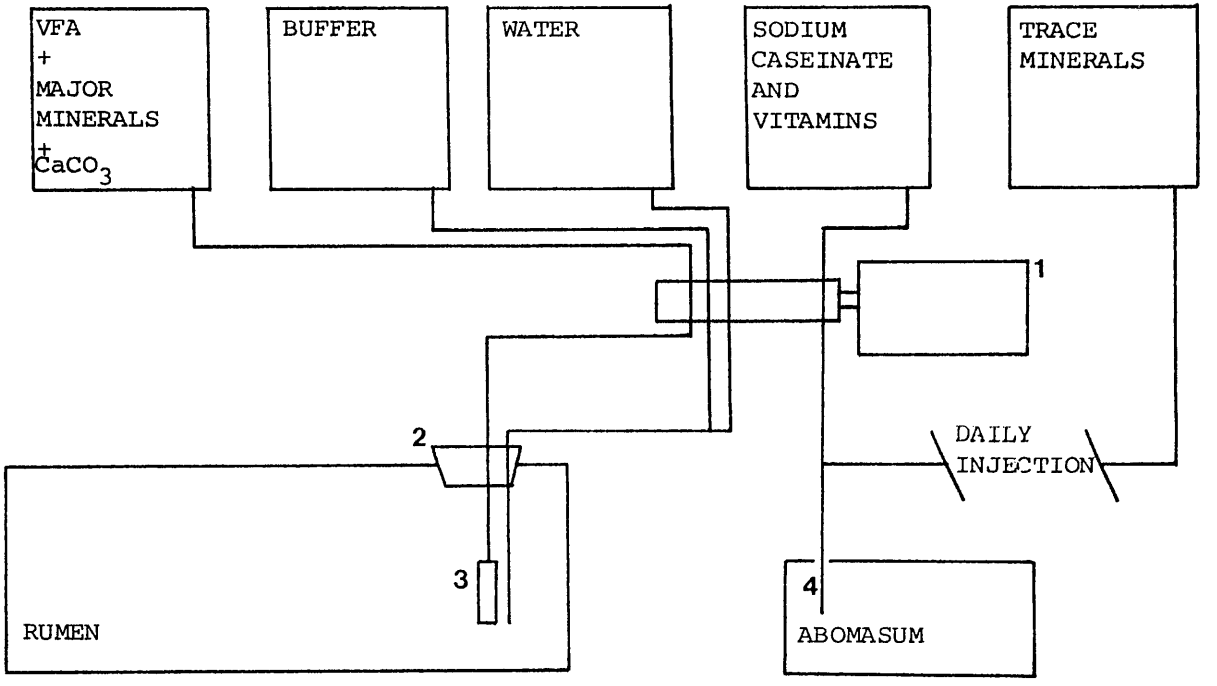


Figure I(7): Diagrammatic representation of the intragastric infusion technique described by Ørskov et al. (1979). 1. Multichannel flow inducer. 2. Pre-bored rubber plug. 3. Protective cuff on VFA infusion line. 4. Abomasal cannula. Total infusion volume 0.6-0.9 L/kg$W^{0.75}$ per d.

Conclusions and Objectives

The difficulties involved in the measurement and comparison of glucose utilization in ruminants, as a result of the dynamic nature of glucose metabolism and the variable accuracy of the existing methods used for its investigation have been discussed. Complete intragastric infusion allows precise control of the nutrients made available for absorption and potentially facilitates a level of controlled perturbation previously unobtainable by conventional means.

The aims and objectives of the work described in this thesis were to establish and perfect the intragastric infusion procedure developed by Ørskov *et al*. (1979) and Macleod *et al*. (1982) to enable the sustenance of cows and sheep for extended periods of time and to investigate the application of the technique in studies of N and glucose metabolism.

SECTION II

MATERIALS AND METHODS

SECTION II

MATERIALS AND METHODS

THE ANIMALS

To facilitate the nutrition of sheep and cows by the the infusion of nutrients animals were surgically prepared with cannulae in the rumen and abomasum.

Animal preparation

Rumen cannulation. Animals were fitted with a modified vulcathene waste drain fitment of 48mm internal diameter (I.D.) (J. McKibbin & Son, Uddingston, Glasgow, Scotland) incorporating a 20mm circular flange at the base. The flange was located within the rumen with the threaded stem exteriorized. An external nylon screw ring was used to secure the cannula which was occluded by a rubber bung and screw cap when not in use.

Surgical preparation involved clipping and washing the left flank of the animal prior to final sterilization of the proposed site of incision with antiseptic solution. Anaesthesia was achieved by an initial, per-jugular injection of sodium barbitone (sheep) or Rompun (cows) (Bayer Ltd., Eastern Way, Bury St. Edmunds) and maintained during surgery with a mixture of fluothane (ICI), nitrous oxide and oxygen administered through a previously inserted, cuffed, endotracheal tube. For cannula insertion a 120mm long incision was made with an electric cautery 60 or 120mm below the transverse processes of the lumbar vertebrae and 50 to 100mm posterior to the

last rib of sheep and cows respectively. The muscle layers of the abdominal wall were separated along the direction of travel of their muscle fibres and retracted to expose the peritoneum which was incised to expose the rumen wall. A pouch of ventral rumen wall was exteriorized and punctured with a 100mm incision to allow the insertion of the cannula flange. Subsequently the incision was closed to the stem of the cannula by a continuous suture and the tissue held tightly around the cannula stem by a purse-string suture to prevent seepage of rumen liquor. The cannnula stem was then temporarily sealed with heavy swabs whilst a 50mm diameter flap of skin was removed 100mm anterior to the initial incision. A scalpel-stab incision was made at this point and the subcutaneous tissue separated to allow the exteriorization of the cannula. At this juncture, the cannula flange was in very close proximity to the liver and manual manipulation ensured no hepatic tissue was trapped between the flange and abdominal wall. The peritoneum and muscle layers were closed by continuous sutures and the skin by single stay sutures. The cannula securing ring was then screwed into position and the heavy swab replaced by a screw cap.

Abomasal cannulation. The abomasal cannula consisted of a 1.0m or 1.5m length of surgical non-toxic, translucent vinyl tube of 5.0mm internal diameter (ID) and 8.0mm external diameter (ED)(Portex, Hythe, Kent). The end was inserted into the abomasum and retained by a silicone rubber collar and washer (20mm ED).

The animals received an identical method of anaesthesia to that described previously. An area of the right hand side, lower flank was shorn and washed prior to sterilization with disinfectant.

A 100-150mm incision was made by cautery extending ventrally from a point 100mm or 200mm posterior to the tip of the last rib of sheep and cows respectively. The abdominal wall and peritoneum were incised and the abomasum drawn back from its position behind the last rib. A 40mm incision was made laterally in the mid-lateral abomasal wall ensuring no digesta leaked into the body cavity. The cuff was inserted and secured with purse-string sutures in the mucosal lining and outer wall of the abomasum. The free end of the cannula was then secured to a 600mm long stainless steel needle through a 10.0 x 3.0mm eye. The needle was then manipulated upwards between the body wall and viscera and exteriorized between the last rib and transverse process of the right flank. At this stage a 1.0M length of 2.0mm ED vinyl tubing was inserted and passed down the cannula and secured at its external end to reduce the risk of subsequent cannula blockage by the abomasal digesta. The abomasal cannula was secured to the rumen cannula with elastic to prevent it being chewed or pulled by the animal. The incision was closed as described for the rumen cannulation.

Jugular catheterization. To facilitate long term, stress-free blood sampling an in-dwelling catheter of single lumen, medical grade polyethylene tubing of ID 1.0mm and ED 1.5mm (Dural Plastics, N.S.W. 2158, Australia) was used. The catheter was passed through a previously inserted intravenous cannula (Medicut, Sherwood Industries, Argyll) until approximately 150mm lay in the jugular vein. An exteriorized portion of 200mm remained which was flushed with sterilized citrate-saline solution (0.3% W.W. sodium citrate, 0.5% W.W. sodium chloride) and sealed by the partial insertion of a

20mm length of 1.0mm diameter stainless steel rod. Adhesive bandage (50mm width) was used to cover and secure the catheter.

Animal management

Surgical preparation was preceeded by a 24h fast to minimise the volume of digesta in the rumen. Access to food and water was resumed 2-3h after surgery and normal intakes were usually observed 3-4 d later. Skin sutures were removed 7-10 d after insertion and weekly removal of wool or hair from around the rumen, and point of entry of abomasal cannulae together with cleaning, disinfection and antibiotic dusting (Acramide, Willington Medical, Shropshire) ensured the minimum of discomfort through insalubrity. Regular observation and disinfection of the area of exteriorization of the jugular catheter obviated the incidence of jugular infection and ensured catheter patency for periods of up to 12 weeks.

Animal accommodation

Cows were accommodated in individual stalls, tethered by the neck to restrict movement. During faeces and urine collection a purpose-designed collection device (Fig. II(1)), to which cows quickly adjusted, was placed in the cubicle. Sheep were housed in individual metabolism cages (Plate II(1)) with excreta collection facilities. During periods of cold weather the animal accommodation was heated by overhead infra-red heaters (Super-Ser, Ross Electrical, Kilmarnock).

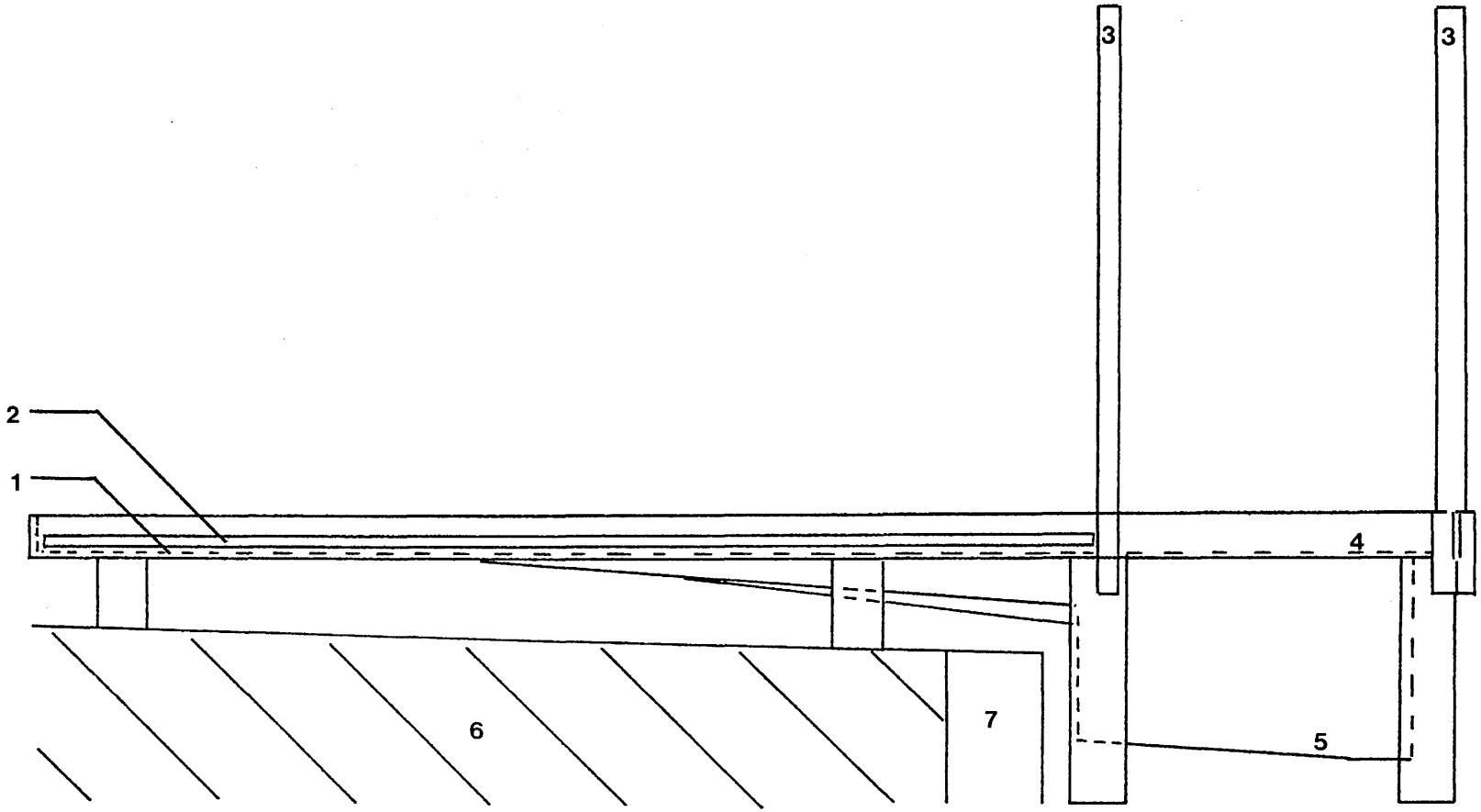


Figure II(1): Urine and faeces separation and collection device used during experimentation with cows. (1-10 scale). 1. 'Expamet' 2489F steel mesh. 2. Rubber matting (20mm thickness). 3. Support for spray curtain. 4. Removable 'Expamet' sections for faeces separation. 5. Collection sump. 6. Existing cubicle standing. 7. Heelstone.

PLATE II(1)

Metabolism cage with urine and

faeces collection facilities for sheep

EXPERIMENTAL TECHNIQUES AND PROCEDURES

Intragastric infusion

The infusion system. The infusion technique was essentially based on the work of Ørskov et al. (1979) and MacLeod et al. (1982). However, certain improvements were necessary to allow the technique to be used as a satisfactory approach to long term experiments. Emphasis on VFA as the major source of energy supplied and the associated dangers of rumen acidosis and hyperosmosis were reduced by the introduction of intra-abomasal infusions of long-chain fatty acids; by pre-mixing of VFA and buffer infusions before their point of entry into the rumen; and by the development of a monitor-regulator of rumen pH . These changes, together with other procedural improvements, permitted the sustenance of sheep by continuous infusions for periods of up to 12 weeks.

The technique comprised a bipartite system of infusion. A single-channel peristaltic flow inducer (Watson-Marlow MC10, Falmouth, Cornwall), utilizing 98.0mm lengths of 2.0mm ID, 2.5mm ED silicone rubber tubing pumped VFA solution from a reservoir, via PVC sleeving, 3.2mm ID, 4.0mm ED (Portex, Hythe, Kent), into the rumen (see Fig. II(2)). Controlling the VFA flow inducer was a 505D pH control module (LH Fermentation, Buckinghamshire) which, in conjunction with an indwelling liquid-proofed pH electrode (Fig. II(3)) (Russell pH, Fife) continuously monitored rumen pH and suspended the acid infusion at pH values below 5.5, recommencing infusion at pH 5.8. The VFA entered the rumen via a protective PVC

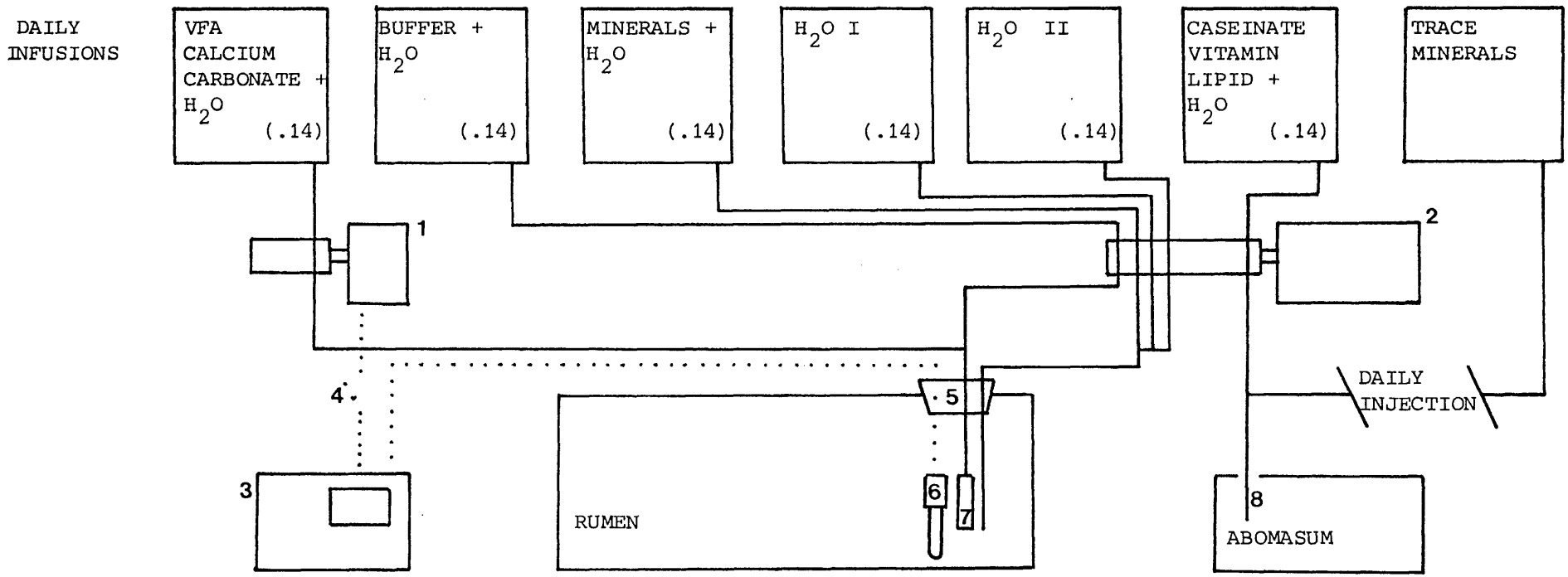


Figure II(2): Diagrammatic representation of the modified intragastric technique used at HRI. 1. Single channel peristaltic flow inducer. 2. Multi-channel peristaltic flow inducer. 3. pH control module. 4. Automated switch: closed when pH < 5.8. 5. Rumen cannula with sealing bung. 6. Moisture proofed pH probe. 7. Protective cuff. 8. Abomasal catheter. Values in parenthesis refer to individual reservoir contents in $L/(kgW^{0.75}/d)$. (.....) electrical connection, (-) polythene pipework.

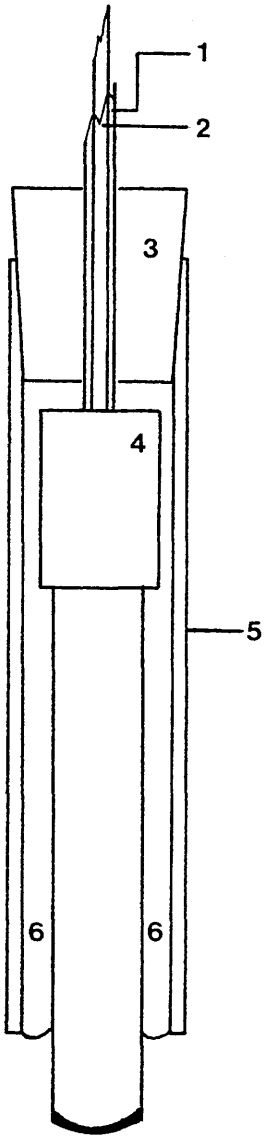


Figure II(3): Liquid-proofed combination electrode used to monitor rumen fluid pH. 1. Translucent vinyl sleeving. 5.0mm. I.D., 8.0mm E.D. 2. Electrical connection to probe. 3. Pre-drilled rubber stopper. 4. Combination pH electrode (glass, bullet tip). 5. Shortened 20ml disposable polythene syringe. 6. Silicone sealant (DOW-Corning). Diagram approximately to scale.

cuff designed to stop direct contact between the acid and the rumen mucosa.

A second infusion pump (Watson-Marlow, 501M) with multi-channel peristaltic pump head utilizing 2.0mm ID, 2.5mm ED and 2.5mm ID, 3.0mm ED silicone rubber tubing infused buffer, major mineral and casein/lipid/vitamin solutions together with additional water. The infusion pipework was designed to ensure mixing of VFA with buffer and mixing of major minerals with water prior to entry into the rumen. All intraruminal infusion pipework and electrical connections to the pH probe entered the rumen via a pre-drilled rubber plug (Fig. II(4)) held in place by the cannula screw cap. In the vicinity of the plug, in-line connectors were used. These were designed to pull open under the influence of any unusual strain on the infusion lines as would be produced, for example, if the lines became entangled with the animal.

The casein mixture was infused directly into the abomasum by means of larger bore silicone pump tubing (2.5mm) to compensate for its high viscosity. An infusion period of 23h was allowed for all infusates and silicone rubber tubing at the peristaltic pump heads was replaced every 7d regardless of apparent condition. Trace minerals were injected via the abomasal cannula on a routine daily basis.

Bulk nutrient solutions. In order to minimise the work load associated with the routine operation of the continuous infusion procedure, large quantities of concentrated infusates were prepared for dilution prior to daily mixing and infusion.

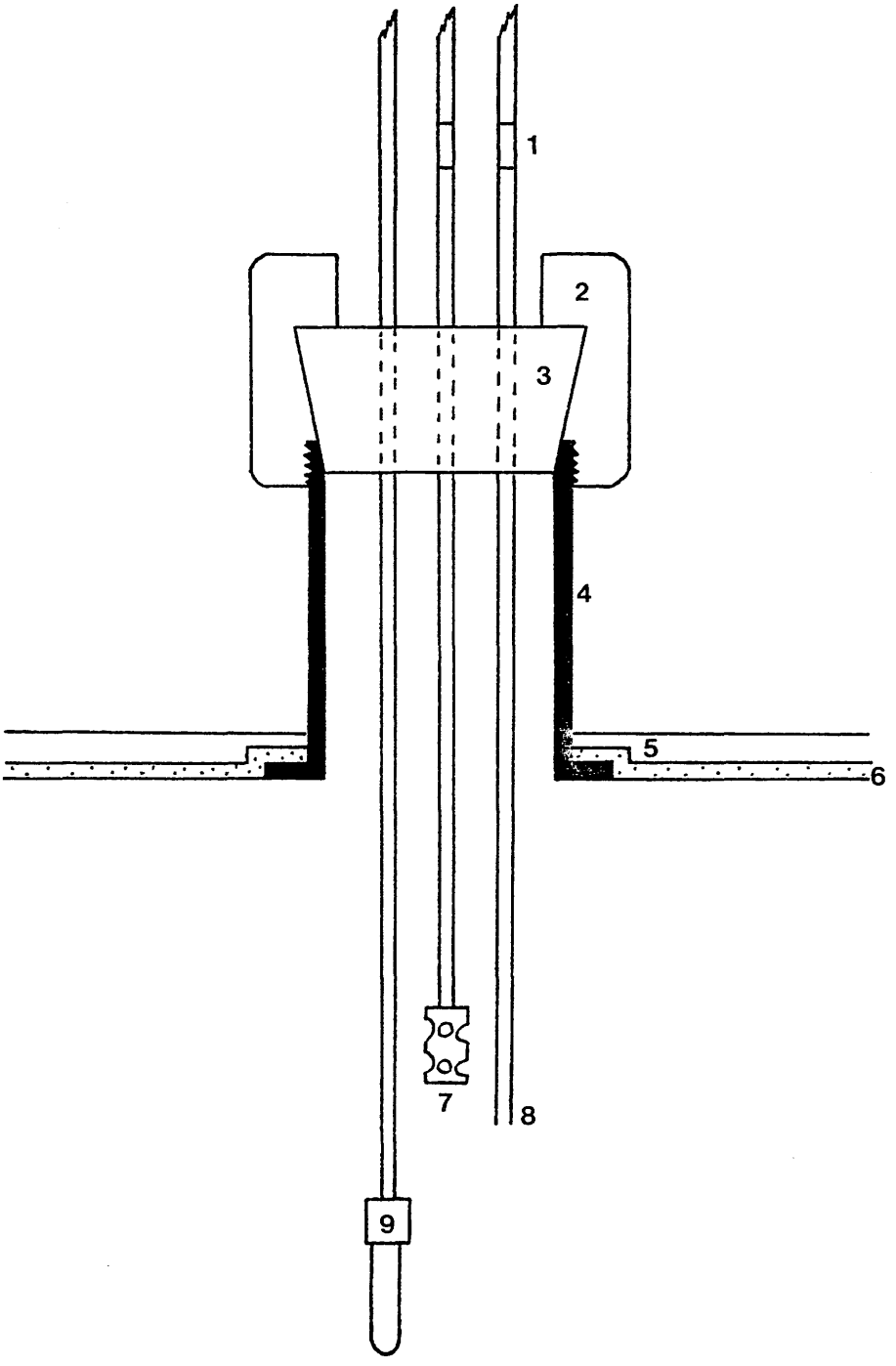


Figure II(4): Diagrammatic representation of the cannula assembly for intragastric infusion and rumen pH measurement in sheep. 1. Infusion line 'snap' connectors. 2. Screw-cap. 3. Drilled rubber plug. 4. Rumen cannula. 5. Body wall. 6. Rumen wall. 7. Protective cuff of the VFA/Buffer infusion line. 8. Major mineral and water infusion line. 9. Water proofed pH probe. Not to scale.

These 'stock' solutions were of fixed composition and daily nutrient inputs were manipulated through varying the dilution of the concentrated solutions measured into the infusion reservoirs.

Preparation of stock solutions.

Volatile fatty acids. Acetic acid (80%) together with 100% propionic and butyric acids (BP Nutrition (UK)) were mixed in chosen molar proportions prior to the addition of calcium carbonate ($CaCO_3$) and water equivalent to 2.7% and 49.0% W/W respectively of the final weight of the VFA mixture. Measurement of all nutrient solutions on a weight, rather than volumetric basis, was logistically preferable. The inclusion of $CaCO_3$ in the VFA solution was designed to supplement calcium inputs from the major-mineral infusion in order to reduce the reliance on expensive water-soluble calcium compounds.

Lipid. The provision of a post-ruminal long-chain fatty acid source with a high stearic acid (C18:0) content in a stable form for infusion was achieved by using an emulsion of beef tallow.

A food grade mono-diglyceride emulsifier was chosen (Emuldan HA 32/S6, Grinsted Products, Denmark) on the basis of its ability to form stable oil-in-water emulsions. During periods of cold weather however, problems of emulsion instability occurred and there was a tendency for infusion lines to become occluded. The development and installation of circulating hot water jackets (40^{o}C) reduced, but did not entirely obviate the problem.

Further improvement in emulsion stability was achieved by the addition of sodium caseinate powder (SMMB, Stranraer) coupled with a more prolonged mechanical agitation of the emulsion during

preparation using a hand held, high-speed, mixer-emulsifier (Silverson, Chesham, Bucks). Subsequent manipulation of the relative proportions of constituents and procedure finally resulted in a highly stable emulsion which would tolerate freezing and thawing.

Preparation of the lipid emulsion involved the dissolution of sodium caseinate in water at 70°C prior to the addition of powdered emulsifier (melting point 60°C) closely followed by liquified beef tallow. Tallow, emulsifier, sodium caseinate and water were mixed in a 1.0:0.15:1.15:26.45 ratio in order to represent 3.5, 0.5, 4.0 and 92% W/W respectively of the final solution. The optional addition of glucose was achieved by direct substitution (W/W) with water. Mechanical emulsification occurred for 3h on the preparation day and for 1h on the following 2 days in order to achieve stability. Cold storage (3°C) was necessary to prolong shelf life during periods of warm weather.

Sodium caseinate. Sodium caseinate solution (10% W/W) was prepared by the gradual addition of dried sodium caseinate (89% DM) (SMMB, Stranraer) to a weak sodium carbonate solution at 70°C. Continuous mixing was used to facilitate complete dissolution and on cooling (<30°C) a vitamin solution (see section below) was added. The quantities of Na_2CO_3, vitamin solution, sodium caseinate and water used represented 0.53, 2.57, 10.0 and 86.9% W/W of the final solution. Continuous cold storage (3°C) was necessary to prolong shelf life.

Vitamin. Constituents of the vitamin solution are shown in Table II(1). Vitamins were dissolved in a solution of water, ethanol and linoleic acid in the proportions shown prior to storage at $3^{o}C$ in a light-proofed container.

Buffer. Potassium bicarbonate ($KHCO_3$), sodium bicarbonate ($NaHCO_3$) and sodium chloride (NaCl) were dissolved consecutively in water ($70^{o}C$) to give a final solution composition of 3.8, 7.3, 0.7 and 88.2% W/W respectively. Mineral deposits settled out during storage and care was taken to avoid their re-suspension and transfer to the infusion reservoirs.

Major mineral. Calcium tetrahydrogen di-orthophosphate ($Ca(H_2PO_4)_2$) and magnesium chloride ($MgCl_2.6H_2O$) were dissolved in water ($70^{o}C$) to give a final solution composition of 1.5, 0.75 and 97.75% W/W respectively. Long-term storage was possible at ambient temperatures.

Trace mineral. Solution composition is shown in Table II(2). Copper was precluded from infusions intended for use with sheep. Long-term storage was possible at ambient temperatures.

Infusion formulation. The withdrawal of solid feed together with infusion of nutrients totally suppresses normal rumen fermentation. Accordingly, calculation of nutrient requirements was based on the recommendations of nutrient requirements for lambs and calves or pigs.

Table II(1): Composition of the vitamin solution.

Constitutent	g/kg
Thiamine hydrochloride	0.09
Riboflavin	0.35
Nicotinic acid	0.35
Choline chloride	13.20
Pyridoxine hydrochloride	0.04
p-Aminobenzoic acid	0.008
Calcium pentothenate	0.26
Folic acid	0.0009
Cyanocobalamine	0.0009
Myo-inositol	13.20
d-Biotin	0.005
2-Methyl-1,4-naphthoquinone	0.04
d-α-Tocopherol acetate	0.35
Linoleic acid	80.84
Ethanol	260.51
Water	610.85

Table II(2): Composition of the trace mineral solution.

Constitutent	g/kg
$FeSO_4.7H_2O$	20.30
$ZnSO_4.7H_2O$	1.20
$MnSO_4.4H_2O$	0.60
$CuSO_4.5H_2O$	0.60
KI	1.10
NaF	0.80
H_2O	975.00

1. Omission of $CuSO_4 5H_2O$ was necessary when the trace mineral mixture was intended for administration to sheep.

Protein requirements. Recommendations for pre-ruminants were derived from the following equation (ARC, 1980):

$$\text{Protein requirement (g/d)} = 6.25 \frac{(R_N + Wool_N + U_{N(e)})}{d_N \times k_{N(u)}}$$

where R_N = N retention in liveweight gain (g/d)

$Wool_N$ = Wool and epidermal N loss (g/d)

$U_{N(e)}$ = Endogenous urinary N (g/d)

d_N = Apparent digestibility of N

$k_{N(u)}$ = Efficiency of utilization of absorbed N.

The protein requirement of an animal fed to meet its energy requirements for maintenance is approximately equal to the endogenous urinary loss divided by the product of d_N (0.92) and $k_{N(u)}$ (0.8) (see Roy et al., 1970; Black et al., 1973). The use of apparent digestibility (ARC, 1980) rather than true digestibility (ARC, 1976) removes the need to make allowance for metabolic faecal N (MFN), the determination of which is complex (Blaxter, 1964; Ørskov et al., 1970), but leads to underestimations of actual N requirements. This coupled with the omission of MFN from estimates of endogenous N excretion points to a considerable error in the ARC (1980) protein recommendations. Hovell et al. (1983), using intragastric infusion gave protein free diets and determined a basal urinary N excretion of 429mg $N/kgW^{0.75}$ per d which was considerably in excess of the 90-100mg $N/kgW^{0.75}$ per d for the same animals derived from ARC (1980) recommendations.

Accordingly in the experiments undertaken here, a base-line tissue N requirement for maintenance of 370mg/kgW$^{0.75}$ per d was adopted prior to adjustment in response to N balance data for individual animals. Coefficients of 1.0 and 0.8 were adopted for casein digestibility and efficiency of utilization of absorbed N respectively (Roy et al., 1970; Black et al., 1973). During the course of the experimental work reported here, ARC revised their recommended tissue N allowances to a new value of 350mg N/kgW$^{0.75}$ per d (ARC, 1984) in response to measurements of UN(E) with ruminants intragastrically infused with N-free nutrient mixtures (Ørskov and Grubb, 1978; Storm and Ørskov, 1979; Ørskov and Macleod, 1982 and 1982a; Hovell et al., 1983).

Energy requirements. Formulation of the energy component of infusions was based on current energy recommendations (ARC, 1980) with the addition of a 4% safety margin. An overall metabolizability (q) of 1.0 was adopted for the infusions together with an efficiency of utilization of absorbed energy for maintenance (k_m) of 0.85 which resulted in a daily maintenance requirement of 430 kJ/kgW$^{0.75}$ per d, adjusted to 450 kJ/kgW$^{0.75}$ per d for cows and 323 kJ/kgW$^{0.75}$ per d for sheep.

Formulation of individual infusates

Sodium caseinate. Requirements for N were met by the infusion of a stock solution of sodium caseinate of known N content. A gross energy value of 19 kJ/gm was adopted for air dried sodium caseinate (88% DM, 3% fat). Lipid emulsion was added to the caseinate stock solution. The mixture was diluted with water to a final weight of

approximately 140g/kgW$^{0.75}$ per d agitated with a mixer-emulsifier and infused over a 23h period.

Lipid. A stock solution of lipid was infused at a rate providing 25 g/d and 180 g/d of post-ruminal long-chain fatty acid for sheep and cows respectively. A gross energy value of 39 kJ/g was adopted for beef tallow and the mono-diglyceride emulsifier together with a coefficient of digestion of 0.95.

Volatile fatty acid. The major proportion of daily energy requirement was met by VFA. Gross energy values of 14.56, 20.74 and 24.9 kJ/g were adopted for acetic, propionic and butyric acids respectively together with a coefficient of digestibility of 1.0. Prior to infusion the VFA stock solution was diluted with water to provide a final infusion weight of 140g/kgW$^{0.75}$ per d.

Buffer. The quantity of buffer necessary to maintain rumen pH within physiological limits (5.5-6.5) was dependent on the quantity of VFA mixture infused, the molar proportions of its constituents and the absorptive capacity of individual animals. Accordingly, an approximate guide (Table II(3)) rather than specific recommendations was used for formulation.

Calculated levels were adjusted in response to the rumen pH and osmotic pressure of individual animals. Dilution with water to a final weight of 140g/kgW$^{0.75}$ per d preceded infusion over a 23h period.

Table II(3): Approximate ratio (W/W) of buffer to VFA required to maintain rumen pH homeostasis in sheep and cows. (From MacLeod, N.A., personal communication).

	Acetic acid content of VFA (mmol/mol total VFA)			
Energy level (multiple of energy maintenance)	450	550	650	750
M	2.0	2.1	2.2	2.3
2M	2.3	2.4	2.5	2.7

Major minerals. Formulation of the mineral mixture was according to the recommendations of Ørskov et al., 1979. These were based on the calcium and magnesium requirements of pigs with adjustments to take account of those levels of supply on the blood concentrations of intragastrically infused sheep. A value of 40g major mineral solution/$kgW^{0.75}$ per multiple of energy requirement at maintenance per d was suggested. Routine blood analyses in the course of the present experiments indicated a degree of mineral deficiency at this level of infusion and a value of 60 $g/kgW^{0.75}$ per multiple of maintenance per d was adopted. Prior to infusion over a 23h period the stock solution of major minerals was diluted to a final weight of 140 $g/kgW^{0.75}$ per d.

Trace minerals. On a once daily basis trace mineral solution was administered through the abomasal cannula at a rate equivalent to 1.0 $ml/kgW^{0.75}$ per d (Ørskov et al., 1979). Analysis of plasma trace mineral concentrations indicated physiological normality except for an excess of copper in the sheep. Accordingly copper sulphate was removed from trace mineral formulations intended for use with sheep.

Vitamins. The stock solution of vitamins was added to the stock solution of sodium caseinate at a rate equivalent to 2.6% W/W of the final vitamin/sodium caseinate stock solution mixture. This was in accordance with the recommendations of Ørskov et al. (1979). Subsequent analysis of blood and plasma and the general health of the animals did not indicate a need for adjustment.

Additional water. To avoid the incidence of high rumen osmotic pressures one or two infusions of water each providing 140 $g/kgW^{0.75}$ per d were given to the animals, bringing the maximum total weight infused to approximately 840 $g/kgW^{0.75}$ per d.

Computer formulation. A computer program was created to facilitate fast and accurate re-formulation of infusions in the event of variation in the composition of stock solutions or planned adjustments in nutrient input. Written in 'BBC Basic' language (Appendix 52) the program considers 16 animal and dietary variables, determines the daily proportions of stock solutions required and offers the option of calculating the precise quantity of individual nutrients infused. An example of the formulations provided is also shown (Appendix 53).

Changeover procedure

Solid to liquid feeding. The transition to intragastric feeding was achieved by the progressive withdrawal of solid food with the concomitant introduction of increments of infusates. Introduction of an infusion regime occurred over at least 5, 2-d periods to allow acclimatization. During the final 2 periods the optional water infusions were introduced if required. A proprietary barley-based concentrate was given during the transition period, initially at a rate of 2.0 kg/d, which was reduced by 0.4 kg/d every other day. Two days after the complete withdrawal of solid feed a liquid proofed pH probe (Fig. II(3))) was introduced into the rumen to facilitate automatic pH control. Also pan-scrubbers (Semi-Chem, Ayr) were placed in the rumen of sheep (8 scrubbers) and cows (30

scrubbers) to help maintain rumen motility and desquamation of the rumen mucosa.

Rumen pH was maintained between 5.5 and 6.5 and the quantity of additional water infused adjusted to ensure rumen fluid osmotic pressures were kept between 250 and 350 mosmol/l (Church, 1979).

Liquid to solid feeding. Following an experiment, the rumen of sheep and cows were drained of rumen liquor. Daily intraruminal infusions of 2.5 l or 10.0 l doses of 'donor' rumen liquor and solids were then given together with 500 g or 3000 g doses of glucose for sheep and cows respectively. Small quantities of good-quality hay, silage and, when possible, fresh-cut grass were made available together with lamb-weaning concentrates (Ewbol Lambweena pellets, BOCM, Basingstoke) or calf-weaning concentrates (Volac Follow-on-calf pellets, Volac, Herts).

Sodium caseinate and major mineral infusions were continued until rumination had re-started (3-4 d) and normal food intake usually resumed within 7 d of introduction. Occasionally animals showed little inclination towards solid food. In those circumstances weaning concentrate and chopped straw cubes were introduced intraruminally until the animals began to eat voluntarily.

Collection, preparation and analysis of body fluids, excreta and infusates

Rumen fluid

Collection. Samples of rumen fluid (50ml) were withdrawn at least twice daily through a 500mm length of PVC tubing (3.2mm, ID;

4.0 ED - Portex, Hythe, Kent) which was inserted into the rumen and connected to a 100 ml syringe.

Analysis of rumen fluid

Rumen pH. Determination of rumen pH was by combination pH electrode (Pye-Unicam, Cambridge).

Rumen osmotic pressure. Fifty µl of rumen fluid was transferred to a 0.5 ml polythene microcentrifuge tube (Alpha Laboratories, Eastleigh, Hants) and inserted into the sample freezing compartment of an automatic osmotic pressure reader (Gonotec, Osmomat 030) previously calibrated to 0 and 300 mosm/l with distilled water and standard sodium chloride solution respectively.

Excreta

Collection and preparation. Urine and faeces were collected in the receptacles provided (see Plate II(1) and Fig. II(1)) into which was placed 2.0 l, 10% N -free sulphuric acid on a daily basis. The collection area was flushed daily with a high pressure/low volume water jet and the receptacle emptied. The urine/faeces collected was weighed, speed-mixed for 5 minutes (Silverson speed mixer/emulsifier, Bucks), and a 500 ml sample was removed for analysis.

Analysis of excreta

Total nitrogen. The total N content of urine and faeces was measured using a macro Kjeldahl method. A Kjeltec system , incorporating a digestion unit, distillation unit and titration unit (Tecator Ltd, Bristol) was used for the analysis.

Samples were first digested with N-free concentrated sulphuric acid, using tablets containing 2.0 g potassium sulphate and 0.02 g selenium catalyst. The digests were distilled and the ammonia collected in 25 ml boric acid solution (40 g/l). The total nitrogen was then determined by titration with 0.01 M hydrochloric acid against a water blank.

Urine creatine. The determination of urine creatine concentration was based on the work of Wong (1971). Creatine reacts rapidly with α-naphthol-diacetyl solution in alkaline conditions and produces a red-coloured complex, the colour intensity of which is proportional to creatine concentration.

To 0.2 ml of creatine standard or sample was added 5.0 ml α-napthol solution. The latter was prepared by dissolving 0.5 g napthol in 100 ml of an alkaline solution containing 20 g sodium hydroxide and 55 g anhydrous sodium carbonate per litre of distilled water. One ml of diacetyl solution (0.05%) was added together with 3.8 ml redistilled water. The mixture was incubated with occasional agitation, for 1.75 hours at $0^{o}C$. Absorbance at 530nm was measured against a water blank using a spectrophotometer (SP6-200, Pye Unicam, Cambridge). Values for samples were compared with those for a standard solution (1.0 mg/ml) to determine creatine concentration.

Urine creatinine. Determination of urine creatinine concentration was by use of the Beckman creatine test combination (Beckman Instruments, USA) which was a modification of the method described by Jaffe (1886). In alkaline conditions creatinine reacts with picric acid to form a red coloured creatinine-alkaline picrate

complex of unknown structural composition. The rate of formation of the complex is proportional to the concentration of creatinine in the original specimen.

Five hundred µl of Beckman creatine reagent (Beckman Instruments, USA) prepared by the addition of 25 ml picric acid solution (50 mM) to a bottle of borate/phosphate buffered sodium hydroxide solution (188 mM) provided, was mixed with 25 µl of sample. The initial rate of complex formation was determined by its effect on absorbance at 520nm (Beckman model 42 spectrophotometer) and compared to that obtained with prepared standard solutions for the determination of the creatinine concentration of samples.

Blood

Collection and preparation. In order to minimise stress all blood sampling was by indwelling jugular catheter. A disposable syringe containing 10 ml sterilized citrate-saline solution (0.3% W/W sodium citrate, 0.5% W/W sodium chloride) was used to withdraw the first 1.0 ml of blood prior to substitution with a plain or heparinized syringe (rinsed in 250 iu heparin/ml solution and dried) to complete sampling. Catheters were then flushed with sterilized citrate-saline solution and closed. Samples were transferred to plain or heparinized McCartney bottles placed in ice. Heparinized samples were centrifuged for 15 minutes at 3500g and the blood plasma was removed. Unheparinized samples were allowed to stand for 30 minutes before centrifugation and removal of serum. Ten µl aprotinin per ml (Sigma Chemicals, Dorset) was added to whole blood samples intended for glucagon anlaysis to inactive enzymes. Plasma and serum were analysed immediately or stored at -20^{o}C.

Blood analysis

Haematocrit. The packed cell volume of heparinized whole blood was determined by partly filling a plain glass capillary tube (Gelman-Hawksley, Sussex) with blood. One end of the tube was sealed (Critoseal) and the tube was spun in a micro-haematocrit centrifuge (Gelman-Hawksley) for 5 mins. The percentage of red cells in the blood was determined using a Hawksley haematocrit reader.

Plasma glucose. Determination of the glucose concentration of plasma was by the GOD-Perid method (Werner et al., 1970). Glucose is oxidised to gluconic acid and hydrogen peroxide by the specific enzyme glucose oxidase (GOD). In the presence of peroxidase enzyme the hydrogen peroxide oxidises a chromogen, Perid (2.2 Azino-di-(3-ethyl-benzthiazoline sulphonate(6)), with the production of a dye, the colour intensity of which is proportional to glucose concentration.

A 1.0 ml aliquot of plasma was deproteinized with 10 ml uranyl acetate solution (0.16% w/v) prior to centrifugation at 3000g for 10 minutes. Either 0.2 ml distilled water (blank) or 0.2 ml of sample supernatant was added to a mixture containing at least 10 iu/ml peroxidase and 1.0 mg chromogen/ml in 100 mM phosphate buffer at pH 7.0. Standard solutions (Boehringer, 1976) were prepared in a similar manner.

After allowing these mixtures to stand for 45 minutes at 20-25^{o}C, away from direct sunlight, the absorbancies of the samples and standards were determined at 640 nm with a SP6-200 spectrophotometer (Pye-Unicam, Cambridge) against the prepared blank.

Comparison of the absorbance of blank-corrected test and standard samples allowed the original plasma glucose concentration to be determined.

Plasma glycerol. Determination of plasma glycerol concentration was as described by Brechany (1984). The method involves the phosphorylation of glycerol by ATP to produce glycerol-1-phosphate and ADP in a reaction catalysed by glycerol kinase. Glycerol phosphate dehydrogenase then catalyses the oxidation of glycerol-1-phosphate in the presence of NAD^+ to produce NADH which subsequently reduces the dye, 2-(p-iodophenyl)-3-p-nitrophenyl-5- phenyl tetrazolium chloride (INT) to a formazan in the reaction catalysed by diaphorase. The intensity of the colour formed is proportional to glycerol concentration in the plasma sample.

To 50 µl of sample, water blank or serially diluted Precimat standard solution (Boehringer) was added 950 µl of a pre-prepared reaction medium. The medium comprised 48% V/V tris buffer, adjusted to pH 8.5; 5.3% V/V of a 2.5 mg/ml NAD solution; 5.3% V/V of a 5.0 mg/ml ATP solution; 2.1% v/v of a 2.5 mg/ml diaphorase solution; 2.1% V/V of a 1.0 mg/ml INT solution; and 1.0% V/V of a triton X:methanol mixture (1:9 V/V). To these were further added 10 µl glycerol phosphate dehydrogenase (110 iu/mg) and 400 µl water. The reaction mixture was allowed to stand for 15 minutes at room temperature and the absorbance at 500 nm was determined (Gilson SP240 Spectrophotometer). Ten iu glycerol kinase was then added as 200 µl of a 1.0 mg/ml solution (Boehringer) together with 400 µl water prior to a 25 minute incubation at room temperature and redetermination of

absorbance at 500 nm. The concentration of glycerol in the samples was determined by comparison of the changes in blank-corrected absorbance of samples and standards.

Plasma L-lactate. A modification of the method devised by Noll (1974) was used to determine the concentration of L-lactate in blood plasma. In the presence of L-lactate dehydrogenase (L-LDH), L-lactic acid is oxidized by NAD to pyruvate. The equilibrium of the reaction is heavily biased towards the left. However, it can be displaced in favour of pyruvate and NADH by trapping pyruvate in a glutamate-pyruvate transaminase (GPT) catalysed reaction with L-glutamate to form L-alanine and α-oxoglutarate. The quantity of NADH formed has a stoichiometric relationship with the concentration of L-lactate and is determined by means of its absorbance at 340 nm (Gilson G-240).

A 0.1 ml aliquot of sample or water (blank) was mixed with 1.0 ml of glycylglycine buffer (pH 10.0) solution containing 14.7 mg L-glutamic acid, 0.2 ml of a 35 mg/ml lyophilized β-NAD solution, 0.9 ml redistilled water and 0.02 ml of GPT solution containing approximately 31.4 iu of enzyme. After 5 minutes, absorbance (A_1) was determined prior to the addition of 0.02 ml of L-LDH solution containing approximately 108 iu of enzyme. On completion of the reaction (10 minutes) absorbance (A_2) was re-read. The blank (B) corrected change in absorbance $(A_2-B)-(A_1-B)$ was used to calculate the concentration of lactic acid in the original sample from the formula:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ (g/l)}$$

where V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of lactic acid

d = light path (cm)

ε = absorption coefficient of NADH at 340 nm.

ΔA = blank corrected change in absorbance at 340 nm.

Serum total protein. Serum proteins are known to react with copper in the presence of alkali to produce purple-coloured copper-protein complexes, the intensity of which are proportional to the original total protein concentration (Gornall et al., 1949).

Five ml of a reagent containing 0.15% (W/V) copper sulphate and 3% (W/V) sodium hydroxide was added to 0.1 ml distilled water (blank), 0.1 ml of a protein solution containing 5.0 g/100 ml human albumin and 3.0 g/100 ml human globulin (standard), and 0.1 ml serum (test). The samples and reagent were vortex-mixed and incubated for 15 minutes at room temperature. Blank corrected absorbances (A) were then subject to a conversion factor (Boehringer) and used to determine the total protein concentration of serum as follows:

$$\text{Serum total protein} = \frac{A \text{ test}}{A \text{ Standard}} \times 8 \text{ (g/100ml)}$$

Plasma amino acids. The amino acid composition of plasma was determined using the modified ion exchange procedure of Moore et al. (1958). The acids, separated by ion exchange were allowed to react with ninhydrin prior to determination of absorbance and comparison

with individual amino acid standards (Sigma Chemicals, Poole, Dorset).

Deproteinization of a 6.0 ml aliquot of blood plasma was achieved by the addition of 2.0 ml of 20% (W/V) sulphosalicyclic acid, and 2.0 ml of norvaline solution (2.5mmol) was added as a internal standard. After thorough mixing the sample and reagents were allowed to stand at $4^{o}C$ for 1 hour prior to centrifugation (Chillspin) at 3000g for 20 minutes. The supernatant was removed and filtered through Whatman No. 1 paper. The filtrate was adjusted to pH 2.0 by addition of saturated lithium hydroxide solution.

The determination of filtrate amino acid composition and concentration was made using an automatic amino acid analyser (Chromaspek J180, Rank Hilger, Kent) fitted with a 350 mm x 2.7 mm bore stainless steel column packed with sulphonated polystyrene ion exchange resin (PA6-particle size 6-7 μ). Amino acids were eluted from the column on a continuous pH gradient formed by the programmed addition of two lithium citrate buffers of pH 1.9 (0.15 M Li^{+}) and pH 11.3 (0.3 M Li^{+}). A temperature program was employed such that acidic amino acids were eluted at a column temperature of $40^{o}C$, neutral amino acids at $60^{o}C$ and basic amino acids at $40^{o}C$, over a 2-hour period. Quantitative determination of amino acids was by measurement of the absorbance of their ninhydrin complexes at 570 nm; the proline/ninhydrin complex was measured at 440 nm. Concentrations of individual amino acids were determined by reference to the absorbance of a standard amino acid mixture.

Plasma albumin. Plasma albumin is known to bind with bromocresol green (BCG), a yellow dye, to produce a green BCG-albumin

complex ,the colour intensity of which is proportional to original plasma albumin concentration (Doumas et al., 1971).

Five ml of an albumin colour reagent (Sigma Diagnostics, St. Louis, USA) comprising BCG, 0.01% (W/V) in a buffer at pH 4.0 was added to either 0.02 ml, 0.85% sodium chloride solution (blank), 0.02 ml of a protein solution comprising 5.0 g/100 ml human albumin and 3.0 g/100 ml human γ-globulin (standard), and 0.02 ml of plasma (test sample). The blank corrected absorbance (A) at 630 nm (SP6-200 Pye-Unicam, Cambridge) of test and standard solution was subject to a conversion factor (Sigma Diagnostics) and used to determine plasma albumin concentration as follows

$$\text{Plasma albumin} = \frac{\text{A Test}}{\text{A Standard}} \times 50 \text{ (g/100ml)}$$

Plasma urea. Determination of the urea concentration of blood plasma was carried out using the urease method of Fawcett and Scott, (1960). Urease cleaves urea in the presence of water with the formation of ammonium carbonate. Ammonium ions react with phenol and hypochlorite solution in the presence of sodium nitroprusside and produce a blue-coloured complex, the intensity of which is proportional to the urea concentration of the sample.

A 0.1 ml aliquot of blood plasma was mixed with 0.9 ml, 0.9% sodium chloride solution. A 0.2 ml aliquot of this diluted sample was added to 0.1 ml of a solution containing at least 10 iu/ml of urease in 50 µl phosphate buffer at pH 6.5. One blank and one standard were also prepared containing 0.1 ml of the urease solution and 0.2 ml distilled water or 0.2 ml of 0.5 mmol urea solution. The

mixtures were vortex-mixed, stoppered and incubated for 10 mins at 37°C prior to the addition of 5.0 ml of a mixture of 0.106 M phenol and 0.17 mmol sodium nitroprusside, and 5.0 ml of a mixture of 11.0 mmol sodium hypochlorite and 0.125 N sodium hydroxide solutions. The mixtures were vortex-mixed and incubated for a further 15 minutes at 37°C. Absorbance was measured at 550 nm with a SP6-200 spectrophotometer (Pye-Unicam, Cambridge). Following correction against blanks, the absorbance of the sample was divided by the absorbance of the standard. This value was multiplied by a conversion factor of 5 (Boehringer test combination) to give plasma urea concentration (mmol/l).

Plasma non-esterified fatty acids. The method used to determine plasma non-esterified fatty acid (NEFA) concentration was that described by Duncombe (1964). NEFA's are converted to chloroform-soluble copper salts. These are measured spectrophotometrically and the NEFA concentration determined from the amount of salts formed.

A 0.2 ml aliquot of blood plasma, distilled water (blank) or 0.5 mmol/l fatty acid standard (12.82 mg palmitate and 14.22 mg stearate/100 ml) were each added to 5.0 ml chloroform. One ml of 0.45 M triethanolamine buffer (pH 7.8) and 1.0 ml of 0.27 M cupric nitrate solution were added and the mixtures mechanically shaken for 10 minutes. The blue-green aqueous layer formed was removed using a fine tipped pippette. Two ml of the remaining chloroform layer was then mixed with 0.2 ml of 9.0 mM diethyldithiocarbonate solution and the absorbance at 436 nm determined with a SP6-200 spectrophotometer (Pye-Unicam, Cambridge). The absorbance of the samples was corrected

for the blank and compared to that of the standard to determine NEFA concentration.

Plasma acetate. The method used for the determination of the acetate concentration in plasma relied on the change in absorbance resulting from the production of NADH during a complex series of reactions. In the presence of acetyl-CoA synthetase (ACS), adenosine-5-triphosphate (ATP) and Co-enzyme A (CoA), acetate is converted to acetyl CoA, which, in the presence of citrate synthetase (CS) reacts with oxaloacetate to form citrate. The oxaloacetate required is formed from malate and NAD in the presence of malate dehydrogenase (MDH), NAD^+ being reduced to NADH. This NADH production can be measured by an increase in absorbance at 340 nm and is proportional to the acetate concentration.

Portions (0.1 ml) of sample solution and distilled water (blank) were mixed with: 1.0 ml of triethanolamine buffer (pH 8.4), containing 4.2 mg of L-malic acid and 2.1 mg magnesium chloride; 0.2 ml of a solution containing 5.0 mg ATP, 0.52 mg CoA and 2.46 mg NAD; and 1.9 ml redistilled water. Absorbances (A_o) at 340 nm were determined by using a Gilson 240G spectrophotometer prior to the addition of 0.01 ml of an enzyme solution containing approximately 275 iu and 6.75 iu of MDH and CS respectively. A second absorbance reading (A_1) was taken 3 minutes after the enzyme addition. A final reading (A_1) was taken after the addition of 0.01 ml of a solution containing approximately 0.4 iu ACS and a 15 minute period of incubation at room temperature. As there was no linear proportionality between the measured change in absorbance and the acetic acid concentration the following formula was used to calculate

the change in acetic acid concentration (ΔAc):

$$\left(\Delta Ac = (A_2 - A_0)_{sample} - \frac{(A_1-A_0)^2_{sample}}{(A_2-A_0)_{sample}}\right) - \left((A_2 - A_0)_{blank} - \frac{(A_1-A_0)^2_{blank}}{(A_2-A_0)_{blank}}\right)$$

from which the acetate concentration in the plasma sample (c) was derived as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta Ac \ (g/l)$$

where V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of acetate

d = light path (cm)

ε = absorbtion coefficient of NADH at 340 nm.

ΔAc = change in acetic acid concentration (g/l)

Plasma D-(-)-3-Hydroxybutyrate. Determination of plasma D-(-)-3-hydroxybutyrate (3-OH butyrate) was by the method of Williamson and Mellanby (1974). 3-OH butyrate is oxidized by NAD in the presence of 3-OH butyrate dehydrogenase to form acetoacetate and NADH. The increase in absorbance at 340 nm as a result of the change in NADH concentration is proportional to 3-OH butyrate concentration.

Equal aliquots (4.0 ml) of plasma and ice cool 10% W/V solution of 60% W/W perchloric acid (SG 1.64) were mixed and centrifuged (Chillspin) at 3000g for 15 minutes. The deproteinized supernatant was then neutralized (pH 7.8) by dropwise addition of 20% W/V potassium hydroxide solution in the presence of 5 µl universal indicator (BDH, Poole, Dorset). Tris buffer (0.1 M) solution at pH 8.5 was prepared containing 1.21% (W/V) tris and 3% V/V hydrochloric

acid (HCl). This was added to 1.0 ml hydrazine hydrate, 20 mg ethylenediaminetetra-acetic acid disodium salt (EDTA-Na$_2$H$_2$.2H$_2$O) and 5.0 ml M HCl to give a final volume of approximately 20 ml and pH of 8.5. One ml of the hydrazine-tris buffer was mixed with 2.0 ml of deproteinized, neutralized sample prior to the addition of 0.10 ml of a 10 mg/ml NAD solution. Ultraviolet absorbance at 340 nm (A_1) was measured against an air blank using a Gilson 240-G spectrophotometer. A quantity (0.01 ml) of 3-OH butyrate dehydrogenase from Rhodopseudomonas spheroides suspended in 3.2 M ammonium sulphate (3 iu/mg)(Sigma Chemicals, Poole, Dorset) was added and an absorbance redetermined (A_2) after 45 minutes. The change in absorbance, corrected for the enzyme suspension was then used to calculate the concentration of 3-OH butyrate in the original sample from the equation:

$$C = \frac{\Delta A \times V}{\varepsilon \times d \times v} \times F \text{ (mmol/l)}$$

Where c = concentration of 3-OH butyrate (mmol/l)

ΔA = corrected change in absorbance

V = assay volume (ml)

v = sample volume (ml)

d = light path (cm)

ε = extinction coefficient ($cm^2/\mu M = 6.22$)

F = dilution factor.

Plasma acetoacetate. Determination of plasma acetoacetate was by the method of Mellanby and Williamson (1974). Acetoacetate is reduced by NADH in the presence of 3-OH butyrate dehydrogenase to form 3-OH butyrate and NAD. The decrease in absorbance at 340 nm as

a result of the change in NADH concentration is proportional to acetoacetate concentration in the original sample.

Deproteinization and neutralization of plasma was as described previously for the determination of 3-OH butyrate. One ml of a 0.1 M phosphate buffer (pH 6.8), prepared by mixing equal volumes of 1.36% W/V potassium dihydrogen phosphate and 1.74% W/V dipotassium hydrogen phosphate solutions was mixed with 2.0 ml of sample and 0.1 ml of a 10 mg/ml solution of NADH (Sigma, Poole, Dorset). Ultraviolet absorbance (A_1) at 340 nm was determined using a Gilson, 240-G spectrophotometer prior to the addition of 0.01 ml 3-OH butyrate dehydrogenase (Sigma Chemicals, Poole, Dorset). The absorbance was redetermined (A2) after 20 minutes. The change in absorbance (ΔA), corrected for the enzyme suspension was then used to calculate the acetoacetate concentration of the original sample from the equation:

$$C = \frac{\Delta A \times V}{\varepsilon \times d \times v} \times F \text{ (mmol/l)}$$

Where C = concentration of acetoacetate in sample mmol

ΔA = corrected change in extinction

V = assay volume (ml)

v = sample volume (ml)

d = light path (cm)

ε = extinction coefficient ($cm^2/\mu M = 6.22$)

F = dilution factor.

Determination of plasma hormone concentrations. Techniques used to determine the concentration of hormones in blood relied on the use of radioimmunoassay (RIA) procedures. RIA is based on the ability of a limited quantity of anti-hormone antibody to bind a

fixed amount of radiolabelled antigen (tracer), and on the inhibition of this reaction by an unlabelled antigen (standard or sample hormone), the percentage of bound radiolabelled antigen is reduced with increased concentrations of unlabelled test sample antigen. Separation of bound and free radiolabelled antigen is necessary for quantitative determination of unlabelled antigen. This is achieved through the precipitation of the antigen-antibody complexes, by chemical means (polyethyleneglycol - PEG), by the addition of a second antibody directed towards the immunoglobulin present in the original antiserum, or by both. The quantity of unlabelled antigen (hormone) in a sample is determined by comparison of the radioactivity of the precipitate, after centrifugation, with values derived from known standards assayed using the same system.

Plasma cortisol. Plasma cortisol concentration was determined using a ^{125}I radioimmunoassay kit (Corning Medical, Medfield, USA). Twenty-five µl portions of lyophilized, defribinated human plasma containing various concentrations of cortisol (Cortisol Standards, Corning, Medfield, USA), cortisol standard or plasma sample were mixed with 100 µl of ^{125}I-labelled cortisol tracer (< 2 µCi) dissolved in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and 8-anilino-naphthalene sulphonic acid (ANS). ANS was used to release cortisol from its corticosteroid binding globulin. One ml of an ovine anti-cortisol antibody covalently bound to glass beads (Corning, USA) and suspended in acetate-buffered saline with BSA was added and vortex mixed for 4 seconds. The tube was incubated for 90 minutes at room temperature, centrifuged at 3000g for 10 minutes and the supernatant removed. The rate of

emission from the remaining precipitate was determined in counts per minute (CPM) using a LKB gamma-counter. From these results the relative % bound cortisol ($B/_{Bo}$) for the controls and standards were calculated.

$$\text{Relative \% bound cortisol} = \frac{\text{Mean CPM of standard}}{\text{Mean CPM of 0ng/ml standard}} \times 100$$

A standard curve of relative % bound cortisol against cortisol concentration (ng/ml) was used for calculation of the results for samples.

Plasma glucagon. Determination of the concentration of pancreatic glucagon in aprotinin de-activated blood plasma was by ^{125}I glucagon radioimmunoassay (Cambridge Medical Diagnostics Inc, Bournemouth). The technique is best described by a procedural flow chart (Table II(4)). Assay buffer contained 0.05 M phosphate buffer with 0.1% human serum albumin (HSA) and trasylol-enzyme inactivator at pH 7.4. Glucagon antiserum (rabbit) was prepared by reconstitution with assay buffer as was normal rabbit serum prior to mixing with ^{125}I porcine glucagon containing 1.0 µCi ^{125}I. The second antibody (goat anti-rabbit gamma globulin) was reconstituted by the addition of 10 ml assay buffer, and the glucagon controls and standards prepared by dilution with 1.5 ml redistilled water.

For duplicate tubes, the mean non-specific binding counts (NSB) were determined and subtracted from the maximum binding counts (B_o), standards, controls and samples to determine the average net counts. The mean net B_o counts were divided by the standard control and sample counts and the ratio of binding to maximum binding ($B/_{Bo}$) calculated.

Table II(4): Procedural flow chart for ^{125}I glucagon assay

	BUFFER µl	STANDARDS µl	CONTROL PLASMA µl	SAMPLE PLASMA µl	TRACER µl	GLUCAGON ANTISERUM µl		SECOND ANTIBODY µl		SALINE ml	
T.C.[1]	-	-	-	-	100	-		-		1.0	
N.S.B.[2]	300	-	-	-	100	-		100		1.0	
B_o[3]	200	-	-	-	100	100		100		1.0	
STANDARDS	-	200	-	-	100	100		100		1.0	
LOW CONTROL	-	-	200	-	100	100		100		1.0	
HIGH CONTROL	-	-	200	-	100	100		100		1.0	
SAMPLE	-	-	-	200	100	100		100		1.0	
							INCUBATE AT 4°C FOR 24h		INCUBATE AT 20°C FOR 2h		CENTRIFUGE AT 3000g FOR 15 MINUTES DECANT SUPERNATANT AND GAMMA-COUNT REMAINING PLUG FOR 60 SECONDS

1. Procedure designed to measure total counts. 2. Procedure designed to measure non-specific binding. 3. Procedure designed to measure maximum binding.

Percentage $B/_{Bo}$ for each standard was plotted against standard concentrations (0.1, 0.25, 0.35, 0.75, 1.3, 2.2, 4.0 mg/ml) on semi-log graph paper to derive a standard curve from which sample glucagon concentrations were determined.

Plasma insulin. Determination of plasma insulin using RIA was by the method of Vernon et al., (1981). Iodination of rat insulin preceded the assay (Madon et al., 1984) and was carried out by the addition of 10 µl, of 0.5 M phosphate buffer (pH 7.2), 5 µg hormone, 500 µCi ^{125}I and 15 µl of 2% w/v glucose to 25 µl of lactoperoxidase-impregnated Enzymobeads (Bio-Rad Laboratories, Richmond, California). The addition of 200 µl sodium azide after 15 minutes arrested the reaction prior to centrifugation at 1000g for 2 minutes. The supernatant was transferred to a Sephacryl S-200/Sephadex G10 column. Two-hundred µl of KI (10 mg/ml) in PBS containing 1% BSA was added to the beads. They were then re-centrifuged and the supernatant transferred to the column once more. The column was eluted with 0.5% BSA:PBS containing 0.1% sodium azide at a flow rate of 6 ml/hour whilst 0.5 ml aliquots of effluent were collected and counted using a LKB Gamma Counter. Ten µl samples of the 6 aliquots with highest counts were then each mixed with 25 µl of 40% trichloroacetic acid (TCAA) to precipitate marked hormone prior to decantation of free ^{125}I and recounting for the calculation of % iodination.

The iodinated insulin was utilized in the procedure shown in Table II(5). The 'first antibody' was an antiserum to bovine insulin-GP-3 capable of cross-reaction with rat insulin. Stored at $-20^{o}C$ in a diluted form (1:1000 distilled water), the antibody was

Table II(5): Procedural flow chart for ^{125}I insulin assay

	FIRST ANTIBODY µl	RIA BUFFER µl	STANDARD µl	SAMPLE µl	INCUBATE OVERNIGHT AT 4°C	^{125}I INSULIN	INCUBATE OVERNIGHT AT 4°C	SECOND ANTIBODY	INCUBATE AT ROOM TEMPERATURE FOR 6h. CENTRIFUGE 3000g FOR 30 MINUTES. REMOVE SUPERNATANT (EXCEPT TOTAL COUNT) AND GAMMA-COUNT PELLET
BLANK	-	200	-	-		100		300	
T.C.[1]	-	-	-	-		100		-	
ZERO	100	100	-	-		100		300	
STANDARDS	100	-	100	-		100		300	
SAMPLE	100	-	-	100		100		300	

1. T.C. Procedure designed to measure total counts.

defrosted and a 100 µl aliquot further diluted with 20.0 ml RIA buffer (0.5% BSA:PBS at pH 7.4). Proprietary insulin standards were diluted with RIA buffer to produce solutions containing 0, 0.16, 0.31, 0.625, 1.25, 2.5 and 5 mg insulin/ml. ^{125}I-insulin was diluted with RIA buffer so that 100 µl produced 10,000 gamma-counts per minute. The 'second antibody' was anti-guinea pig precipitating serum. Fifteen ml RIA buffer was mixed with 140 mg EDTA and the pH adjusted to 7.4 prior to the addition of 10 µl normal guinea pig serum, 120 µl anti-guinea pig precipitating serum and 15 ml, 16% PEG in order to provide a second-antibody reaction medium. A standard curve of gamma counts per minute corrected for blank against insulin concentration was used to determine the insulin concentration of the test samples.

Plasma growth hormone. The determination of plasma growth hormone (GH) was similar to that for the insulin RIA of Vernon et al. (1981). Iodination of growth hormone was as described previously for insulin iodination (Madon et al., 1984), and the subsequent assay procedure is shown in Table II(6).

The 'first antibody' was an antiserum to ovine GH (anti-OGH-2) raised in rabbits. It was stored at -20^{o}C in a diluted form (1:100 in distilled water) and defrosted prior to the dilution of a 100 µl aliquot with 30 ml RIA buffer (0.5% BSA:PBS at pH 7.4) for use. Proprietary GH standards were diluted with RIA buffer to provide GH standards containing 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 ng/ml. ^{125}I-ovine GH was diluted with RIA buffer so that 100 µl produced 10,000-20,000 gamma CPM. The 'second antibody' was anti-rabbit precipitating serum. Fifteen ml RIA buffer was mixed

Table II(6): Procedural flow chart for ^{125}I growth hormone assay

	FIRST ANTIBODY µl	RIA BUFFER µl	STANDARD µl	SAMPLE µl	INCUBATE AT ROOM TEMPERATURE FOR 4h	^{125}I-GH µl	INCUBATE AT ROOM TEMPERATURE OVER-NIGHT	SECOND ANTIBODY µl	INCUBATE AT ROOM TEMPERATURE FOR 2h. CENTRIFUGE 3000g FOR 30 MINUTES. REMOVE SUPERNATANT (EXCEPT TOTAL COUNT) AND GAMMA-COUNT REMAINING PELLET.
BLANK	-	200	-	-		100		300	
T.C.[1]	-	-	-	-		100		-	
ZERO	100	100	-	-		100		300	
STANDARDS	100	-	100	-		100		300	
SAMPLE	100	-	-	100		100		300	

1. Procedure designed to measure total counts.

with 140 mg EDTA and the pH adjusted to 7.4 prior to the addition of 5 µl normal rabbit serum, 125 µl anti-rabbit precipitating serum and 15 ml 16% PEG to provide a second-antibody reaction medium.

A standard curve of gamma counts per minute corrected for blank against GH concentration was used to determine the plasma GH concentration of test samples.

Plasma glutamic oxaloacetic transaminase (GOT). In a GOT catalysed, reversible reaction α-oxoglutarate and L-aspartate give rise to L-glutamate and oxaloacetate. Oxaloacetate, in the presence of malate dehydrogenase (MDH), is reduced by NADH resulting in the formation of L-malate and NAD^+. The rate of change in absorbance at 340 nm due to the oxidation of NADH to NAD^+ is used to determine the activity of GOT in the sample.

One Boehringer GOT test combination tablet was dissolved in 3.0 ml of phosphate buffer (80 mmol) at pH 7.4, containing L-aspartate (200 mmol/l). This resulted in the formation of a composite reaction medium containing 0.6 iu/ml MDH, 1.2 iu/ml lactate dehydrogenase (LDH), 0.18 mmol/l NADH and 12 mmol/l α-oxoglutarate. A 0.5 ml aliquot of sample was vortex-mixed with 3.0 ml of the reagent at room temperature. After approximately 60 seconds absorbance at 340 nm was determined against an air blank. Absorbance was redetermined at precisely 60, 120 and 180 seconds later. The mean change in absorbance (ΔA) per minute was calculated and multiplied by a factor of 1111 (specified by Boehringer Corp.) to derive the GOT activity in the sample. Activity was then corrected for temperature by multiplying by a factor of 2.08 (specified for temperatures between 25°C and 37°C).

Plasma L-γ-Glutamyltransferase (γ-GT). Plasma γ-GT activity was determined by the method of Persijn and Slik (1976). γ-GT catalyses the reaction of L-γ-glutamyl-3-carboxy-4-nitroanilide (GCN) with glycylglycine (GG) which leads to the production of L-γ-glutamylglycyclglycine and 5-amino-2-nitrobenzoate. The resultant rate of change in absorbance at 405 nm is used to determine the γ-GT activity in the sample.

A substrate/glycylglycine tablet (Boehringer, 1976) was dissolved in 2.0 ml of tris-buffer (100 mmol) at pH 8.5 to produce a reagent solution comprising 2.9 mmol/l GCN and 100 mmol/l GG. A portion of sample (0.2 ml) was vortex-mixed with the reagent and the absorbance at 405 nm immediately determined using a Gilson, 240G spectrophotometer and redetermined after 60, 120 and 180 seconds. The product of mean change in absorbance per minute and a conversion factor (1158) gave γ-GT activity in the sample which, further corrected for temperature resulted in plasma γ-GT activity.

Sorbitol dehydrogenase. Determination of plasma sorbitol dehydrogenase (SDH) activity was based on the work of Gerlach (1957) and Gerlach and Schurmeyer (1960). SDH catalyses the reversible reaction between fructose and NADH which yields NAD^+ and sorbitol. The change in spectrophotometric absorbance at 340 nm is proportional to NAD^+ production and SDH activity

A 1.0 ml aliquot of sample was added to 2.0 ml of 1.12 M triethanolamine buffer at pH 7.4 and 1.0 ml of 0.18 mM/l NADH. The mixture was incubated for 45 minutes at 25°C prior to the addition of 0.2 ml of 102 mM/l fructose solution. Absorbance at 340 nm was

measured immediately using Gilson G240 spectrophotometer and remeasured precisely 10 minutes later. The change in absorbance was related to plasma SDH activity (iu/l) by means of prepared tables (Boehringer, 1976).

Plasma mineral ion analyses

Plasma calcium, magnesium and copper. The determination of plasma calcium, magnesium and copper concentration was by atomic absorption spectrophotometry (see Price, 1972). Assay mixtures containing 0.1 ml plasma and 0.5 ml lanthanum chloride (1% W/V) solution diluted to 5.0 ml with de-ionised water, were injected into the air/acetylene flame of an Instrumentation Laboratory AA/AE atomic absorption spectrophotometer (Model 157). The absorption of light passing through the ensuing atomic vapour was determined against blanks at 422.7, 285.2 and 324.7 nm wavelengths respectively for Ca, Mg and Cu and was compared with the absorption of proprietary standard solutions (British Drug Houses Ltd., Poole, Dorset) for the evaluation of the mineral content of the samples.

Plasma phosphate. The determination of phosphate concentration relied on the molybdate/vanadate reaction described by Zilversmit et al. (1950). Phosphate reacts with molybdate and vanadate in nitric acid to give a coloured complex, the absorbance of which is proportional to the phosphate concentration.

A sample of plasma (0.2 ml) was vortex-mixed with 2.0 ml trichloroacetic acid (1.2 M) and centrifuged at 3000 g for 10 minutes. One ml of the sample supernatant or of trichloroacetic acid (blank) were each mixed with 1.0 ml of a solution of ammonium

vanadate (21.0 mM) and nitric acid (0.28 N), and 1.0 ml of a solution of ammonium molybdate (40.0 mM) and sulphuric acid (2.5 N) (Boehringer, 1976). The reactants were vortex-mixed, and allowed to stand for 10 minutes at room temperature. The absorbance at 405 nM was determined against the blank using a SP6-200 spectrophotometer (Pye-Unicam, Cambridge) and multiplied by a correction factor (13.6 mM, Boehringer, 1976) to determine the phosphate concentration of the original sample.

Infusates

Collection and analysis. The requirement for infusate collection and analysis was minimised by the use of uniform, pre-assayed proprietary chemicals. However, 10 ml aliquots were taken from individual caseinate and lipid stock mixtures for total nitrogen analysis using the Macro-Kjeldahl technique previously described.

SECTION III

EXPERIMENTAL

SECTION III

EXPERIMENTAL SECTION

General Introduction

Glucose utilization, together with the relative contribution of different precursors for gluconeogenesis depends on various animal, dietary and environmental factors. Quantitative estimates of glucose-precursor supply to the tissues have largely been derived from short-term, tracer-kinetic studies. The problems involved with isotopic procedures together with the limited scope for perturbation of precursor supply afforded by conventional means of manipulation of the diet have been discussed in the Introduction. These factors restrict the range of investigations for which the techniques may be used.

In view of the above limitations an attempt was made to estimate glucose requirements by alternative means. It was considered that this might be done using the complete intragastric infusion procedure to perturb the supply of exogenous glucose-precursors. With a normally-fed animal, shortfalls in such exogenous glucose-precursors as propionate or amino acids are off-set by gluconeogenesis, predominantly from amino acids and triacylglyceride glycerol from the tissues. In such circumstances, however, it is difficult to differentiate between the quantity of glucose used for the animal's absolute needs and that used as a non-specific energy substrate. It is equally as difficult to determine the precise nature of the precursor supply. Theoretically, it would appear that these problems could be overcome or avoided by

use of intragastric infusion to effectively restrict exogenous glucose-precursor supply to protein alone whilst sustaining above-maintenance energy status. Lipolysis with the associated release of glycerol would then be limited and circumstances would be created where the predominant glucose precursor would be protein. The protein catabolism occurring to meet the animal's glucose needs might then be determined from N excretion. This, in turn, could be related to the quantity of glucose which could be formed from deaminated amino acids. Potentially the procedure might also be used to compare the gluconeogenesity of selected substrates on the basis of their ability to 'spare' protein.

EXPERIMENT 1

Introduction

Initially a series of preliminary trials (Experiment 1a) were conducted to investigate the effects on non-pregnant, non-lactating dairy cows of the intragastric infusion of their nutrient requirements. This was done using the technique described by Ørskov et al. (1979). Experiment 1a was also designed to evaluate and develop the technique for use at the Hannah Research Institute. This took approximately 5 months and involved the sustenance of 3 cows for a total period of 140d.

The work undertaken culminated in a further experiment (Experiment 1b) designed to test modifications made to the infusion procedure during Experiment 1a together with an assessment of the metabolic consequences of restricting exogenous glucose-precursor supply.

Experimental

Animals and their management.

Experiment 1a. Three non-pregnant, non-lactating Freisian cows (Nos 26, 317 and 257) of LW 460 to 500kg were surgically modified and allowed to convalesce. They were then introduced to complete intragastric infusion over a 10d period (see 'Changeover procedure', Section II).

Experiment 1b. Cow 257, on completing Experiment 1a was used in a further experiment designed to test the modifications made to the technique resulting from the findings of Experiment 1a and to

assess the effects of perturbation of nutrient supply on N utilization.

Experimental plan and procedures

Experiment 1a. Infusions comprising 650:250:100mmol/mol VFA mixture (acetate:propionate:butyrate), (Ac:Pr:Bu), sodium caseinate, emulsified tallow, buffer, vitamins and minerals were formulated. These were intended to supply metabolizable energy (ME) at 495kJ/kgW$^{0.75}$ per d (1.1 x the energy required to meet maintenance requirements) and 420mg casein N/kgW$^{0.75}$ per d supplied to the tissues (1.0 x the casein N required to meet tissue N requirements for maintenance) (see Ørskov et al., 1979; Macleod et al., 1982). Vitamins and minerals were also infused to meet requirements and buffer was infused in quantities calculated to maintain the pH balance of the rumen (see 'Infusion formulation', Section II).

Rumen liquor was sampled twice-daily and samples of blood were taken at 3-day intervals. Urine and faeces collections were made daily and the sodium caseinate and lipid solutions were sampled periodically.

Experiment 1b. Cow 257 was subjected to the same experimental procedures as are described above. The quantities of nutrients that were initially infused (see Table III(1)). Protein adjustment period), resulted in a positive N retention. This was reduced to approximately 0g/d by reducing the supply of casein N to the tissues from 420mg to 370mg/kgW$^{0.75}$ per d. This quantity of N was subsequently adopted as the approximate maintenance requirement of the tissues for N. Following a control period (P1) of 6d in which

Table III(1): Experiment 1b. The quantities of stock solution (g/d) infused into Cow 257.

[1]Stock solutions infused	Protein adjustment period	Control period P1	Propionate withdrawal period P2	Final control period P3
[2]VFA (650:250:100)	3624	3858	-	3858
(860:0:140)	-	-	2730	-
[3]Sodium caseinate	3990	2560	2560	2560
Duration (d)	12	6	5	3

Throughout the experiment 3600g lipid, 12000g buffer, 9500g major minerals and 130ml of the trace mineral stock solution was infused per d. 1. Composition of stock solutions are shown in Section II. 2. mmol/mol. 3. The vitamin allowance was mixed with the sodium caseinate stock solution.

this level of N was infused the 650:250:100 mmol/mol VFA mixture was replaced with a 860:0:140 mmol/mol mixture (P2; 5d) prior to the reintroduction of the initial mixture (P3; 3d). Throughout the experiment the infusions were isoenergetic and isonitrogenous.* Vitamin and mineral infusions were also continued throughout. Sampling of infusates and body fluids were as described in Experiment 1a, except that 2-hourly blood sampling over the period 1000h to 2200h was undertaken on days immediately before and during the isoenergetic withdrawal of propionate from the infusions (P2).

Chemical Analysis. The sodium caseinate and lipid stock solutions were analysed for their N content as were 500ml sub-samples of speed-mixed urine and feaces slurry.

Blood samples in Experiment 1a were analysed for the concentrations of calcium (Ca), magnesium (Mg), phosphorus (P), urea, glucose and 3-OH butyrate in plasma. Blood samples from Experiment 1b were analysed for the concentration of glucose.

Statistical analysis. Data from Experiment 1a was summarised by calculation of means and standard errors for the observations. Experiment 1b was subject to completely randomised analysis of variance (ANOVA). The statistical significance between the differences in treatment effects was determined by F-test whilst the significance of the differences between individual means was by t-test.

*Refer to Appendix 55.

Results

Experiment 1a. During Experiment 1a cow 257 underwent two periods of intragastric infusion lasting 18d and 47d. Cow No. 317 received nutrients by infusion for 20d whilst cow no. 26 was sustained for a period of 55d. In each case the infusions were terminated following operational breakdowns in the infusion system. This led to the animals becoming metabolically unstable as judged from rumen pH and osmotic pressure measurements. However, when the experiment progressed according to protocol the animals showed no signs of abnormality, other than those normally shown by animals on low-roughage diets.

A summary of the effects of the intragastric infusion of nutrients on the concentration of blood metabolites in the cows are shown in Table III(2). Also shown are values from the literature for normally fed cows and cows sustained by the infusion procedure of Macleod et al. (1982).

Experiment 1b. The effects of treatments on the N excretion and glucose concentration in blood plasma are summarised in Table III(3), the raw data for which is shown (Appendix (1)). The results of more close monitoring of the glucose concentration in blood plasma during the experiment are shown in Figure III(1). During (P1) the adjusted dose of casein that was infused resulted in slightly negative N retention (Figure III(2)). The effect of isoenergetic removal of propionate (P2) was to reduce the glucose concentration in blood plasma a small degree (see Figure III(1)) and increase N excretion from 47g/d to 54g/d (Figure III(2). Subsequent reintroduction of propionate (P3) resulted in a return of the glucose

Table III(2): Experiment 1a. Comparison of the concentrations of metabolites measured in the blood of cows given normal rations and those sustained by intragastric infusions.

Item	[1]Measured value (mean ± SE)	MacLeod et al. (1982)	[2]Normal range
Cu(μmol/l)	19.62±0.62 (n=9)[3]		9.4-23.6
Ca(mmol/l)	2.35±0.09 (n=9)	2.4 ± 0.16	2.0-3.0
Mg(mmol/l)	0.93±0.04 (n=9)	1.3 ± 0.08	0.65-1.23
P(mmol/l)	1.53±0.14 (n=9)	1.8 ± 0.17	1.16-2.32
3-OH butyrate (mmol/l)	0.16±0.02 (n=9)	Negative (qualitative test)	<0.8
Urea(mmol/l)	2.18±0.25 (n=9)		2.0-6.64
Glucose(mmol/l)	4.2±0.03 (n=9)		2.2-3.3
Vitamin B(ng/l)	290±0.57 (n=6)		<20
PCV (%)	28.2±0.21 (n=9)	30 ± 3	32-35

1. Values measured during Experiment 1a. 2. Recommended 'normal' values from the Veterinary Investigation Centre (West of Scotland Agricultural College). 3. 'n' refers to the number of single observations from which the mean values were calculated. All values are means with standard errors.

Table III(3): Experiment 1b. The effect of treatments on the N excretion and blood plasma glucose concentration of Cow 257.

[1]Treatment	N excretion ($g/kgW^{0.75}$ per d)	Plasma glucose (mmol/l)
Period 1	[2]$0.46\pm0.01^{a,3}$	4.96 ± 0.15^{a}
2	0.52 ± 0.02^{b}	4.73 ± 0.34^{b}
3	0.43 ± 0.03^{a}	4.93 ± 0.17^{a}

1. Treatments are as per Table III(1). 2. Means with standard errors. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

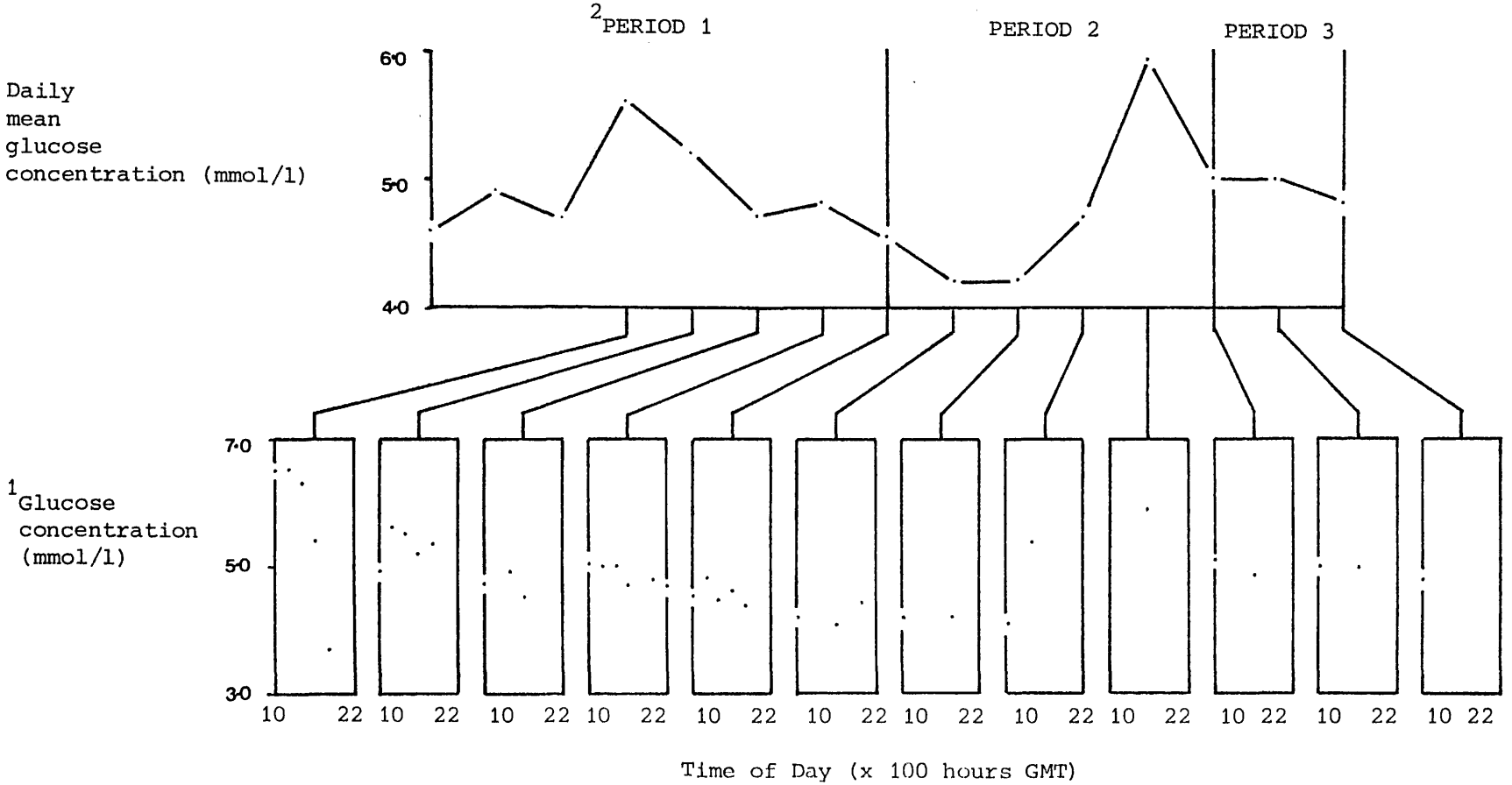


Figure III(1): Experiment 1b. The effect of glucose-precursor supply on the concentration of glucose in the blood plasma of sheep. 1. Plasma glucose concentration of spot samples (n=2) taken at specified times during the day. 2. See Table III(1) for the treatments administered.

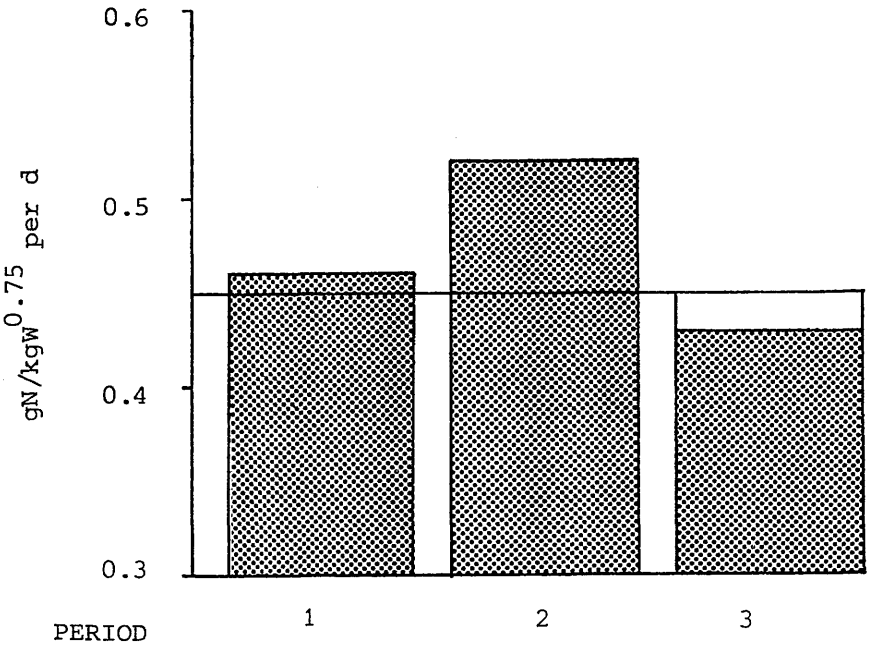


Figure III(2): Experiment 1b. N intake (—), N loss (▨) and positive N retention (□) in cow 257 (480kg LW) sustained by intragastric infusions of 462mg casein. N/kgW$^{0.75}$ per d, 180g/d tallow and VFA equivalent, in total, to 495kJ ME/KgW$^{0.75}$ per d. Minerals, buffer and vitamins infused as per Table III(1).

concentration in blood plasma to normal levels and a reduction in N excretion to the extent that protein was retained at 2.37g/d.

Discussion

The blood metabolites measured in Experiment 1a were comparable with similar measurements made in normally fed animals and those sustained by intragastric infusions of nutrients in other laboratories. It was concluded, therefore, that the substenance of the animals by the intragastric infusion of their nutrient requirements caused no physiologically adverse reactions in the short term.

In Experiment 1b the infusions during P1 resulted in a small negative retention of nitrogen (Figure III(2)). On the basis of these results it was calculated that 369mgN/kgW$^{0.75}$ per d would be required at the tissue level to maintain the animal at zero nitrogen balance. This represents a value of 38g N/d for cow 257 (480kgW).

The isoenergetic removal of propionate from the infusions (P2) resulted in a total excretion of 54g N/d (Appendix 1A) which caused the animal to suffer a net daily loss of 7.5g N/d. The total N excreted would result from the deamination of approximately 380g amino acid/d (ARC, 1980) which, if completely utilized for gluconeogenesis would yield around 220g/d or 2.15g/kgW$^{0.75}$ per d of glucose.* This quantity of glucose would represent that used when glucose-precursors are in short supply and would therefore be equivalent to the minimum glucose requirement of the animal (see Krebs, 1965; Leng, 1970a; Lusk, 1928). When propionate was reintroduced (P3) N excretion was reduced to a level at which a small

*Refer to Appendix 56.

positive retention of nitrogen occurred, even though the quantity of N infused per d was the same as in P1.

Isoenergetic removal of propionate (P2) also caused a fall in the glucose concentration measured in blood plasma (see Figure III(1)) but the fall was gradual and the overall effect was small despite its statistical significance (see Table III(3)). This level of hypoglycaemia was well within normal physiological limits, which indicated that alternative glucose sources were adequate to meet the animal's requirement. This suggests that similar treatments might be used routinely without adverse physiological reactions, at least in the short term. When propionate was re-introduced (P3) the glucose concentration of blood plasma was immediately increased to values similar to those measured during the initial control period (P1) (see Figure III(1).

The studies described above indicate that the perturbation of glucose-precursor supply was possible using intragastric nutrition and that isoenergetic withdrawal of propionate was associated with marked effects on the metabolism of N.

EXPERIMENT 2

Introduction

In Experiment 1 it was shown that the perturbation of exogenous glucose-precursor supply did not result in adverse physiological reactions. It was also shown that such perturbation could be used to estimate the glucose requirements of cows by measuring the effect on their utilization of N. In order to further investigate the effects of glucose-precursor supply on N metabolism and to increase the statistical precision of the experimental data it was decided to carry out a more extensive study with a larger group of animals.

During the operation of the intragastric infusion system in Experiment 1 difficulties were encountered. A particular problem was the metabolic instability shown by cows and to overcome this problem an automatic pH control device was developed (see Section II). This reduced the risk of instability and acidosis but it was considered that the system would initially be easier to apply and the infusions be more manageable if the volumes of infusates were smaller. Accordingly, it was decided to continue the experiments using sheep as the experimental animals.

Experimental

Animals and their management. Four Finnish Landrace X Dorset Horn wethers (nos. 463, 466, 467 and 468) of LW 55.4 $\pm$ 1.78 kg were surgically prepared and allowed to convalesce. They were then introduced to complete intragastric nutrition during a 10d transition period (see 'Changeover procedure', Section II).

Experimental plan and procedures. Infusions comprising 650:250:100mmol/mol VFA mixture (Ac:Pr:Bu), sodium caseinate and emulsified tallow (25g/d) were formulated. These were designed to provide 495kJ ME/kgW$^{0.75}$ per d (1.5 x the ME required to meet the energy requirement for maintenance) and 370mg casein N/kgW$^{0.75}$ per d supplied to the tissues (1.0 x the casein N required to meet the tissue N requirements for maintenance - Experiment 1). Minerals and vitamins were also infused to meet requirements and buffer was infused in quantities calculated to maintain the pH balance of the rumen (see 'Infusion formulation', Section II).

Measurements of N balance taken when the above quantities of nutrients were infused showed a slightly negative retention of N. Accordingly the casein dose was increased during the subsequent control period (P1) to a level calculated to allow 0g/d retention of N. This was equal to 384mg N/kgW$^{0.75}$ per d at the tissues which was provided by 490mg casein N/kgW$^{0.75}$ per d from the caseinate infusion if an overall efficiency of utilization of casein N of 80% was assumed. Throughout the subsequent experimental treatments 480mg casein N/kgW$^{0.75}$ per d was adopted as the casein N requirement of the animals and the equivalent quantity of sodium caseinate solution was infused.

Following the 4d control period (P1) a 860:0:140mmol/mol VFA mixture was infused which isoenergetically replaced the 650:250:100mmol/mol mixture (P2, 8d). The protein catabolism resulting from the withdrawal of propionate from the infusions (P2) was then used to calculate the theoretical minimum glucose requirement of the animals.* In four subsequent 5d periods (P3, 4, 5

*Refer to Appendix 56.

and 6) the 860:0:140mmol/mol VFA mixture was supplemented with intra-abomasal infusions of glucose which were equivalent to approximately 50, 75, 100 and 0% of the calculated minimum glucose requirement. The nutrient infusions were isonitrogenous and isoenergetic throughout the treatments (see Table III(4)). At the end of P6 the animals were reintroduced to solid food (see 'Changeover procedure', Section II).

Rumen liquor was sampled twice-daily and samples of blood were taken daily. Faeces and urine collections were made daily and the sodium caseinate and lipid solutions were sampled periodically.

Chemical Analysis. The sodium caseinate and lipid stock solutions were analysed for their N content, as were 500ml sub-samples of speed-mixed urine and faeces.

The packed cell volume (PCV) % of whole heparinized blood was measured prior to the separation of blood plasma and measurement of the concentration of glucose, urea, 3-OH butyrate and non-esterified fatty acids (NEFA) therein. Samples of blood were taken at the end of selected periods (P1, 2, 5 and 6) and the quantities of amino acid, Ca, Mg, P and Cu were measured. Amino acid analysis included determination of the concentrations of aspartate, threonine, serine, glutamate, proline, glycine and citrulline combined, alanine, cystine, valine, methionine, isolucine, leucine, tyrosine, phenylalanine, histidine, ornithine, lysine, arginine and total amino acids in blood plasma.

Statistical analysis. All data were subject to ANOVA for randomised block designs. An ANOVA for completely randomised block designs was

Table III(4): Experiment 2. Treatment infusions administered to 4 wethers of 55.4±1.78 kg LW.

Period	Duration (d)	Infusion	
		Ac:Pr:Bu (mmol/mol)	Glucose (g/d)
1	4	650:250:100	0
2	8	860: 0 :140	0
3	5	860: 0 :140	26
4	5	860: 0 :140	38
5	5	860: 0 :140	52
6	5	860: 0 :140	0

The sheep were sustained by the above infusates with the addition of 480mg casein $N/kgW^{0.75}$ per d and 25g/d emulsified tallow equivalent, in total, to 495 kJ $ME/kgW^{0.75}$ per d. Minerals, buffer and vitamins were infused to requirement (see 'Infusion formulation', Section II).

used to summarise the amino acid data. The remaining data, i.e. the metabolites other than amino acids were subject to Duncan's multiple range testing.

Results

N Utilization. The effects of treatments on the quantity of N excreted by the sheep are shown in Figure III(3). The daily data from which the results are derived are shown in Appendices 2, 4, 6 and 8.

During the initial control period (P1) the quantity of casein N required to maintain the sheep at 0g/d N retention was 480mg casein N/kgW$^{0.75}$ per d. The isoenergetic withdrawal of propionate from the infusions (P2) resulted in an increase in N excretion by the sheep from 0.48 $\pm$ 0.02 to 0.61 $\pm$ 0.04g N/kgW$^{0.75}$ per d which resulted in a mean net N loss of approximately 2.64g N/d (Figure III(3)). A minimum glucose requirement of 2.47 $\pm$ 0.12g glucose /kgW$^{0.75}$ per d was calculated from the N excretion measured in P2 on the basis that 1g catabolised protein was equivalent to 0.58g glucose.* This quantity of glucose was then used as the basis for the glucose doses used in P3, 4 and 5.

The isoenergetic re-introduction of glucose in these graded doses (see Figure III(3)) during P3, 4 and 5 resulted in incremental reductions in N excretion (i.e. 0.5, 0.44 and 0.42g/kgW$^{0.75}$ per d respectively). In P5, 52g glucose/d was infused. This was approximately equal to the value of minimum glucose requirement which was calculated from the N excreted in P2 but the infusion resulted in the retention of 1.42g N/d. The subsequent isoenergetic withdrawal

*Refer to Appendix 56.

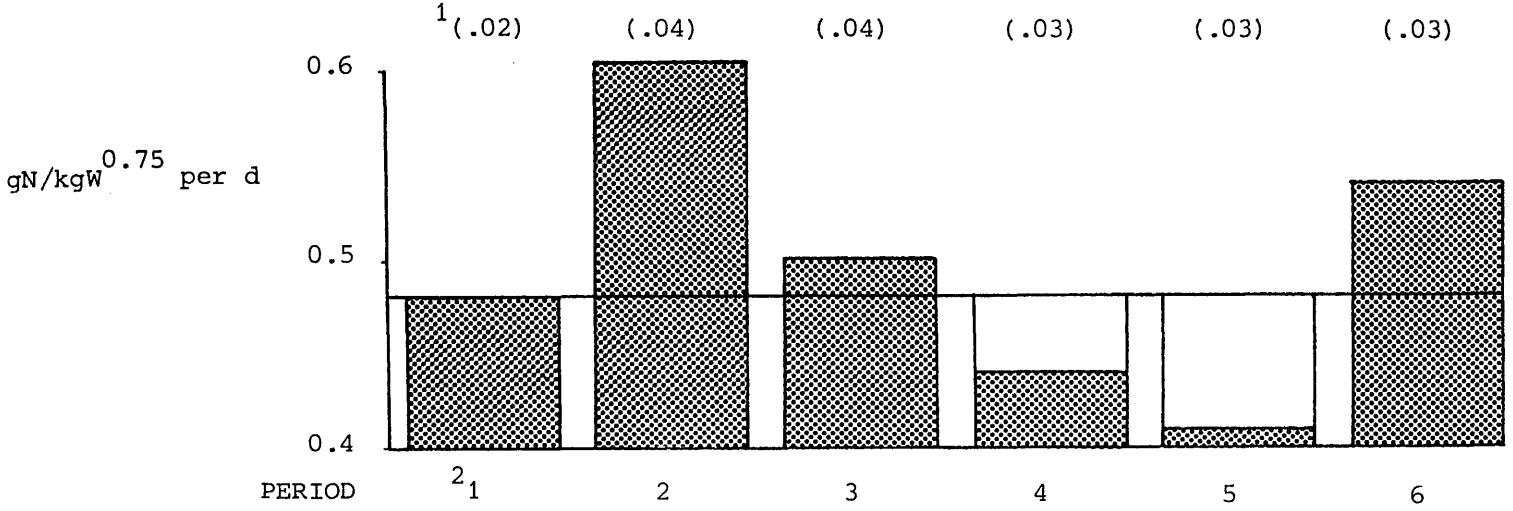


Figure III(3): Experiment 2. N intake (—), N loss (▨) and positive N retention (□) in 4 wethers (LW 55±1.78kg) sustained by intragastric infusions of 480mg casein N/kgW$^{0.75}$ per d, 25g tallow and VFA equivalent, in total, to 495 kJ ME/kgW$^{0.75}$ per d. Minerals, buffer and vitamins were infused to requirement (see 'Infusion formulation', Section II). 1. SEM of N loss. 2. Refer to Table III(4) for treatments.

of propionate (P6) was associated with an increase in N excretion to 0.54 ± 0.03g $N/kgW^{0.75}$ per d which was equivalent to the net loss of 1.22g N/d as compared to 0.61g $N/kgW^{0.75}$ per d during P2.

Amino acids. The effects of the treatments in which adequate glucose-precursors were supplied by the infusions (i.e. P1 and P5) and those in which glucose-precursor supply was restricted (i.e. P2 and P6) are shown in Tables III(5), (6), (7) and (8). The data from which these results are derived are shown in Appendices 10 and 11.

Non essential amino acids (NEAA). The isoenergetic withdrawal of propionate from the infusions (P2) had a marked effect on the concentrations of the NEAA measured in blood plasma (Table III(5)). There was a 55% reduction in alanine concentration ($P < 0.01$) together with a 45% reduction in serine concentration. Smaller reductions (< 20%) were also measured in citruline and glycine, proline and aspartate concentrations. The concentration of glutamate remained constant whilst the concentrations of cystine and ornithine tended to increase.

The differences in the concentrations of NEAA's measured during P5 and P6 were largely of the same order as those measured in P1 and P2. However, the concentrations of cystine and ornithine measured during P6 tended to be lower than in P5.

Essential amino acids (EAA). The isoenergetic withdrawal of propionate from the infusions (P2) had marked effects on the concentrations of EAA measured in the blood plasma (Table III(6)). There were substantial increases in the concentrations of valine ($P <$

Table III(5): Experiment 2. The effects of treatments on the concentration (μmol/100ml) of non-essential amino acids measured in the blood plasma of sheep.

Amino acid (μmol/100ml)	Treatment Period[1]				
	1	2	5	6	SEM
Alanine	[2]22.21[a3]	10.02[b]	13.99[b]	8.15[b]	1.94
Citrulline & Glycine	69.40	51.00	80.00	61.00	9.68
Serine	7.80	4.30	12.60	6.60	2.95
Cystine	0.75	0.91	0.84	0.82	0.16
Glutamate	28.20	26.00	24.70	21.10	2.32
Proline	8.10	6.70	10.20	9.20	0.95
Aspartate	1.50	1.20	1.50	1.10	0.16
Ornithine	10.50	11.20	12.60	10.20	1.45

Measurements taken from 4 wethers (55.4±1.78kg LW) sustained by intragastric infusions of nutrients. 1. Refer to Table III(4) for treatments. 2. Mean values of 4 sheep. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(6): Experiment 2. The effects of treatments on the concentrations (μmol/100ml) of essential amino acids measured in the blood plasma of sheep.

Amino acid (μmol/100ml)	Treatment period[1]				
	1	2	5	6	SEM
Arginine	[2]5.90	9.10	7.40	7.40	1.37
Histidine	7.70	9.90	7.60	8.50	0.64
Methionine	1.70	1.30	1.40	1.00	0.16
Valine	14.48[a3]	32.59[b]	24.75[b]	29.73[b]	2.65
Threonine	4.90	5.50	5.10	5.10	0.98
Isoleucine	5.80	9.90	8.80	8.50	1.43
Phenylalanine	4.50[a]	5.23[a]	4.48[a]	3.37[b]	0.35
Tyrosine	3.46[a]	3.47[a]	2.95[ab]	2.17[b]	0.26
Leucine	11.40	19.50	13.90	16.20	2.43
Lysine	10.60	10.70	9.90	9.40	1.36
Total amino acid concentration	218.90	218.40	245.40	209.70	9.16

Measurements taken from 4 wethers (55.4±1.78kg LW) sustained by intragastric infusions of nutrients. 1. Refer to Table III(4) for treatments. 2. Mean values of 4 sheep. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(7): Experiment 2. The effects of treatments on the proportions (mol/100mol) of non-essential amino acids measured in the blood plasma of sheep.

Amino acid (mol/100mol)	Treatment Period[1]				
	1	2	5	6	SEM
Alanine	[2]10.22[a3]	4.55[b]	5.71[be]	3.89[bd]	0.87
Citrulline & Glycine	31.69	23.06	32.33	29.17	3.57
Serine	3.54	1.96	5.11	3.18	1.26
Cystine	0.34	0.42	0.34	0.39	0.06
Glutamate	12.83	11.91	10.16	10.07	0.98
Proline	3.73	3.04	4.19	4.40	0.45
Aspartate	0.70	0.54	0.60	0.54	0.07
Ornithine	4.82	5.11	5.09	4.86	0.56

Measurements taken from 4 wethers. (55.4±1.78kg LW) sustained by intragastric infusion of nutrients. 1. Refer to Table III(4) for treatments. 2. Mean values of 4 sheep. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(8): Experiment 2. The effects of treatments on the proportions (mol/100mol) of essential amino acids measured in the blood plasma of sheep.

Amino acid (mol/100mol)	Treatment period[1]				
	1	2	5	6	SEM
Arginine	[2]2.71	4.22	3.09	3.53	0.62
Histidine	3.53	3.23	3.13	4.05	0.52
Methionine	0.78	0.61	0.60	0.50	0.07
Valine	6.64[a3]	15.15[b]	11.20[bc]	14.11[bd]	1.61
Threonine	2.22	2.52	2.09	2.42	0.43
Isoleucine	2.63	4.55	3.61	4.05	0.61
Phenylalanine	2.05[bc]	2.39[b]	1.85[ac]	1.60[ac]	0.16
Tyrosine	1.58[b]	1.60[b]	1.21[ab]	1.03[a]	0.13
Leucine	5.18	9.03	5.63	7.70	1.02
Lysine	4.83	4.89	4.07	4.50	0.54

Measurements taken from 4 wethers (55.4±1.78kg LW) sustained by intragastric infusions of nutrients. 1. Refer to Table III(4) for treatments. 2. Mean values of 4 sheep. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

0.01), arginine, leucine, isoleucine and histidine with smaller increases measured in phenylalanine and threonine. The concentrations in blood plasma of lysine and tyrosine tended not to respond to propionate withdrawal (P2) whilst the mean concentration of methionine tended to be reduced slightly. This trend of change was repeated in P6 except that no changes were observed in the concentrations of arginine and threonine and there was a slight reduction in the concentration of lysine in comparison to the values measured in P5.

Total amino acid concentration. The isoenergetic removal of propionate from the infusions (P2) had little effect on the total plasma amino acid concentrations. The same treatment administered later (P6) did, however, result in the total concentration of amino acids measured in the blood plasma being reduced. This response to propionate withdrawal was not statistically significant ($P > 0.05$).

Amino acid composition. As a result of the small and statistically insignificant differences measured between the mean plasma total amino acid concentrations measured during the treatments, changes in proportions of amino acids closely reflected the changes in absolute concentrations that were measured (Tables III(7) and (8)).

Blood Metabolites. During the control period (P1) the metabolites measured (Table III(9)) were found to be within the normal physiological range (Appendix 54). The isoenergetic withdrawal of propionate from the infusions (P2) resulted in some marked changes. There was a slight reduction in blood packed cell volume and in the concentration of glucose in blood plasma ($P < 0.01$) together with

Table III(9): Experiment 2. The effect of treatments on N excreted, blood PCV% and the concentration of glucose, 3-OH butyrate, NEFA and urea in the blood plasma of sheep.

Metabolite	Treatment Period[1]						
	1	2	3	4	5	6	SEM
Total N output ($g/kgW^{0.75}$ per d)	[2]0.48^{bc}[3]	0.61^{e}	0.50^{cd}	0.44^{ab}	0.41^{a}	0.54^{d}	0.01
Glucose (mmol/l)	4.02^{d}	3.00^{ab}	3.42^{abc}	3.57^{bcd}	3.78^{cd}	2.90^{a}	0.18
3-OH butyrate (mmol/l)	0.32^{a}	2.98^{c}	1.81^{b}	1.11^{ab}	0.90^{ab}	3.75^{c}	0.35
NEFA (mmol/l)	0.47^{a}	0.95^{b}	0.67^{a}	0.57^{a}	0.52^{a}	0.96^{b}	0.09
Urea (mmol/l)	2.50^{a}	3.80^{b}	2.26^{a}	2.23^{a}	2.18^{a}	2.74^{a}	0.30
PCV %	29.47^{c}	27.46^{b}	25.64^{a}	25.90^{ab}	25.45^{a}	24.95^{a}	0.54

Values measured in 4 wethers (LW 55.4±1.78kg) sustained by the intragastric infusion of nutrients. 1. Refer to Table III(4) for treatments. 2. Mean values of 4 sheep. 3. Within-parameter means with like or no superscripts are not significantly different ($P > 0.05$).

large increases in the concentrations of 3-OH butyrate ($P < 0.01$), NEFA ($P < 0.01$), and urea ($P < 0.01$) (Table III(9)). Changing the level of glucose infusion resulted in 'step-wise' increases in plasma glucose concentration and associated reductions in 3-OH butyrate, NEFA and urea. In the final period (P6) in which propionate was isoenergetically withdrawn, a response similar to that observed in P2 was measured. There was a small reduction in plasma glucose concentration together with marked increases in plasma 3-OH butyrate and NEFA concentration. The concentration of urea measured in the blood plasma also increased but to a level that was significantly lower ($P < 0.05$) than that measured in P2. The daily data from which these results were taken are found in Appendices 2 to 9.

Discussion

The quantity of N calculated to meet the tissue needs of the sheep in the control period (P1) was 384mg $N/kgW^{0.75}$ per d. This was similar to the equivalent requirement for cows (370mg $N/kgW^{0.75}$ per d) which was calculated in Experiment 1a.

The isoenergetic withdrawal of propionate from the infusions which occurred in P2 and P6 had different effects on N utilization in the sheep. The quantity of N excreted in P6 presumably, as a result of the deamination of amino acids for gluconeogenesis was approximately 10% less than that excreted in P2. This together with the fact that the rate of N retention measured in P5 was higher than in P1 (Figure III(3)), despite constant N infusions suggested that an increase in the efficiency of casein N utilization had occurred. It appeared likely that the increase in efficiency was associated with the previous restriction of exogenous glucose-precursor supply.

Measurements of the concentrations and % composition of amino acids in blood plasma showed a general trend of reductions in NEAA's with concomitant increases in the EAA's. The majority of the NEAA in blood plasma are gluconeogenic (see 'Gluconeogenesis' Section I) and their removal from the circulating pool may be construed as circumstantial evidence for their use in gluconeogenesis (see also Bergman, 1973; Baird, 1977; and Wolf & Bergman, 1972). There is particular justification for the assumptions here because of the steady-state conditions induced in animals which are sustained by intragastric infusion of nutrients of constant composition at constant rates. The removal of the gluconeogenic NEAA's from the circulating pool would also result in a proportional increase in the concentration and % composition in blood plasma of essential amino acids which have a more restricted availability for gluconeogenesis.

It may be argued that the gluconeogenic NEAAs most affected by isonergetic propionate withdrawal from the infusions i.e. alanine, serine together with glycine and citrulline, proline and asparate would be the ones most readily available and most extensively used for gluconeogenesis. It may also be argued that those gluconeogenic amino acids least affected by the treatments imposed on the animals, of which the essential amino acids constitute the majority, are spared for more critical purposes. Methionine concentrations in blood plasma however, were seen to fall in response to propionate withdrawal. Such a response may be indicative of a specific requirement for this amino acid which is a reflection of its role as a limiting amino acid for tissue deposition (Bender, 1985).

The other metabolites measured in blood plasma responded incrementally to the treatments in which graded doses of glucose were infused (P3, 4 and 5). Glucose concentration increased in the blood plasma when compared to the concentration measured in P2 whilst plasma 3-OH butyrate, NEFA and urea decreased. The plasma urea concentration measured in P6 was significantly different to that measured in P2. A similar trend was observed with N excretion in those periods.

PCV% remained fairly constant throughout the experiment except for a significant fall during P2. PCV% was measured during the experiment in order to monitor the electrolyte balance of the animal's blood which responds to the electrolyte balance of the infusions. PCV% was also used to indicate any incidence of anaemia which may result from chronic malnutrition. Accordingly, the effects of the treatment imposed during P2 on the PCV% may be anticipated but not with a treatment of such short duration.

The response of the other blood metabolites measured to the isoenergetic withdrawal of propionate (Table III(9)) was similar to the responses shown in underfed ruminants which are mobilizing adipose tissue reserves. For these effects to be measured in animals sustained by infusions formulated to provide above-maintenance energy supply is of considerable interest and reasons for this response are discussed later.

In summary the results from this experiment point to the fact that when animals in positive energy balance suffer an inadequate glucose-precursor supply from exogenous sources they are readily able to utilize body reserves to meet their needs. This appears to occur in a selective manner in an attempt to rectify the deficiencies in

nutrient supply and in consequence there appears to be an adaptation whereby the efficiency of utilization of N is improved. If this is so it is clear that results from similar perturbative procedures designed to measure and compare the gluconeogenisity of selected infused substrates would need to be corrected to allow for the adaptation in N utilization.

EXPERIMENT 3

Introduction

In both Experiment 1 and Experiment 2 there was evidence that restriction of the supply of infused exogenous glucose-precursor (propionate or glucose) led to an adaptation in metabolism, with a sparing of the requirement for glucose and/or an increase in the efficiency of utilization of amino acids. However, the experiments provided little information about the 'chronic' or 'acute' nature of the response, about its timescale or about its possible mechanism(s). With these points in view therefore, an experiment was undertaken with the specific aim of investigating the occurrence of the adaptive response in animals subjected to a short period of complete propionate withdrawal followed by a period of persisting shortage in the availability of propionate. An extensive series of blood analyses were made, which were designed to provide information about the timescale and nature of the metabolic response. The analyses included measurements of blood plasma substrates, 'key' circulating hormones involved in the regulation of metabolism and several enzymes, whose release from the liver and plasma concentration may be used as an index of the breakdown of liver tissue.

Experimental

Animals and their management. Three Finnish-Landrace x Dorset Horn wethers (nos. 463, 466 and 468) of 71 $\pm$ 2.9kg LW were surgically prepared and allowed to convalesce. They were then introduced to complete intragastric nutrition during a 10d transition period (see 'Changeover procedure' Section II).

Experimental plan and procedures. Infusions comprising 650:250:100mmol/mol VFA mixture (Ac:Pr:Bu), sodium caseinate and emulsified tallow (25g/d) were formulated. These were designed to provide ME at 495kJ/kgW$^{0.75}$ per d (1.5 x the ME required to meet the energy needs for maintenance) and 370mg casein N/kgW$^{0.75}$ per d supplied to the tissues (1.0 x the casein N required to meet the tissue N requirements for maintenance measured in Experiment 2).

Minerals and vitamins were also infused in meet requirements and buffer was infused in quantities calculated to maintain the pH balance of the rumen (see 'Infusion formulation', Section II).

Measurements of N balance taken when the above quantities of nutrients were infused showed that the sheep were not being maintained at the intended 0g/d N retention. Accordingly, during a 5d control period (P1) the casein dose was changed to 413 $\pm$ 5mg N/kgW$^{0.75}$ per d. This would supply 330mg/kgW$^{0.75}$ per d to the tissues if an overall efficiency of utilization of casein N of 80% is assumed. Throughout the subsequent experiment this level of casein was given.

During the control period (P1, 5d) a 650:250:100mmol/mol VFA mixture was infused which was then isoenergetically replaced (P2, 8d) with a 860:0:140mmol/mol mixture. The propionate content of the infusions was then readjusted to 50% of the quantity given in P1. This was achieved by infusing a 760:120:120mmol/mol VFA mixture (P3, 32d). Finally, the propionate content of the infusions was readjusted to 100% of the quantities given in P1 (P4, 6d). The nutrient infusions were isonitrogenous and isoenergetic throughout

the treatments (see Table III(10)). At the end of P4 the animals were reintroduced to solid food (see 'Changeover procedure', Section II).

Blood was sampled at 4d intervals throughout the experiment. Faeces and urine collections were made daily and the sodium caseinate and lipid solutions were sampled periodically.

Chemical analysis. The sodium caseinate and lipid stock solutions were analysed for their N content. Sub-samples (500ml) of speed-mixed urine and faeces were analysed for N, creatine and creatinine.

The PCV% of whole heparinized blood was measured prior to the separation of blood plasma and the measurement of the concentrations of glucose, glycerol, lactate, amino acids, total protein, albumin, globulin, urea, acetate, 3-OH butyrate, acetoacetate, cortisol, insulin, glucagon, growth hormone, Ca, Mg, P, Cu, glutamic oxaloacetic transaminase (GOT), L-γ-glutamyl transferase (γ-GT) and sorbitol dehydrogenase (SDH).

Statistical analysis. All data were subject to ANOVA for experiments of randomized block design. Data from P3 were subjected to 'GENSTAT' optimized regression analysis against time (d). This was carried out with the intention of identifying possible physiological adaptations occurring in response to the isoenergetic partial-withdrawal of propionate from the infusions during that period.

Table III(10): Experiment 3. Treatments given to sheep sustained by intragastric infusions of nutrients.

Treatment period	Duration (d)	VFA molar mixture infused Ac:Pr:Bu (mmol/mol)
1	5	650:250:100
2	8	860: 0 :140
3	32	760:120:120
4	6	650:250:100

3 wethers of 71±2.9kg LW were sustained by the infusion of 413mg+5mg casein N/kgW$^{0.75}$ per d, 25g/d emulsified lipid and VFA equivalent, in total, to 495 kJ ME/kgW$^{0.75}$ per d. Minerals, buffer and vitamins were infused to requirement (see 'Infusion formulation', Section II).

Results

Animal health. All three animals completed P1 but during P2, sheep no. 463 was found dead. A post-mortem examination conducted at the West of Scotland Agricultural College, Veterinary Investigation Centre (W.O.S.A.C., V.I.C.) concluded that there were no abnormalities in the brain, heart, liver or kidneys of the animal and no obvious cause of death could be identified.

N Utilization. The effects of treatments of the N excreted by sheep 468 and 466 of LW 65.5 $\pm$ 7.8 (SD) kg are summarised in Figure III(4). The daily data from which the above results are derived are shown in Appendices 12 and 13.

During P1 the quantity of casein N required to sustain a daily retention of 0g/d was calculated to be 413 $\pm$ 5mg/kgW$^{0.75}$ per d. Subsequent N balance measurements confirmed this to be correct. Assuming the overall efficiency of utilization of N to be 80% this represents a tissue casein N requirement for maintenance of approximately 330mg casein N/kgW$^{0.75}$ per d.

The isoenergetic withdrawal of propionate from the infusions (P2) resulted in an increase in N excretion from 0.41 to 0.59g N/kgW$^{0.75}$ per d. This resulted in a net loss of N of approximately 4.1g/d. Reintroduction of propionate at 50% of the quantity infused in P1 was associated with a reduction in N excretion to 0.38g N/kgW$^{0.75}$ per d (P3). This resulted in a net gain of N of approximately 0.76g/d. There was a significant difference between the N excreted in P3 and P2, and between the N excreted in P3 and P1 (see Table III(11)). The treatment P4, in which propionate was infused in quantities equal to those given during the control period

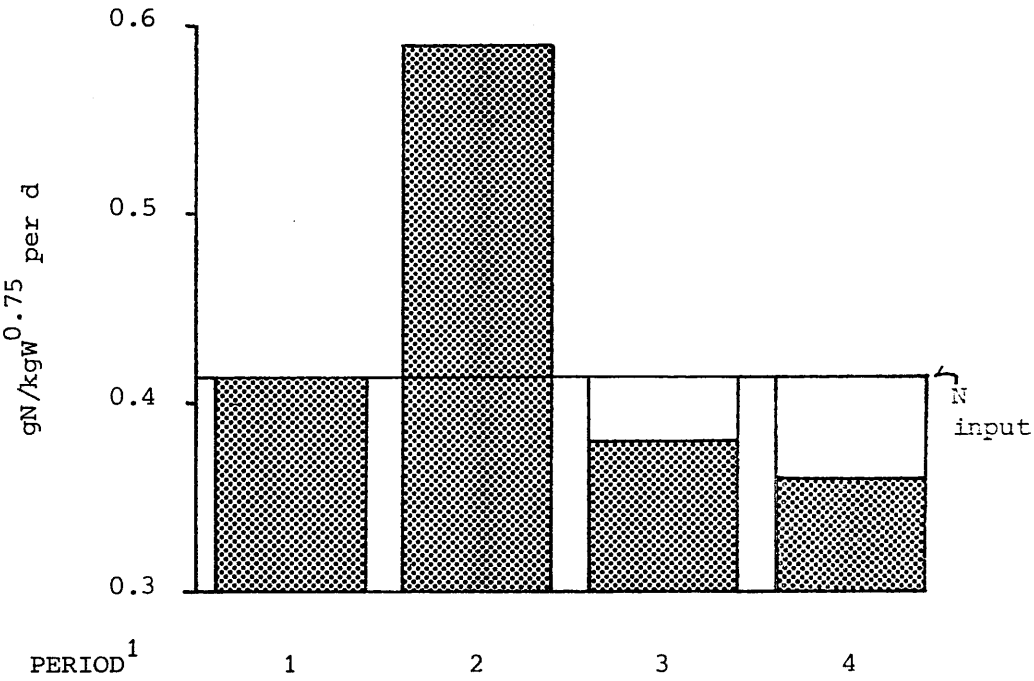


Figure III(4): Experiment 3. The effect of treatments on the N excreted by sheep 466 and 468. 1. For treatments refer to Table III(10).

Table III(11): Experiment 3. The effects of glucose-precursor restriction on the excretion of metabolites by sheep sustained by intragastric infusions of nutrients.

Item	Treatment period[1]				
	1	2	3	4	SEM
N ($g/kgW^{0.75}$ per d)	0.41^{a2}	0.59^{b}	0.38^{c}	0.36^{c}	0.01
Creatine ($g/kgW^{0.75}$ per d)	0.05	0.08	0.05	0.04	0.01
Creatinine ($g/kgW^{0.75}$ per d)	0.07	0.07	0.07	0.06	0.00

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

(P1) resulted in a further reduction in N excretion which led to the retention of 1.22g N/d. The N excreted by the sheep in P4 was significantly lower than that excreted in P1 and P2 (Table III(11)). The differences between the N excreted in P3 and P4 were not statistically significant. Creatine and creatinine concentration were also measured in the urine/faeces mixture. Creatinine tended to be excreted at a uniform rate throughout the experiment whereas creatine peaked during P2 (see Appendices 12 and 13). Changes in the excretion of these metabolites were not, however, statistically significant (Table III(11).

Amino acids. The effects of treatments on the concentrations (μmol/100ml) of the amino acids measured in blood plasma are shown in Tables III(12) and (13). The proportions of the amino acids (mol/100mol) are shown in Tables III(14) and (15). The daily data to which these results refer are shown in Appendices 14 and 15.

Non-essential amino acids (NEAA). The isoenergetic withdrawal of propionate from the infusions (P2) had a considerable effect on the concentrations of the NEAA measured in blood plasma (Table III(12)). There was almost a 70% reduction in alanine concentration ($P < 0.01$) together with large reductions in the concentrations of citruline and glycine, serine and aspartate. The concentrations of glutamate and proline also tended to fall whilst the concentration of cystine ($P < 0.05$) and ornithine tended to rise in response to propionate withdrawal. Reintroduction of propionate at 50% (P3) appeared to have the opposite effect to the treatment of P2. The result of this was that the concentrations of the majority of NEAA's

Table III(12): Experiment 3. The effects of glucose-precursor supply on the concentrations of non-essential amino acids in the blood plasma of sheep sustained by intragastric infusions of nutrients.

Amino acid (μmol/100ml)	Treatment period[1]					
	1	2	3	4	SEM	F
Alanine	28.87[a2]	9.77[b]	36.37[c]	43.30[d]	1.39	***
Citrulline & Glycine	73.80[a]	51.26[a]	150.37[bc]	184.23[c]	12.88	***
Serine	12.44[bcd]	5.77[bc]	24.46[ad]	30.04[a]	2.75	*
Cystine	0.62[a]	1.09[b]	1.50[c]	2.05[d]	0.08	***
Glutamate	22.25	20.51	25.17	27.45	2.16	NS
Proline	18.40	14.45	24.99	31.55	2.49	NS
Aspartate	1.88[bc]	1.16[b]	2.13[ac]	2.77[ac]	0.21	*
Ornithine	8.65	12.32	12.85	14.60	1.72	NS
Total amino acids	270.4[a]	280.5[ac]	392.0[bc]	471.0[b]	24.79	*

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(13). Experiment 3. The effects of treatments on the concentrations of the essential amino acids measured in the blood plasma of sheep.

Amino acid (µmol/100mol)	Treatment period[1]					
	1	2	3	4	SEM	F
Arginine	9.23	12.36	7.76	9.05	1.80	NS
Histidine	8.49	9.39	10.84	16.19	1.75	NS
Methionine	3.00	2.97	2.65	3.24	0.41	NS
Valine	19.84[c2]	40.53[b]	25.00[ac]	28.02[a]	1.39	**
Threonine	9.13	11.77	8.61	9.53	1.23	NS
Isoleucine	8.63[c]	18.28[b]	10.08[ac]	12.37[a]	0.63	**
Phenylalanine	6.03	7.14	5.57	6.11	0.64	NS
Tyrosine	7.79	6.77	7.21	8.73	0.44	NS
Leucine	13.83[c]	31.46[b]	16.38[ac]	19.55[ac]	1.81	*
Lysine	12.59	19.85	15.75	19.13	2.22	NS
Tryptophan	4.97	3.83	4.40	3.52	0.23	NS

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(14): The effect of glucose precursor supply on the proportions (mol/100mol) of non-essential amino acids in the blood plasma of sheep sustained by intragastric infusions of nutrients.

Amino acid (mol/100mol)	Treatment Infusions[1]				
	1	2	3	4	SEM
Alanine	10.70^{a2}	3.52^{b}	9.38^{a}	9.18^{a}	0.76
Citrulline & Glycine	27.31^{a}	18.21^{b}	38.05^{c}	38.99^{c}	0.90
Serine	4.60^{a}	2.06^{b}	6.17^{c}	6.36^{c}	0.33
Cystine	0.20	0.40	0.40	0.40	0.04
Glutamate	8.20	7.40	6.40	5.80	0.43
Proline	6.80	5.00	6.40	6.70	0.72
Aspartate	0.70^{a}	0.41^{b}	0.54^{bc}	0.59^{ac}	0.03
Ornithine	3.20	4.40	3.20	3.10	0.24

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P < 0.05$).

Table III(15): Experiment 3. The effect of glucose-precursor supply on the proportions (mol/100mol) of essential amino acids in the blood plasma of sheep sustained by intragastric infusions of nutrients.

Amino acid (mol/100mol)	Treatment Infusions[1]				
	1	2	3	4	SEM
Arginine	3.41	4.38	2.03	1.96	0.35
Histidine	3.10	3.40	2.80	3.50	0.32
Methionine	1.10	1.00	0.70	0.70	0.10
Valine	7.33^{a2}	14.59^{b}	6.46^{a}	5.98^{a}	0.42
Threonine	3.37^{a}	4.18^{a}	2.21^{b}	2.04^{b}	0.19
Isoleucine	3.19^{a}	6.54^{b}	2.60^{a}	2.63^{a}	0.15
Phenylalanine	2.23^{a}	2.55^{a}	1.45^{b}	1.31^{b}	0.09
Tyrosine	2.88^{a}	2.42^{b}	1.87^{c}	1.86^{c}	0.08
Leucine	5.11^{a}	11.23^{b}	4.22^{c}	4.17^{cd}	0.01
Lysine	4.66^{a}	6.98^{b}	3.99^{a}	4.07^{a}	0.42
Tryptophan	1.84	1.39	1.16	0.76	0.14

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

were returned to values similar to those measured in P1. Citruline and glycine ($P < 0.05$) and cystine ($P < 0.01$) together with serine exhibited significant 'overshoot' in this respect.

There was little change in the concentrations of the NEAA's compared to their values in P3 when propionate was fully reintroduced into the infusions (P4). Alanine ($P < 0.05$) and cystine ($P < 0.05$) concentrations were, however, significantly higher in P4 than in P3.

The proportions of the NEAA's measured in the blood plasma (Table III(14)) followed similar patterns of change during the experiment as their concentrations. However, as a result of the significant increases in total amino acid concentrations measured in P3 (+ 45%, $P < 0.05$) and P4 (+ 74%, $P < 0.05$) compared to P1 the changes in proportions of the NEAA's were relatively small in those periods. Consequently there was a tendency for the differences in the mean proportion of NEAA's in P3 and P4 to be small and not statistically significant. Also for more than 50% of the non-essential amino acids measured there were no statistically significant differences between the proportions measured in P1 and in P3 and P4.

Linear regression analysis of the data in P3 (Table III(16)) showed significant positive correlations between the % composition of glutamate (mol/100mol) and time (d) ($P < 0.05$) and the concentrations (μmol/100ml) of aspartate ($P < 0.05$), proline ($P < 0.05$) and cystine ($P < 0.01$) each with time.

Essential amino acids (EAA). The effects of the treatments on the concentration and relative proportions of the EAA in blood plasma are shown in Tables III(13) and (15). The isoenergetic withdrawal of

Table III(16): Experiment 3. Changes in the amino acid content of blood plasma from sheep resulting from prolonged glucose-precursor restriction[1].

Amino acid	Regression Equation[2]	R
Glutamate (mol/100mol)	$y=6.35-0.56t+0.117t^2-0.007t^3+0.00011t^4$	0.96^{a3}
Cystine (mol/100mol)	$y=0.266-0.011t+0.0025t^2-0.00007t^3$	0.89^{a}
Cystine (µmol/100ml)	$y=0.83+0.048t$	0.72^{b}
Aspartate (µmol/100ml)	$y=1.419+0.04t$	0.60^{a}
Proline (µmol/100ml)	$y=18.47+0.47t$	0.51^{a}
Tryptophan (mol/100mol)	$y=1.432-0.0183t$	0.55^{a}
Threonine (µmol/100ml)	$y=6.74+0.13t$	0.56^{a}
Valine (µmol/100ml)	$y=21.37+0.23t$	0.52^{a}
Histidine (µmol/100ml)	$y=6.46+0.312t$	0.66^{b}

1. Propionate was reduced to 50% of control values for 32d. 2. y = amino acid concentration or proportion in blood plasma. t = time (d) from first day of treatment. 3.a. $P < 0.05$. b. $P < 0.01$. No significant relationships were found between the duration of treatment and the concentration or proportion of the other amino acids that were measured.

propionate from the infusion(s) was associated with substantial increases in the concentrations of valine ($P < 0.01$), leucine ($P < 0.01$), isoleucine ($P < 0.01$) and lysine. There also tended to be smaller increases in the concentrations of histidine, threonine, arginine and phenylalanine together with reductions in the concentration of tyrosine and with tryptophan and a small reduction (9%) the concentration methionine.

Isoenergetic reintroduction of propionate at 50% (P3) of the level infused in P1 was associated with reductions in the concentrations of valine ($P < 0.01$), threonine, isoleucine ($P < 0.01$), phenylalanine, tyrosine, leucine ($P < 0.01$), lysine and tryptophan. There also tended to be smaller reductions in the concentrations of arginine, histidine and methionine. The concentrations of all the EAA's measured in P3 excepting valine ($P < 0.05$) and isoleucine ($P < 0.05$) were not significantly different to those measured in P1.

Isoenergetic reintroduction of propionate to the infusions at the levels given in P1 was associated with small, non-significant changes in all the EAA concentrations measured when compared to values measured in P3.

Linear regression analysis of the data in P3 (Table III(16)) showed significant positive correlations between the concentrations (µmol/100ml) of threonine ($P < 0.05$), valine $P <).05$) and histidine ($P < 0.01$) each with time (d) and a negative correlation between the proportion of the EAA's respresented by tryptophan in the blood plasma (mol/100mol) with time (d) ($P < 0.05$).

The proportions of essential amino acids in blood plasma (Table III(15)) largely followed the same patterns of change during

the experiment as their concentrations (Table III(13)) the significant changes in total amino acid concentrations in blood plasma measured between periods (Table III(12)) resulted in the changes in the proportions of the amino acids being small. Consequently, there were no statistically significant changes in the proportions during P3 and P4. Also, for half of the essential amino acids measured there were no significant changes between P1, P3 and P4.

Blood metabolites

The effects of treatments on the metabolites measured in blood are summarised in Tables III(17), (18), (19) and (20). The data to which these relate are shown in Appendices 16-27 inclusive.

During P1 the concentrations of the majority of the metabolites measured in blood plasma were within the normal physiological range (see Appendix 54). The concentration of copper in the blood plasma however, was high, despite the fact that little copper was given to the animals in the infusions. The reasons for this anomaly are discussed later (see 'The acute and chronic effects of intragastric infusion').

When propionate was isoenergetically withdrawn from the infusions plasma urea tended to increase but the total protein and albumin concentration together with the albumin:globulin ratio of the blood plasma were unaffected (Table III(17)). During this period plasma glucose concentration fell as did lactate whilst glycerol, NEFA ($P < 0.05$), acetate ($P < 0.001$), acetoacetate ($P < 0.05$) and 3-OH butyrate ($P < 0.001$) concentrations rose considerably (Table III(18)). Plasma insulin and growth hormone concentrations were seen

Table III(17): Experiment 3. The effect of treatments on the concentrations of blood plasma constituents of sheep sustained by intragastric infusions of nutrients.

Blood plasma constitutent	Treatment Infusions[1]				
	1	2	3	4	SEM
Urea (mmol/l)	2.20	3.50	2.50	2.50	0.34
Total Protein (g/l)	78.00	77.00	80.00	82.00	1.19
Albumin (g/l)	36.50	36.00	33.80	33.00	0.82
Alb:Glob[2]	0.91	0.90	0.74	0.68	0.03

1. Refer to Table III(10). 2. Ratio of the concentration of albumin (g/l) to globulin (g/l). Values shown are period means.

Table III(18): Experiment 3. The effect of treatments on the concentrations (mmol/l) of blood plasma constituents of sheep sustained by intragastric infusions of nutrients.

Blood plasma constituents (mmol/l)	Treatment Infusions[1]				
	1	2	3	4	SEM
Glucose	4.10	2.90	4.00	3.90	0.25
Lactose	1.40	0.80	2.40	2.60	0.29
Glycerol	0.10	0.20	0.30	0.40	0.07
NEFA	0.07^{a2}	0.42^{b}	0.07^{a}	0.06^{a}	0.03
Acetate	1.49^{a}	6.66^{b}	1.82^{a}	1.34^{a}	0.12
Acetoacetate	0.56^{a}	1.07^{b}	0.52^{a}	0.25^{a}	0.09
3-OH butyrate	0.12^{a}	2.50^{b}	0.44^{a}	0.22^{a}	0.11

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(19): Experiment 3. The effect of treatments on the concentrations of blood plasma hormones (ng/ml) and enzymes (iu) in sheep sustained by intragastric infusions of nutrients.

Blood plasma constitutent	Treatment Infusions[1]				
	1	2	3	4	SEM
Insulin	0.86	0.16	0.79	0.97	0.21
Glucagon	0.30	0.30	0.40	0.50	0.20
Growth hormone	2.80	2.00	2.90	2.50	0.52
Cortisol	6.64[a2]	15.22[b]	4.66[a]	4.90[a]	1.33
γGT	70.00	91.00	114.00	133.00	36.20
GOT	396.00	309.00	331.00	163.00	46.40

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(20): Experiment 3. The effect of treatments on the concentrations of blood plasma minerals (mmol/l) and the blood packed cell volume in sheep sustained by intragastric infusions of nutrients.

Blood plasma constituent (mmol/l)	Treatment Infusions[1]				
	1	2	3	4	SEM
Ca	2.40[a2]	2.05[b]	2.36[a]	2.31[a]	0.05
Mg	0.93[a]	0.80[b]	0.94[a]	0.98[a]	0.02
P	2.40	3.10	2.60	2.90	0.21
Cu	28.28[a]	27.75[ab]	25.28[bc]	22.85[c]	0.58
PCV	29.80	31.80	25.50	25.00	1.40

1. Refer to Table III(10). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

to fall whilst glucagon remained unaffected and the concentration of cortisol in the blood plasma rose significantly ($P < 0.05$). Plasma γ-GT concentration tended to rise whilst the SGOT concentration of blood plasma tended to fall (Table III(19)). The concentrations of Ca ($P < 0.05$), Mg ($P < 0.05$) and Cu fell in the blood plasma and the concentration of P was increased. The packed cell volume of the blood also tended to increase but the effect was not statistically significant (Table III(20)).

When the propionate was reintroduced into the infusions at 50% of its initial inclusion rate (P3) there was a general reversal of the reactions which occurred in response to complete propionate withdrawal. Urea concentration fell once more, as did NEFA ($P < 0.01$), acetate ($P < 0.001$), acetoacetate ($P < 0.05$) and 3-OH butyrate ($P < 0.001$). Meanwhile, the concentrations of glucose, lactate and glycerol tended to rise. There were also effects on the plasma hormones that were measured. The concentrations of insulin, glucagon and growth hormone tended to rise whilst the plasma cortisone concentration fell significantly ($P < 0.05$). The hepatic enzymes γ-GT and GOT tended to rise. Also the increases in the concentrations of plasma Ca ($P < 0.05$), Mg ($P < 0.05$) were measured together with non-significant falls in the concentrations of P and Cu. There also tended to be a fall in the PCV% in P3.

Regression analysis of the data obtained from P3 (Table III(21)) identified significant positive relationships between glucagon and duration of the treatment ($P < 0.05$) as well as between growth hormone, and treatment duration ($P < 0.05$) and total protein concentration in the blood plasma with duration ($P < 0.05$). There was also a significant negative relationship between GOT

Table III(21): Experiment 3. Adaptive changes in metabolite concentrations measured in the blood of sheep resulting from prolonged glucose-precursor restriction[1].

Plasma constitutent	Regression equation[2]	R
Glucagon (ng/ml)	y = 0.253 + 0.0001t	0.97[a3]
Growth hormone (ng/ml)	y = 1.611 + 0.066t	0.96[a]
Total protein (g/l)	y = 74.99 + 0.263t	0.61[a]
GOT (iu)	y = 772.1 - 23.21t	0.83[b]

1. Propionate was reduced to 50% of control values for 32d. 2. y = concentration of blood constituent, t = time (d) from first day of treatment. 3. a. $P < 0.05$. b. $P < 0.01$. No significant relationships were found between the duration of treatment and the concentration or activity of the other blood constituents that were measured.

concentration and time ($P < 0.01$). There were no other statistically significant adaptive effects identified between the metabolites measured and duration of the treatment in P3 by the regression analysis.

The isoenergetic reintroduction of propionate to the infusions at the doses given in P1 (i.e. P4) caused few effects in addition to those already resulting from the treatment used in P3. The exceptions to this trend were the albumin:globulin ratio (Table III(17)) and γ-GT (Table III(19)) which both appeared to change progressively throughout the experiment regardless of treatment. Packed cell volume % also tended to follow a downward trend from the beginning to the end of the experiment (see Appendices 19 and 25).

Discussion

The quantity of N required by the sheep to meet their tissue needs for maintenance was 330mg casein N/kgW$^{0.75}$ per d. This was approximately 15% below the equivalent values calculated for cows and sheep in Experiments 1 and 2.

Isoenergetic withdrawal of propionate from the infusions (P2) resulted in the excretion of 0.58g N/kgW$^{0.75}$ per d. This was calculated to be the result of 4.06g amino acid/kgW$^{0.75}$ per d which, if completely utilized for gluconeognesis would provide the animal with 2.35g glucose/kgW$^{0.75}$ per d.* The significant reductions in the concentrations of alanine, citrulline, arginine, serine and aspartate in blood plasma which were measured during P2 provide circumstantial evidence for their use for gluconeogenesis. This may also explain the increases measured in the relative proportions (mol/100mol) and concentrations (μmol/100ml) in plasma of the EAA's,

*Refer to Appendix 56.

valine, leucine, isoleucine and lysine as well as histidine, threonine, arginine and phenylalanine. The increased concentrations and proportions of the above EAA's in blood plasma suggests they are not as readily available for gluconeogenesis as the NEAA's. In contrast to the above observations, methionine concentration and composition in the blood plasma tended to fall slightly in response to propionate withdrawal from the infusions. Such a response may be indicative of an obligatory requirement for the amino acid which reflects its role as a limiting amino acid for tissue synthesis (Bender, 1985). The concentration of total amino acids measured in P2 was not significantly different to that measured in P1. This indicates that the uptake of amino acids from the blood was equilibrated by their release into the blood as a result of tissue mobilization. The net effect of this nutrient flux, however, was a loss of N from the tissues.

The quantity of creatinine excreted throughout P2 and for the whole experimental period remained relatively constant (Appendices 12 and 13). This observation is in accord with the belief that creatinine secretion occurs independently of diet and is constant for individual animals although dependent on their muscle mass. The sharp rise in creatine excretion associated with P2 is indicative of an increase in muscle protein degradation (Scott-Allen, 1977).

The effect of isoenergetic propionate withdrawal on the other metabolites measured were, in some instances, quite marked. Urea concentration in the blood plasma was increased slightly whilst the total protein, albumin and globulin concentrations remained constant (Table III(17)). Urea entry and excretion rates are well correlated with plasma urea concentration (Lindsay, 1978), and increased plasma

urea N has been associated with, amongst other things, the gluconeogenesis of amino acids from muscle protein (see Sykes, 1978). Accordingly an interpretation of increases in plasma urea concentration is that the gluconeogenic NEAA's were being used for glucose production in order to make good the deficit resulting from propionate withdrawal from the infusions.

Albumin concentration together with the ratio of albumin:globulin were unaffected by propionate withdrawal. Albumin is considered to be an index of long-term protein adequacy (Sykes, 1978) which may explain the absence of any marked response to short periods of net N loss which occurred in P2. The concentration of globulin remained constant which resulted in an unchanging albumin:globulin ratio. As the total protein fraction of the blood is the sum of the albumin and globulin fractions (Payne et al., 1970) this also did not respond to propionate withdrawal.

The metabolites most often used as indicators of energy adequacy, namely plasma glucose, lactate, glycerol, NEFA, acetate, acetoacetate and 3-OH butyrate (see Table III(18)) all responded to isoenergetic propionate withdrawal in a manner similar to that observed in animals suffering energy and protein undernutrition who, in consequence metabolize body tissue. This occurred despite the infusion of nutrients sufficient to provide an above-maintenance energy supply to the animals.

The hormones measured in blood plasma also responded to propionate withdrawal (Table III(19)). Plasma insulin concentration tended to fall whilst plasma glucagon was unchanged. The reduction in insulin:glucagon ratio which resulted was similar to that observed in fasted sheep by Gow et al. (1981). The change in insulin:glucagon

ratio is also thought to promote the utilization of gluconeogenic amino acids released from tissue protein for gluconeogenesis in the liver (McDowell, 1983). Growth hormone concentration tended to fall during P2. A similar response to a sudden fall in plasma glucose concentration was measured in wethers by Trenkle (1971). The exact role of growth hormone in glucose homeostasis in ruminants is still the subject of much controversy however. Cortisol concentrations measured in the blood plasma increased significantly ($P < 0.05$) in response to propionate withdrawal. Cortisol is known to stimulate gluconeogenesis and protein catabolism and is secreted in response to a decrease in blood glucose concentration (Bassett, 1968).

The enzymes measured in P2 were not significantly affected by propionate withdrawal (Table III(19)) although plasma γ-GT concentration tended to rise. The enzymes measured were chosen because of their association with breakdown of hepatic tissue (see Manston & Allen, 1981). It appears that the duration of P2 was too short to cause the significant effects to the liver associated with long periods of nutrient imbalance (Hughes et al., 1973).

The concentrations of the minerals measured in blood plasma during P2 (Table III(20)) were seen to decrease in the case of Ca ($P < 0.05$), Mg ($P < 0.05$) and Cu and were increased in the case of P. The reasons for these changes are not known.

The effect of isoenergetically reintroducing propionate (P3) at 50% of the dose given in P1 resulted in N excretion significantly lower than that excreted in P1 ($P < 0.05$) and P2 ($P < 0.001$). There also appeared to be an overriding trend for the metabolite concentrations measured in P3 to be similar to those measured in P1. Additionally, there were quite marked differences between the

concentrations of metabolites measured in P2 and P3, many of which were statistically significant.

Clearly the isoenergetic withdrawal of 50% of the propionate infused in P1 did not cause the animal to suffer the same metabolic effects as occurred in P2. The treatment of P3 was therefore insufficient inducement for the continuation of the adaptation which appeared to occur when propionate was isoenergetically removed from the infusions (P2). The majority of metabolites monitored during P3 did not change significantly with time (see Tables III(16) and (21)) which adds further support to the idea that the adaptive process did not continue in P3. The mean excretion measured in P3 was however 0.04g $N/kgW^{0.75}$ per d ($P < 0.05$) less than in that measured in P1 which could be associated with an increase in the efficiency of utilization of casein N. The quantity of N excreted in P4 which was 0.06g $N/kgW^{0.75}$ per d lower than that measured in P1 ($P < 0.01$) suggests that an increase in efficiency of utilization of casein N was still measureable in P4.

This was, presumably, a response to the isoenergetic removal of propionate from the infusions during P2 which persisted throughout P3 and P4. The adaptation in N utilization occurring in P2 was associated with marked changes in many of the metabolites and hormones which were monitored in the blood plasma. The residual effects which were measureable in P3 and P4 were not, however, associated with such changes. This would indicate that either the metabolic changes occurring in P3 and P4 were too subtle for detection by the blood analyses used or that the changes in N utilization were independent of the other parameters measured. The former option appears to be the most realistic.

The P3 treatment which was imposed for 32d resulted in the concentrations and activities of the metabolic parameters measured to generally be no different to when they were measured in P1. These observations suggest that when the propionate supply was reduced by 50% of that given in the control period there still remained adequate glucose precursors for marked changes in blood composition of the type observed in P2 to be avoided. This observation suggests that either the glucose requirements of the sheep had been reduced considerably as a result of the P2 treatment or that the yield of glucose from the gluconeogenic amino acids had been greatly improved. The former option would appear to be the most tenable.

It was clear from the results obtained from this experiment that the prolonged period of propionate restriction from the infusions had not produced the adaptive effects on N utilization that were intended.

EXPERIMENT 4

Introduction

Changes in the efficiency of utilization of N by ruminants were measured during Experiments 1 and 2 and these changes appeared to correspond to periods of inadequate exogenous glucose-precursor supply. Experiment 3 was designed to further investigate these metabolic adaptations by subjecting the sheep to a prolonged period (32d) in which exogenous glucose-precursor supply was restricted so that it was inadequate for the animal's requirements.

The degree of perturbation of the propionate supply imposed on the sheep in Experiment 3 was intended to perpetuate the adaptive response resulting from an initial period in which propionate was completely withdrawn from the infusions. However, in view of the animal's response to the period of restricted propionate infusion it appears that the circumstances in which the adaptive changes were initiated in Experiments 1 and 2 had not been reproduced.

With this in mind Experiment 4 was designed to subject animals to a more severe regime than was used in Experiment 3 and to totally withhold propionate from isoenergetic infusions for as long as was practicable for the animals to maintain glucose homeostasis and avoid clinical hypoglycaemia.

Experimental

Animals and their management. Three Finnish Landrace x Dorset Horn wethers (nos. 462, 6693 and 6696) of 59.3 $\pm$ 6.4kg LW were surgically prepared and allowed to convalesce. They were then introduced to

complete intragastric nutrition during a 10d transition period (see 'Changeover procedure', Section II).

Experimental plan and procedures. Infusions comprising 650:250:100mmol/mol VFA mixture (Ac:Pr:Bu), sodium caseinate and emulsified tallow (25g/d) were formulated. These were designed to provide ME at 495kJ/kgW$^{0.75}$ per d (1.5 x the energy required to meet the energy needs for maintenance - Ørskov et al., 1979) and 350mg casein N/kgW$^{0.75}$ per d at the tissues (1.0 x the casein N required at the tissues to meet N needs for maintenance (Experiment 3). Minerals and vitamins were also infused in the quantities required and buffer was infused in quantities calculated to maintain the pH balance of the rumen (see 'Infusion formulation', Section II).

Measurements of N balance taken when the above quantities of nutrients were infused showed that a net loss of N had occurred. In order to reduce this net loss of N to 0g/d the casein dose was adjusted to 473 $\pm$ 42mg casein N/kgW$^{0.75}$ per d. This would supply 378 $\pm$ 34mg casein N/kgW$^{0.75}$ per d at the tissues if an overall efficiency of utilization of casein N of 80% is assumed. This quantity of casein N was given throughout all subsequent treatments.

Following an 8d control period (P1) in which the adjusted quantities of above were infused, a 860:0:140mmol/mol VFA mixture (Ac:Pr:Bu) was given (P2) in place of the 650:250:100mmol/mol mixture given in P1. On the basis of previous results the length of P2 was restricted to 12d. It was considered that clinical hypoglycaemia would result from further extension of the period beyond this time. Finally, the propionate content of the infusions was isoenergetically reintroduced (P3, 5d) in the same quantities as were given in P1.

The nutrient infusions were isoenergetic and isonitrogenous throughout the experiment (see Table III(22)).

Blood was sampled at 2d intervals during P1 and at daily intervals during P2 and P3. Faeces and urine collections were made daily and the sodium caseinate and lipid solutions were sampled periodically.

Chemical analysis. The chemical analyses carried out in this experiment were the same as those described in Experiment 3.

Statistical analysis. All data were subject to ANOVA for randomised block designs. Data from P2 underwent linear or quadratic regression analysis. The N data from P2 also underwent analysis using the 'cusum' technique (BSI, 1980 and 1980a).

Results

Animal health. The experimental animals were maintained in a state of good health throughout P1 and P2. On the final day of P3, however, sheep no. 6693 died and a post-mortem examination revealed a severe staphylococcal infection within the sinus in the body wall which surrounded the abomasal catheter (see 'Acute and chronic effects of infusion'). Four days after the completion of Experiment 4, sheep 6696 became inappetant. The animal was distroyed and post-mortem examination revealed that the reticulum was obstructed by a polythene pan scourer. There was also an infection within the sinus around the abomasal catheter similar to, but less severe than that found in sheep 6693.

Table III(22): Experiment 4. Treatments given to 3 sheep sustained by intragastric infusions of nutrients.

Treatment period	Duration (d)	VFA molar mixture infused Ac:Pr:Bu (mmol/mol)
1	8	650:250:100
2	12	860: 0 :140
3	5	650:250:100

3 wethers of 59.3 ± 6.4kg LW were sustained by the infusion of 473 ± 42mg casein $N/kgW^{0.75}$ per d, 25g/d emulsified tallow and VFA equivalent, in total, to 495kJ $ME/kgW^{0.75}$ per d. Minerals, buffer and vitamins were infused to requirement (see 'Infusion formulation', Section II).

N utilization. The quantity of casein N required in the infusions to sustain a daily retention of 0g/d was calculated to be 473 ± 42mg casein $N/kgW^{0.75}$ per d. This represents a tissue N requirement for maintenance of 378 ± 34mg $N/kgW^{0.75}$ per d. The isoneregetic withdrawal of propionate from the infusions (P2) resulted in an increase in the N excreted by the sheep from 0.47 to 0.64g $N/kgW^{0.75}$ per d (Figure III(5)) which resulted in a net loss of 3.6g N/d. The isoenergetic reintroduction of propionate into the infusions (P3) was associated with a reduction in N excretion to $0.46g/kgW^{0.75}$ per d which was equivalent to a net retention of 0.2g N/d. There was a significant difference ($P < 0.001$) between the N excreted in P1 and P2 and between P2 and P3. The difference in the mean N excretion which occurred in P1 and P3 was not significant. Examination of the N data obtained in P1 and P3 for individual sheep revealed changes in N status from -0.18g/d to 0.21g/d and from 0.45g/d to 0.84g/d for sheep 462 and 6696 respectively. In contrast, the corresponding P1 and P3 values for sheep 6693 were 0.47g/d and 0.43g/d.

Daily measurements of the N excreted by the sheep during P2 (see appendices 28, 35 and 42) were also used to determine mean daily N balance. The N balance data were then subject to quadratic regression analysis against time but no statistically significant relationship was revealed.

Cusum analysis of N data. The N data of P2 were also subjected to analysis using the cumulative sum technique (cusum) (BSI, 1980 and 1980a) which was used to identify the point in time where adaptive changes in N metabolism may have occurred.

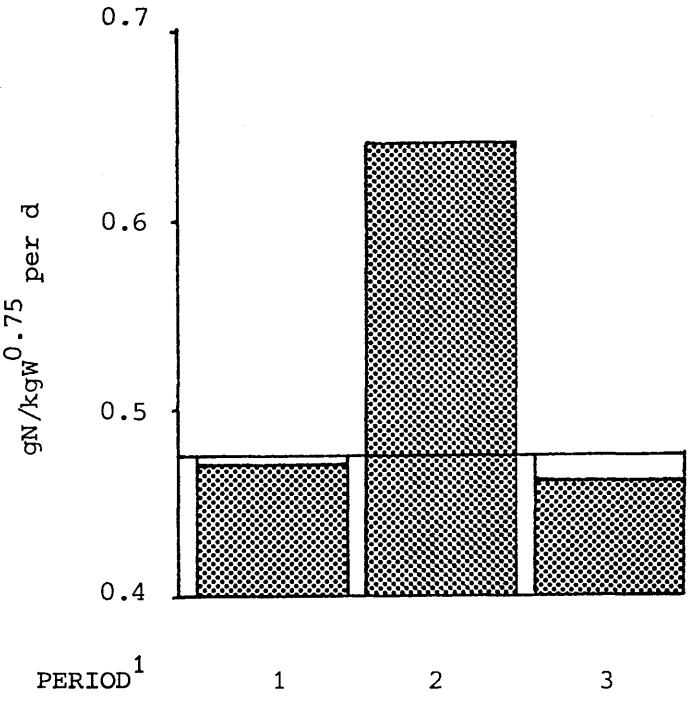


Figure III(5): Experiment 4. N input (—), retention (□) and loss (▨) in 3 sheep sustained by the intragastric infusion of nutrients. 1. See Table III(22) for period treatments.

This technique is a novel procedure. Consequently, the manner in which the N balance data from P2 was analysed will be described. The mean N balance for the 3 sheep over the last 10d of period 2 was calculated and used to represent a cusum target value (T). For each datum the deviation from T was calculated as well as the cumulative sum of the deviations from T. Finally, the successive differences between deviations from T were calculated (see Table III(23).

The cumulative sum of deviations from T were plotted against their corresponding observation number (see Figure III(6)). From this data measurements of variation together with an assessment of the significance of the results obtained were determined.

Measurements of variation

1. The Standard error: The standard error (SE) of the N excretion data obtained in P2 is defined as:

SE = Mean of the successive differences x y

where y = a conversion factor of 1/1.128 (BSI, 1980a)

therefore SE = 1/1.128 x 40.2

= 35.6

2. V. max. When observing cusum charts (Figure III(6)) for possible points of change, the basis for segmentation within the chart are local maxima and minima. The criterion used to assess the significance of such changes is the maximum extent (V_{max}) to which the cusum plot deviates from a straight line joining the ends of the sequence of data within which the change is measured (BSI, 1980a).

Table III(23): Experiment 4. Cusum calculation from N balance measruements taken from 3 sheep during P2.

Observation No.	[1]N balance mg N/kgW$^{0.75}$ per d (x)	[2]Deviation from T mg N/kgW$^{0.75}$ per d	Cumulative sum of deviations from T	Successive differences between deviations from T
1	-211	-61	-61	57
2	-154	-4	-65	7
3	-147	3	-62	81
4	-228	-78	-140	70
5	-168	-8	-148	31
6	-127	23	-125	16
7	-143	7	-118	52
8	-91	59	-59	1
9	-92	58	-1	53
10	-145	5	+4	
Mean	-150.6	-	-	40.1

1. Mean daily N balance of 3 wethers. 2. T = x = -150.

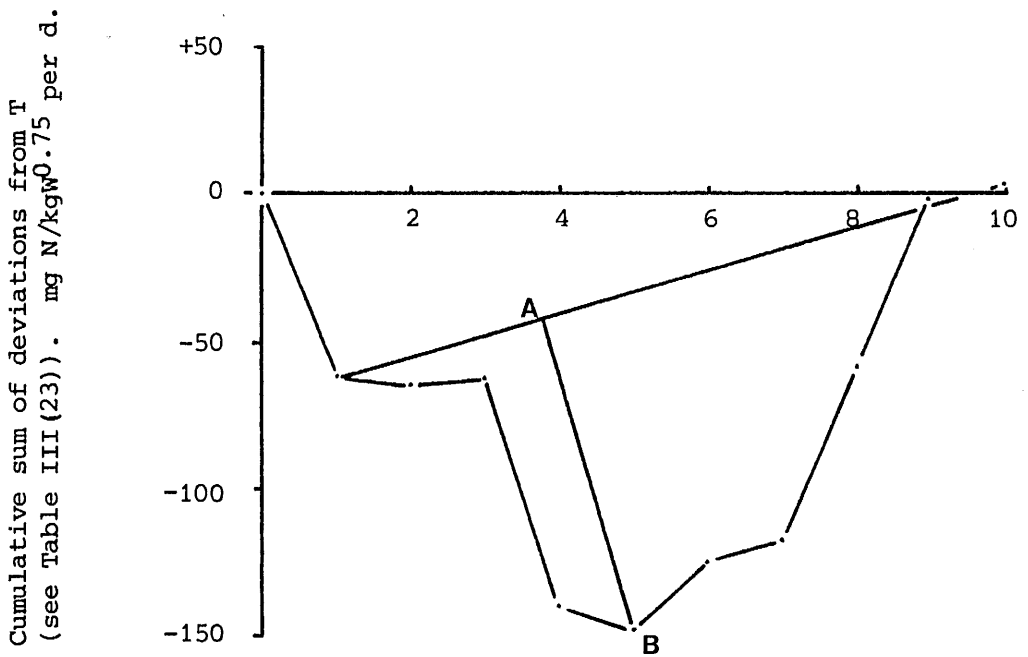


Figure III(6): Experiment 4. Cumulative sum of deviations from the mean value of daily N balances (mg $N/kg^{0.75}$ per d) measured during P2. Line AB = V_{max}.

V_{max} is calculated from the formula:

$$V_{max} = (C_r - C_i) + \frac{r-i}{j-i} (C_j + C_i)$$

where:

r = the sample no. corresponding to a suspected change point in data sequence

i = the sample number corresponding to the beginning of the sequence in which the change is suspected

j = the sample number corresponding to the end of the sequence in which the change is suspected

C_i = the cusum value of sample no. i

C_j = the cusum value of sample no. j

C_r = the cusum value of sample no. r

M = Length of sequence or span in which a change is suspected i.e. j-i.

Having calculated V_{max} it is then standardized by dividing by the SE value. The standardized V_{max} value is then referred to a nomogram (Figure III(7)) for the appropriate span (the number of observations contained within the sequence in which the change is suspected). The P-scale on Figure III(7)) is used to give the probability of exceeding V_{max} in a sequence of length M from the series of observations.

Accordingly: i = 1 C_i = 61 M = j-i

j = 10 C_j = +4

r = 5 C_r = -148

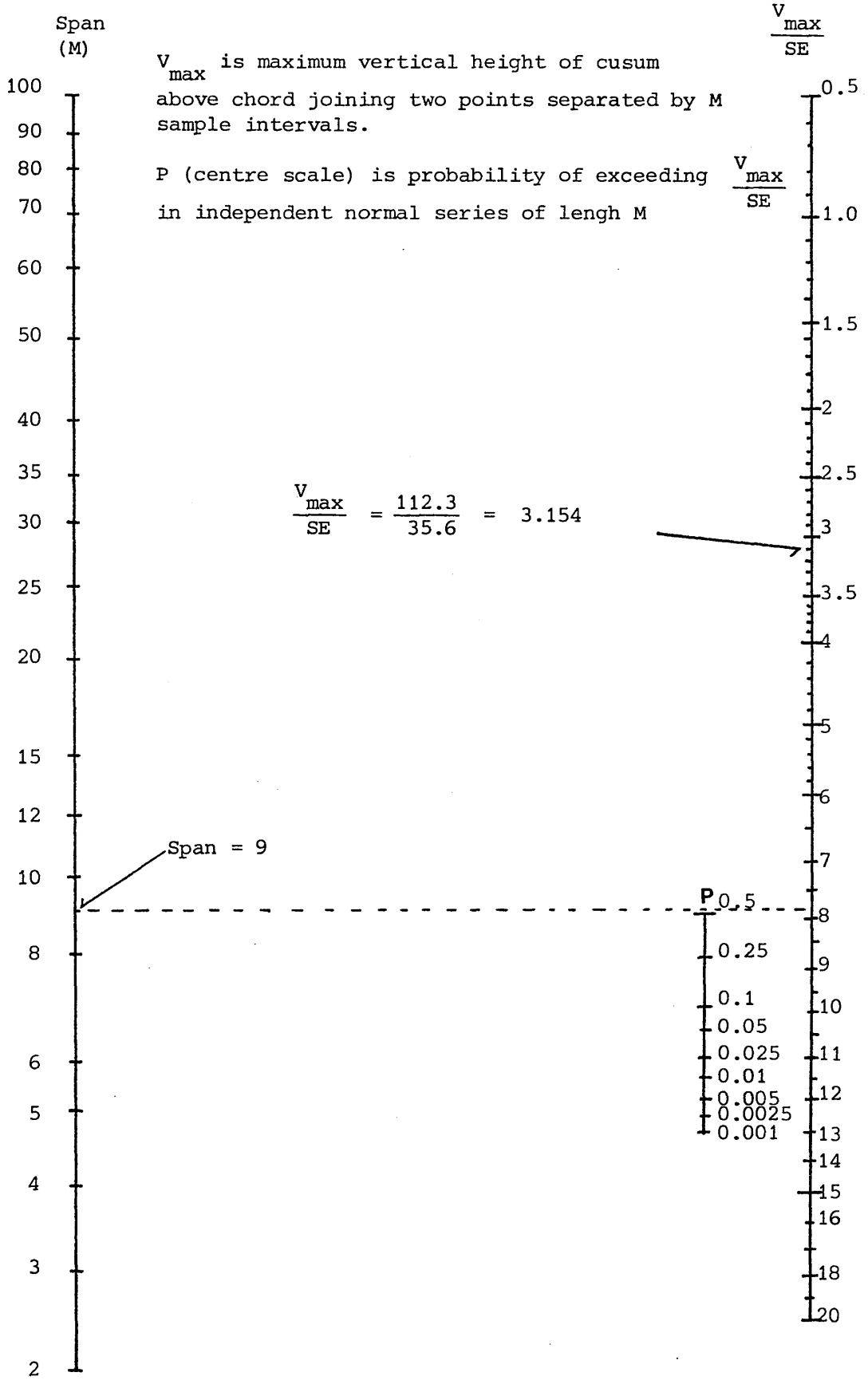


Figure III(7): Experiment 4. Nomogram for evaluating maximum vertical height (V_{max}) of cusum over a span of M samples.

$$V_{max} = (-148 - -61) + (4 \times \frac{(4 + -61)}{9})$$

$$= 112.3$$

$$\text{The standardized } V_{max} = \frac{112.3}{35.6} = 3.154$$

$$\text{The span (M)} = 10-1 = 9$$

From the nomogram (Figure III(7)) the change occurring at observation no. 5 (P2, day 6) was not statistically significant. There was, however, a tendency for the cusum to change at this point in time which is an indication that the adaptive process began to positively affect the efficiency of utilization of N during days 5 to 6 of period 2.

Amino acids

Non-essential amino acids (NEAA). The effects of the treatments on the concentrations (µmol/100ml) and relative proportions (mol/100mol) of the non-essential amino acids are shown in Tables III(24) and (25). The data from which these results were derived are found in Appendices 49, 50 and 51.

The concentrations of the NEAA's measured in P1 were similar to those measured in the equivalent treatments in Experiments 2 and 3.

The isoenergetic withdrawal of propionate from the infusions (P2) had marked effects on the concentrations of the NEAA's. There were large reductions in the concentrations of alanine (-64%), serine (-45%), citrulline and glycine (-42%) and aspartate (-38%, $P < 0.01$).

Table III(24): Experiment 4. The effect of treatments on the concentrations of non-essential amino acids measured in the blood plasma of sheep.

Amino acid (µmol/ml)	Treatment Period[1]			
	1	2	3	SEM
Alanine	21.79	7.78	18.74	3.35
Citrulline & Glycine	82.79	47.75	94.01	12.10
Serine	9.52	5.22	11.24	1.55
Cystine	0.81	1.07	0.85	0.15
Glutamate	17.02	13.35	15.20	1.69
Proline	14.43	14.64	19.19	6.99
Aspartate	1.64[a]	1.02[b]	1.47[a]	0.09
Ornithine	[2]7.77[ab]	10.90[b]	5.11[a]	0.81
Total amino acids	261.20	256.00	251.60	37.60

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different. ($P > 0.05$).

Table III(25): Experiment 4. The effect of glucose-precursor supply on the proportions (mol/100mol) of non-essential amino acids in the blood plasma of sheep sustained by intragastric infusions of nutrients.

Amino acid (mol/100mol)	Treatment Infusions[1]			
	1	2	3	SEM
Alanine	8.45[a2]	3.04[b]	7.29[a]	0.40
Citrulline & Glycine	31.89[a]	18.68[b]	37.17[c]	0.68
Serine	3.64[a]	2.03[b]	4.52[c]	0.16
Cystine	0.32	0.42	0.33	0.03
Glutamate	6.32	5.20	6.60	0.60
Proline	5.40	5.67	6.71	1.82
Aspartate	0.63	0.40	0.65	0.09
Ornithine	2.98[a]	4.29[b]	2.00[c]	0.20

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

A smaller reduction in the concentration of glutamate in blood plasma was also measured. Plasma cystine and ornithine concentrations rose by 30-40% in the blood plasma whilst plasma proline tended to be unaffected by the treatment.

The total amino acid concentration in blood plasma was unchanged in P2 (Table III(26)) and there were statistically significant changes in the relative proportions of alanine ($P < 0.001$) citrulline and glycine ($P < 0.001$), serine ($P < 0.01$) and ornithine ($P < 0.05$) (see Table III(25)). There was a significant reduction in the concentration and relative proportion of alanine measured in blood plasma with time during the P2 treatment ($P < 0.05$ - see Table III(28)).

The isonergetic reintroduction of propionate to the infusions during P3 resulted in a return of the concentrations of the NEAA's to values similar to those measured in P1 (see Table III(24)). The total amino acid concentration in blood plasma was again unchanged during P3 and there were statistically significant increases in the relative proportions of alanine ($P < 0.01$), citrulline and glycine ($P < 0.001$), serine ($P < 0.001$) together with reductions in the proportion of ornithine ($P < 0.01$) and cystine in the blood plasma.

Essential amino acids (EAA). The effects of the treatments on the concentrations (μmol/100ml) and relative proportions (mol/100mol) of the essential amino acids measured in blood plasma are shown in Tables III(26) and (27). The data from which these results were derived are found in Appendices 49, 50 and 51.

The concentrations of the EAA's measured in P1 were similar to those measured in the equivalent treatments in Experiments 2 and 3.

Table III(26): Experiment 4. The effects of treatments on the concentrations of essential amino acids measured in the blood plasma of sheep.

Amino acid (μmol/100ml)	Treatment Period[1] 1	2	3	SEM	F
Arginine	7.94	10.27	7.01	1.51	NS
Histidine	9.52	10.06	8.62	0.83	NS
Methionine	2.24	2.05	1.70	0.35	NS
Valine	[2]24.64[a]	46.59[b]	21.21[a]	2.41	**
Phenylalanine	6.00	7.65	5.77	0.74	NS
Tyrosine	7.49	7.47	5.75	1.20	NS
Leucine	15.16[a]	30.50[b]	13.14[a]	1.78	**
Isoleucine	8.67[ac]	15.83[b]	6.80[a]	1.09	**
Lysine	2.61	2.35	1.54	0.29	NS
Total amino acids	261.20	256.00	251.60	37.61	NS

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different ($P < 0.05$).

Table III(27): Experiment 4. The effect of glucose-precursor supply on the proportions (mol/100mol) of essential amino acids in the blood plasma of sheep sustained by intragastric infusions of nutrients.

Amino acid (mol/100mol)	Treatment Infusions[1]			
	1	2	3	SEM
Arginine	[2]2.99[a]	4.00[b]	2.58[a]	0.25
Histidine	3.62	3.91	3.68	0.27
Methionine	0.89	0.81	0.65	0.09
Valine	9.33[a]	18.17[b]	9.08[a]	0.86
Threonine	3.04	3.08	1.93	0.26
Isoleucine	3.29[a]	6.21[b]	2.68[a]	0.25
Phenylalanine	2.33	3.01	2.39	0.31
Tyrosine	2.86	2.94	2.21	0.22
Leucine	5.76[a]	11.90[b]	5.41[a]	0.34
Lysine	4.99	5.34	3.77	0.18
Tryptophan	1.06	0.94	0.57	0.14

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(28): Experiment 4. Changes with time, in the amino acid content of blood plasma from sheep resulting from prolonged glucose-precursor restriction[1].

Amino acid	Regression equation[2]	R
Alanine (mol/100mol)	$y = 2.88 + 0.23t - 0.027t^2$	0.94[a]
Alanine (µmol/100ml)	$y = 10.4 - 0.39t$	0.63[a]
Valine (µmol/100ml)	$y = 60.46 - 2.07t$	0.66[a]
Isoleucine (µmol/100ml)	$y = 23.01 - 1.07t$	0.68[a]
Leucine (µmol/100ml)	$y = 40.92 - 1.53t$	0.65[a]

1. Propionate was isoenergetically withdrawn from the infusions for 12d. 2. y = amino acid concentration or proportion in blood plasma. t = time (d) from first day of treatment. a. $P < 0.05$. No significant relationships were found between the duration of treatment and the concentration or proportion of the other amino acids that were measured.

The isoenergetic withdrawal of propionate from the infusions (P2) resulted in large increases in the concentrations of leucine (101%, $P < 0.01$), isoleucine (83%, $P < 0.01$), valine (90%), arginine (30%) and phenylalanine (30%). There were smaller changes (< 10%) in the concentrations of the other EAA's measured (Table III(26)), of particular interest though was a reduction (9%) in the concentration of methionine. Since there was no changes in the total amino acid concentration of the blood plasma measured in P1 and P2 changes in the relative proportions of the amino acids largely followed the same patterns of change as their concentrations. There were however significant increases in the proportions of arginine ($P < 0.05$) and lysine ($P < 0.001$) measured in the blood plasma (see Table III(27)).

During P2 there were also significant reductions in the concentrations of valine ($P < 0.05$), isoleucine ($P < 0.05$) and leucine ($P < 0.05$) each with time (d) (Table III(28)). This adaption was not observed with the concentrations or relative proportions of the other amino acids that were measured.

The isoenergetic reintroduction of propionate to the infusions during P3 resulted in a return of the concentrations of the EAA's to values similar to those measured in P1. Measurements of the relative proportions of the amino acids responded in the same manner as their concentrations excepting lysine which was found to be lower in P3 than in P1 ($P < 0.01$).

Metabolites excreted in the urine. The concentrations of creatine and creatinine were monitored on a daily basis throughout the experiment. The results of the analysis are shown in Table III(29)) and the data from which the results were derived are shown in

Table III(29): Experiment 4. The effect of treatments on the excretion of metabolites by sheep sustained by intragastric infusions of nutrients.

Metabolite	Treatment Infusions[1]			
	1	2	3	SEM
N (g/kgW$^{0.75}$ per d)	0.47^{a2}	0.64^{b}	0.46^{a}	0.01
Creatine (g/kgW$^{0.75}$ per d)	0.08^{ab}	0.11^{a}	0.04^{b}	0.005
Creatinine (g/kgW$^{0.75}$ per d)	0.06^{a}	0.07^{a}	0.06^{b}	0.0001
Creatine (g/d)	1.43^{a}	2.08^{b}	0.86^{c}	0.14
Creatinine (g/d)	1.28^{a}	1.29^{a}	1.11^{b}	0.12

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Appendices 28, 35 and 42. Total creatinine excretion together with the quantities excreted on a metabolic liveweight basis remained fairly constant throughout the experiment with a 14% reduction ($P < 0.001$) between P1 and P3. Total creatinine excretion (g/d) was increased by 43% ($P < 0.05$) during P2 when propionate was isoenergetically withdrawn from the infusions.

Blood metabolites. The effects of the treatments on the blood metabolites measured are shown in Tables III(30), (31), (32) and (33). The data from which these results were derived are shown in Appendices 28-48 inclusive.

During P1 the concentrations of the metabolites measured were similar to measurements taken in the equivalent treatment period in Experiment 3.

Isoenergetic propionate withdrawal (P2) resulted in an increase in the mean concentrations of NEFA ($P < 0.05$), 3-OH butyrate ($P < 0.001$), acetoacetate ($P < 0.001$) and acetate ($P < 0.05$) in the blood plasma. The concentrations of urea, growth hormone, cortisone, γ-GT, SGOT and Mg tended to rise to a smaller degree than the above whilst the concentrations of glucose, lactate, glycerol, insulin and SDH together with PCV% tended to fall. The mean concentrations of total protein, albumin, glucagon, Ca, P, Cu and the albumin:globulin ratio of the blood plasma were unaffected by the treatment.

A significant negative correlation existed between the concentrations of glucose ($P < 0.05$), urea ($P < 0.01$) and lactate ($P < 0.05$) and the length of the period of isoenergetic propionate withdrawal. Similarly, significant positive correlations existed

Table III(30): Experiment 4. The effect of treatments on the concentrations of blood plasma constituents of sheep sustained by intragastric infusions of nutrients.

Blood plasma constituent	Treatment Infusions[1] 1	2	3	SEM
Urea (mmol/l)	3.52[ab3]	4.35[a]	2.70[b]	0.34
Total protein (g/l)	78.77	76.40	71.67	2.10
Albumin (g/l)	28.50	29.67	27.50	0.88
Alb:Glob[2]	0.59	0.67	0.65	0.03

1. Refer to Table III(22). 2. Ratio of the concentration of albumin (g/l) to globulin (g/l). 3. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(31): Experiment 4. The effect of treatments on the concentrations (mmol/l) of blood plasma constituents of sheep sustained by intragastric infusions of nutrients.

Blood plasma constitutent (mmol/l)	Treatment Infusions[1]			
	1	2	3	SEM
Glucose	4.00^{ab2}	3.34^{a}	4.44^{b}	0.23
Lactose	1.53	0.87	2.77	0.42
Glycerol	0.18^{ab}	0.14^{a}	0.22^{b}	0.01
NEFA	0.06^{a}	0.19^{b}	0.13^{ab}	0.02
Acetate	1.94^{a}	6.05^{b}	2.31^{a}	0.67
Acetoacetate	0.18^{a}	0.38^{b}	0.15^{a}	0.01
3-OH butyrate	0.18^{a}	1.26^{b}	0.30^{c}	0.02

1. Refer to Table III(22). 2. Means with like or no superscripts are not singificantly different ($P > 0.05$).

Table III(32): Experiment 4. The effect of treatments on the concentrations of blood plasma hormones (ng/ml) and enzymes (iu) in sheep sustained by intragastric infusions of nutrients.

Blood plasma constitutent	Treatment Infusions[1]			
	1	2	3	SEM
Insulin	[2]1.18	0.30	0.64	0.28
Glucagon	0.44	0.44	0.43	0.03
Growth hormone	0.99	2.63	2.55	0.53
Cortisol	4.60	7.44	4.97	1.02
γGT	33.53	42.87	44.16	5.11
GOT	69.67	105.10	91.00	11.34
SDH	9.51	6.53	4.98	2.00

1. Refer to Table III(22). 2. Means with like or no superscripts are not significantly different ($P > 0.05$).

Table III(33): Experiment 4. The effect of treatments on the concentrations of blood plasma minerals (mmol/l) and the blood packed cell volume in sheep sustained by intragastric infusions of nutrients.

Blood plasma constitutent	Treatment Infusions[1]			
	1	2	3	SEM
Ca	2.36	2.32	2.34	0.03
Mg	0.76	0.81	0.69	0.06
P	2.46	2.08	2.33	0.21
CU[2]	35.20	37.90	47.90	3.50
PCV	27.10	24.00	24.10	1.09

1. Refer to Table III(22). 2. Cu measured in µmol/l. Means with like or no superscripts are not significantly different ($P > 0.05$).

between the concentrations of 3-OH butyrate ($P < 0.05$) and acetoacetate ($P < 0.05$) and time (Table III(34)).

The isoenergetic reintroduction of propionate to the infusions (P3) generally had the opposite effect on the blood metabolites than was caused by its withdrawal. There were reductions in the concentrations of urea ($P < 0.05$), acetate ($P < 0.05$), 3-Oh butyrate ($P < 0.001$) and acetoacetate ($P < 0.001$). There was also a tendency for the concentrations of total protein, albumin, NEFA and cortisone in the blood plasma to be reduced. Only in the case of 3-OH butyrate ($P < 0.05$) were the concentrations measured in P3 significantly different to those measured in P1.

The concentrations of glucose ($P < 0.05$) and glycerol ($P < 0.05$) were increased as a result of the reintroduction of propionate and there was a tendency for the concentrations of lactate, insulin, P and Cu to rise also. The concentrations of these metabolites in P3 were not significantly different to their values when measured during P1.

The ratio of albumin:globulin concentrations in the blood plasma did not respond to the reintroduction of propionate to the infusions, neither did the concentrations of glucagon, growth hormone, γ-GT, Cu and Mg.

Discussion

The quantity of N required by the sheep to meet their tissue needs for maintenance was 378 $\pm$ 34mg $N/kgW^{0.75}$ per d. This value was approximately equal to the corresponding values calculated in the control periods of the previous experiments.

Isoenergetic withdrawal of propionate from the infusions (P2) resulted in the excretion of 0.64g $N/kgW^{0.75}$ per d which gave a mean

Table III(34): Experiment 4. Adaptive effects on the concentration (mmol/l) of metabolites in the blood of sheep sustained by intragastric infusion of nutrients from which propionate was removed.

Plasma metabolite	Regression equation[1]	R
Glucose	y = 3.67 -0.04t	0.55[a]
Urea	y = 7.59 -0.49t	0.70[b]
3-OH butyrate	y = 0.97 + 0.4t	0.56[a]
Acetoacetate	y = 3.77 + 0.35t	0.48[a]
Lactate	y = 1.17 -0.05t	0.60[a]

1. y = metabolite concentration (mmol/l) in blood plasma. t = time (d) from first day of treatment. a. $P < 0.05$. No significant relationships were found between the duration of treatment and the concentration of the other metabolites that were measured.

net loss of N of 3.63g N/d. This was calculated to have been the result of the catabolism of 4.48g amino acid/kgW$^{0.75}$ per d which, if completely utilized for gluconeogenesis would provide 2.6g glucose/kgW$^{0.75}$ per d.*

Reduced concentrations of alanine, citrulline and glycine, and serine were measured in the blood plasma during P2 together with statistically significant reductions in their relative proportions provide circumstantial evidence for the use of these amino acids for gluconeogenesis. Concomitant increases in the concentrations and relative proportions of the essential amino acids suggests they are not as readily available for gluconeogenesis as the NEAA. In contrast to the above observations, methionine concentration and composition in the blood plasma tended to fall slightly in response to propionate withdrawal. This response may be indicative of an obligatory requirement for the amino acid which reflects its role as a limiting amino acid for tissue synthesis (Bender, 1985).

There was no change in the total amino acid concentrations measured in P1 and P2 which suggests that the release of amino acids into the blood plasma was in equilibrium with their removal. As was observed in Experiment 3, the result of the above nutrient flux was a net loss of N from the tissues.

The linear and quadratic regression analyses of the N and amino acid data taken from P2 identified a few statistically significant time-dependent relationships (Table III(34)). Specifically the concentration and relative proportions of alanine was reduced with time as were the concentrations of valine, isoleucine, and leucine. The unusual situation in which the concentrations of these four non-essential or essential amino acids

*Refer to Appendix 56.

in the blood plasma appear to follow the same patterns of change with time in response to the isoenergetic propionate withdrawal from the infusions is difficult to reconcile with the findings of the previous experiments.

The cusum analysis of the N balance measurements made during P2 identified a tendency for N excretion to be reduced after a period of 5-6d (Figure III(6)) in which glucose-precursor supply was limited. The change identified above was not, however, statistically significant. This was likely to have been due to the variation found between N excretion measurements taken on a daily basis from animals sustained by intragastric infusions.

Quadratic regression analyses of the other metabolites measured in P2 did not identify any significant curvilinear response with time. The frequency of blood sampling for analysis or the degree of replication within the experiment may have been responsible for those findings.

The isoenergetic reintroduction of propionate to the infusions (P3) resulted in a significant reduction in N excretion ($P < 0.001$) compared to P2. The mean quantity of N excreted in P3 was not however significantly different to that excreted in P1 although it tended to be lower.

Examination of the individual N data for the sheep revealed that in P3 sheep 6693 had excreted N at a higher rate than the other animals which influenced the average excretion for that period. It may be argued that the elevated levels of N excretion measured in sheep 6693 (Appendix 35) were associated with the Staphylococcus aureus infection (Scott-Allen, 1977) which was discovered during the

examination of the animal subsequent to its death (see 'Acute and chronic effects of infusion').

Creatinine and creatine excretion during this experiment followed much the same patterns of change as were described in previous experiments. Creatinine excretion remained fairly constant but was reduced significantly during P3. this response would be expected of an animal with a diminishing tissue protein mass as would result from the treatments imposed on the animals during this experiment. Creatine excretion was closely related to N excretion and rose significantly during the period when negative retention occurred (P2).

The effects of treatments on the concentrations of the other metabolites measured during the experiment were in some instances quite marked.

P1 resulted in metabolite concentrations of the same order as those measured in the equivalent treatment period of Experiment 3.

The isoenergetic withdrawal of propionate from the infusions resulted in some major changes in plasma metabolite concentrations. Urea concentration and the albumin:globulin ratio in blood plasma tended to rise whilst the total protein and albumin concentrations were unchanged. The increased concentration of urea is likely to have been associated with the deamination of gluconeogenic amino acids for glucose production in order to meet the deficit resulting from propionate withdrawal. Albumin concentration was not significantly changed by the treatment and the mean globulin concentration for the period tended to fall. The albumin:globulin ratio measured in sheep 6693 was at the lower end of the normal physiological range during P2 and P3. This may have been the result

of elevated globulin levels which are often associated with bacterial infection (Manston & Allen, 1981). Total protein, the sum of the albumin and globulin fractions of the blood (Payne et al., 1970) fell slightly when propionate was withdrawn from the infusions, indicating that the animals had suffered a reduction in their protein status during the treatment (P2).

The concentrations of glucose, lactate, glycerol, NEFA, acetate, acetoacetate, and 3-OH butyrate in the blood plasma responded in exactly the same manner as in the equivalent treatment of Experiment 3. These responses were also similar to those observed in animals suffering energy and protein under-nutrition which in consequence were required to mobilize body tissue to make good the nutrient deficiency. This response to isoenergetic propionate withdrawal from the infusions occurred despite the provision of nutrients which were sufficient to provide an above-maintenance supply of energy to the animals.

The hormones measured in the blood plasma responded to propionate withdrawal from the infusions. The concentration of insulin tended to be reduced whilst plasma growth hormone, and cortisone tended to be increased. The concentration of glucagon remained unchanged which resulted in a reduction of the insulin:glucagon ratio in the blood plasma. The latter response is similar to that observed in fasted sheep (Gow et al., 1981) and is associated with the utilization of gluconeogenic amino acids for gluconeogenesis in the liver (McDowell, 1983).

The concentration of cortisol was increased by over 60% when propionate was withdrawn from the infusions. Cortisol is known to stimulate gluconeognesis and protein catabolism and is secreted in

response to reduced concentrations of glucose in the blood plasma (Bassett, 1968). The concentration of growth hormone was increased when propionate was isoenergetically withdrawn from the infusions. This is a similar response to that obtained in sheep that had been deprived of food (see Bassett, 1974; Gow et al., 1981).

Of the enzymes measured during P2 (Table III(32)) γ-GT and GOT activity tended to be increased in comparison to their activities in P1, whereas SDH activity tended to fall. The prolonged period (12d) in which propionate was isoenergetically removed from the infusions (P2) appears, therefore, to have had a degenerative effect on the liver resulting in a release of liver enzymes. The response of SDH activity to the treatment is more difficult to explain but may be associated by the fact that it is not exclusively produced in the liver and that release from other tissues may influence the plasma activity.

Linear and quadratic regression analyses of the results obtained in P2 identified certain statistically significant relationships between metabolite concentrations measured in the blood plasma and the time that sheep had been exposed to isoenergetic withdrawal of propionate from the infusions. There were significant negative correlations between glucose ($P < 0.05$), urea ($P < 0.01$) and lactate ($P < 0.01$) each with time. There were also significant positive correlations between 3-OH butyrate ($P < 0.05$) and acetoacetate ($P < 0.05$) each with time (Table III(34)).

These findings suggest that the hypoglycaemic/hyperketonaemic state induced in the animals when propionate was isoenergetically withdrawn from the infusions become more severe as the treatment continued. The adaptation in N status measured in the sheep was not

therefore associated with any similar improvements in glucose or gluconeogenic precursor status in the blood plasma. The progressive hyperketoaemia also suggests the animals to have suffered metabolic effects similar to those expected if there had been a continuous deficiency of energy during the experimental treatment.

The isoenergetic reintroduction of propionate to the infusions (P3) affected the concentrations of the metabolites measured when compared to their values in (P2). Urea, total protein and albumin concentrations were reduced together with the albumin:globulin ratio. The urea concentration was indicative of the associated reduction in N excretion measured during this period. There was also a tendency for total protein and albumin concentrations to fall progressively throughout the experiment which is associated with a long term reduction of protein status. The fall in albumin concentration may also have been partly responsible for the effects on albumin:globulin ratio that were observed.

During P3 the concentrations of the other metabolites measured in the blood plasma largely returned to values which were not significantly different to those measured in P1. The one exception to this trend was the concentration of 3-OH butyrate in blood plasma which remained significantly higher in P3 than in P1 ($P < 0.05$). It is difficult to reconcile this finding with the effects of the treatments on the other metabolites that were monitored during this experiment.

The prolonged isoenergetic withdrawal of propionate from the infusions was intended to facilitate the identification of possible adaptive trends in N utilization. The experiment succeeded in this task in so far as a tendency for N excretion to be increased during

the first 5d of the treatment and then to fall over the remaining period. Cusum analysis identified this trend but the effects were not statistically significant.

By extending the duration of the experimental treatment still further it may have been possible to have identified a statistically significant adaptation in N utilization. The period could not, however, be safely extended because of the potential risk of clinical hypoglycaemia and the additional problem of the suspected association between propionate withdrawal and immunological deficiency (see 'Acute and chronic effects of infusion').

The absence of adaptive effects on the other metabolites measured during the P2 treatment is an indication that the adaptation in N utilization was independent of the other metabolites measured or that the changes were too subtle to have been measured from their effects on blood plasma metabolite concentrations.

THE ACUTE AND CHRONIC EFFECTS OF INTRAGASTRIC INFUSION

During the development and use of the intragastric infusion procedure sheep and cows were wholly sustained by the infusion of nutrients for a cumulative period of 730d and 176d respectively. The infusions were carried out over a 2-year period and during this period the maximum period of continuous infusion undergone by an individual animal was 79d and the maximum cumulative period for an individual was 188d. Despite the effectiveness with which the technique was able to sustain animals for long periods of time, operation of the system was not without risks or side effects.

Acute complications

Rumen acidosis and hyperosmolarity. Prior to the development of equipment designed to control rumen pH, the most common cause of experimental failure was rumen acidosis and hyperosmolarity. The VFA solution designed to be infused into the rumen exerted an osmotic pressure (OP) of approximately 1700mosm/l and had a pH of 3. The composite solution flowing into the rumen, which comprised the VFA solution together with additional water, buffer and major minerals did so at pH 4.3 and OP 594mosm/l. This indicated the need for considerable VFA absorption by the rumen if maintenance of rumen homeostasis (pH 5.5-6.5, OP 250-350mosm/l - Englehart, 1969) was to be achieved. If disparity between the VFA infused and that absorbed did occur it resulted in a progressive reduction of the pH of the rumen fluids together with increased OP. When they are associated, these factors are known to inhibit reticulo-rumen motility (Bruce & Huber, 1973) and cause rumen stasis and reduced absorptive efficiency

(Ahrens, 1967). When this occurred the result was mucosal erosion and haemorrhage which was associated with dehydration of the blood, due to osmosis of tissue water into the rumen; seepage of rumen fluids from the cannula; and the excretion of blood stained, fluid diarrhoea. Post mortem investigation of the alimentary tract of animals suffering this disorder revealed large areas of the mucosa which had undergone necrosis and haemorrhage. Similar lesions and considerable erosion were also observed in the abomasum.

The major cause of the rumen acidosis and hyperosmolarity was cessation of the buffer or water infusions which was the result of occluded infusion lines. The pH control equipment introduced to restrict the infusion of VFA to periods when rumen pH was above 5.8 effectively eliminated the risk of acidosis.

Despite the improvements it was found that a finite limitation on the gross energy value of infusions existed. This was because of the relationship between the acidity and the osmotic pressure of the fluids in the rumen and the volume of fluid the rumen could contain before seepage would occur from the cannula. Increments in the quantities of VFA infused into the rumen resulted in increased OP and decreased pH. The additional buffer required to restore pH to normal further increased OP and consequently there was a need for more water in order to maintain the OP homeostasis of the rumen. A finite limit to the volume of infusions of approximately 0.8-1.0L/kg$W^{0.75}$ per d thus had a regulatory effect on the potential quantity of VFA which could be administered.

Medium term effects

The sustenance of sheep and cows with infused liquid nutrient solutions over long periods of time resulted in various complications.

Hypercupraemia. Transition from normal to intragastric nutrition resulted in a consistent effect on the concentration of copper in blood plasma (see Appendices 21, 27, 34, 41 and 48). Previously normal concentrations of copper measured in blood plasma (9.4-19μmol/l) (Doxey, 1977) were increased to non-physiological concentrations (30.73 $\pm$ 1.36μmol/l, n = 40). Measurement of the copper concentration of all infusates was undertaken but no probable causal agents were identified. To discover whether the source of copper causing the hypercupraemia was of endogenous origin, tissue samples were obtained by biopsy and post-mortem surgery. Tissue obtained from the brain, kidney and liver of sheep given normal rations and sheep sustained by the infusion of nutrients contained copper in physiological concentrations (Table III(35)). Analysis of blood revealed no indications of haemolytic crisis or haemoglobinurea despite the doubling of the concentration of copper in the blood plasma. There were also no consistent indications of liver necrosis as reflected in associated leakage of liver-bound enzymes into the blood (SDH, γ-GT and GOT - see Appendices 19, 25, 32, 39 and 46) nor was there indications of kidney disfunction as indicated by changes in plasma urea concentration.

Post-mortem examination of 7 animals revealed only one instance where toxicity may have occurred. Perivascular oedema of

Table III(35): The concentration of copper measured in selected tissues from sheep given normal rations and those by the intragastric infusion of nutrients.

Tissue	No. Samples	Copper concentration[2] µmol/kg DM or µmol/l	Normal range[1] µmol/kg DM or µmol/l
Liver (biopsy)[4]	3	1143 ± 236	314 - 7850
(post mortem)[5]	7	3729 ± 939	314 - 7850
Kidney[5]	5	264 ± 11	141 - 314
Brain[5]	2	358 ± 53[3]	157 - 314
Blood (Plasma)[5]	40	30.73 ± 1.36	9.4 - 19.0

1. As per the West of Scotland Agricultural College Veterinary Investigation Centre. 2. Means ± SE. 3. Mean ± SD. 4. Sheep given normal rations. 5. Sheep sustained by intragastric infusions.

the brain together with tubular nephrosis, interstitial fibroplasia and glomerular atrophy of the kidney together with congestion of the liver by fatty vacuolation of the hepatocytes may have been indicative of the disorder (Church, 1979a; Ishmael et al., 1971). However, of these symptoms, the fat infiltration into the liver may have been associated by hyperketonaemia experienced during treatments where propionate was withdrawn from the infusions (Payne, 1977). Despite this isolated case where symptoms of toxicity were found the majority of animals suffered few adverse physiological effects on those tissues most vulnerable to injury from copper. The effects of, in most instances, an eight-fold increase in fluid intake associated with the changeover to intragastric feeding may have also contributed to the observed kidney deterioration.

During treatments where exogenous glucose-precursor supply was restricted it is possible that hepatic proteolysis could have been responsible for the hypercupraenima. nearly 80% of the total copper found within the body is located in the liver (Dick, 1954); largely incorporated into mitochondria, microsomes, nucleii and the soluble fraction of parenchymal cells. A hepatic release of copper into the circulation of 2.6mg/d would be all that is necessary to cause the observed elevation in the plasma copper concentration (i.e. from 15µmol/l to 30µmol/l). The liver of a sheep with a normal copper concentration of approximately 254mg/kg DM (W.O.S.A.C. - Veterinary Investigation Centre) and a dry matter content of 330g/kg fresh weight (Altman, 1972) need only to undergo a 5% reduction in freshweight (Kauffman, 1978) to allow such a release of copper into the blood. Since, during hypophagia, up to 40% of liver protein can be deaminated within the first few days (Vernon & Peaker, 1983) it is

possible that hepatic proteolysis could be responsible for the observed hypercupreamia during periods of isoenergetic withdrawal of propionate from the infusions.

An explanation of the elevated concentration of copper in blood plasma during periods of protein balance or accretion, is more difficult to find. Hypercupraemia is, however, characteristic of most acute and chronic infections found in humans (Underwood, 1977) and is also associated with a leukocytic endogenous mediator (LEM), which when released, stimulates hepatic synthesis of ceruloplasmin (Beisel et al., 1974), a blue copper-protein in which form, 90% of the copper in blood exists. Elevated ceruloplasmin levels have also been observed by McCosker (1968) in several disease conditions of sheep and similar increases were observed with other 'stressors' including ACTH and hydrocortisone. Starcher and Hill (1965) went further and suggested that any stresses or any condition resulting in elevated corticosteroid concentrations in the blood could increase ceruloplasmin concentration. It may be possible then that the 'stress' resulting from the 'infection' of sheep with surgical implants which did bring about an immune response (see later section) together with the continuous microbiological challenge associated with such preparations were enough to initiate the action of LEM's. Of the corticosteroids, only cortisol concentration in the blood plasma was determined in the course of the experiments and values remained within the physiologically normal range of 15-65ng/ml (Doxey, 1977) except when glucose precursor supply was restricted. The concentrations of other corticosteroids in the blood were not determined but it is possible that these substances may have been elevated and that an explanation for the observed hypercupreamia may

result from the stress induced by the surgical modifications and the dietary and behavioural changes imposed on the experimental animals.

Immunodeficiency. There were indications that prolonged periods of exogenous glucose-precursor restriction were associated with an increased susceptibility to bacterial infection of the sheep. It was discovered on post-mortem examination that 3 from a total of 4 sheep subjected to prolonged malnutrition through propionate exclusion treatments suffered, to varying degrees from the formation of abscesses. The proliferation of Staphylococcus aureus infections in the region of the abomasal catheter sinus which was located within the intramuscular facia of the right flank was responsible and subsequently lead to a systemic infection in one of the animals. Animals subjected to longer periods of infusion per-se but with short or intermittent periods of propionate exclusion remained free from such infections.

Malnutrition and the catabolic state resulting have been closely associated with reduced disease resistance (Sheffy & Schultz, 1977). The ensuing corticosteroid release associated with energy undernutrition release was implicated in immunosuppression and the promotion of thymic atrophy (Scott et al., 1976).

Long term complications

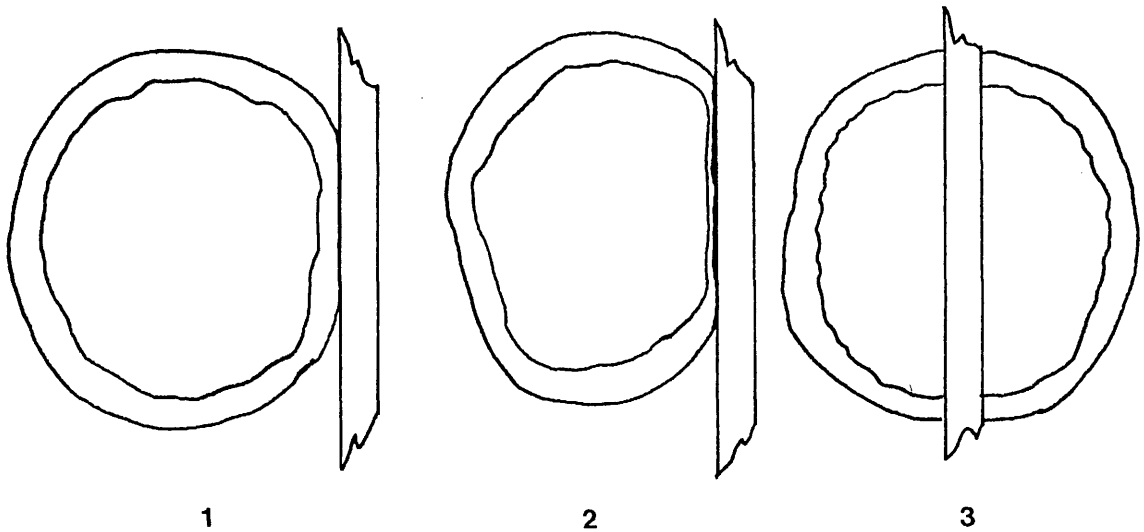
Mucosal atrophy. Post-mortem examination of the alimentary tract of animals subject to prolonged periods of intragastric infusion revealed a degree of mucosal atrophy in the rumen which appeared not to be related to the effects of acute acidosis. Areas of papillae were loosely associated with the submucosal tissue and readily

sloughed by the force of finger pressure.

Considerable quantities of mineral sediment were also observed in the rumen and both complications may have been associated with the low rumen motility that was observed throughout the experiments and the small extent to which epithelial desquamation had occurred in the rumen despite the inclusion of abrasive polythene pan scourers for that purpose.

Changes within the morphology of the lower gut were also observed. The walls of the small and large intestines had become atrophied, the tissue from which had become thin and transluscent.

Cannula rejection. Abomasal cannulation involved the insertion of a thin cannula into the pyloric region of the abomasum which subsequently proceded vertically between the viscera and the body wall to a point of exteriorization between the last rib and transverse processes of the right flank. Varying degress of tissue rejection were observed in response to the insertion. Usually the abomasal cannula was rejected outwards into the intermuscular fascia of the right flank with the formation of a discrete sinus running between the point of exteriorization and an area close to the abomasal entry point. In some instances rejection in the outward direction was considerable and this resulted in the exteriorization of part of the cannula. Also observed were instances of ingrowth where the cannula invaded and had become enclosed by, the small intestine (Figure III(8)). Despite the severity of the lesion there were no indications of digesta leakage or peritonitis. However, the possibility of the occurrence of such disorders should not be ignored. Consequently, rejection of the abomasal cannula imposes



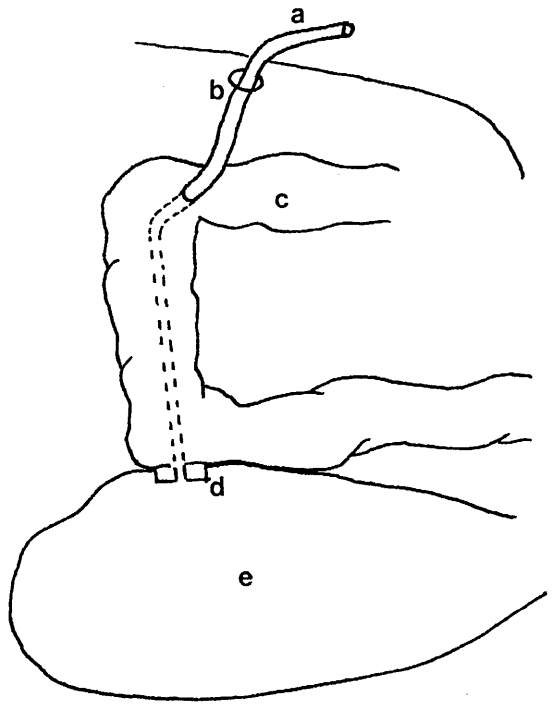


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Figure III(8): Diagrammatic representation of the invasion of the small intestine of a sheep by an implanted abomasal cannula.

1. Intestine and cannula in close proximity.
2. Erosion of intestine wall as a result of contact.
3. Invasion of intestine by the cannula.
4. Overview: *a*. Abomasal cannula. *b*. Exteriorization point.
   c. Small intestine. *d*. Cannula cuff.
   e. Abomasum.

obvious limitations on the patency of the preparation and lifespan of the prepared animals. There were no obvious indications of rejection observed in animals prepared with rumen cannulae.

SECTION IV

GENERAL DISCUSSION

SECTION IV

GENERAL DISCUSSION

Using the intragastric infusion technique it was possible during the experiments reported in this thesis to make selective and precise changes in the animals' nutrient supply. This allowed the metabolic responses to specific changes in nutrient supply to be studied and provided a novel method for investigating aspects of gluconeogenesis and of the intimate interrelationships between energy and protein metabolism in the ruminant.

Energy Metabolism

When the animals were given 'control' infusions containing acetate, propionate, butyrate, casein and lipid, with or without glucose, they apparently maintained their homeostasis and, as judged from the analysis of various blood constituents (see experimental results) were physiologically normal (see Appendix 54) and in positive energy balance. However, the isoenergetic withdrawal of propionate from the infusions had marked and consistent effects on the concentrations of many of the metabolites and hormones that were measured in the blood plasma. These changes were indicative of changes in the animals' utilization of substrates and possibly in the animals effective energy status. Specifically, the concentrations of glucose, lactate and insulin were reduced and there was a fall in the insulin:glucagon ratio. The concentrations of acetate, acetoacetate, 3-OH butyrate, NEFA, urea and cortisone were increased as was the activity of γGT. These changes were similar to those observed in

ruminants suffering from energy undernutrition (see Baird et al., 1972; Blum et al., 1981; Bouchet & Paquay, 1981; Munro et al., 1964; Faulkner & Zammit, 1985 and Barakat et al., 1983), where it is thought that a reduction in propionate supply (Brockman, 1978; Istasse et al., 1985) or falling concentrations of glucose (Tao & Asplund, 1975) in blood plasma may lead to a reduced insulin:glucagon ratio and a mobilization and catabolism of tissue fat and protein. Lipolysis of adipose tissue results in a release of NEFA into the blood, and their partial oxidation in the liver leads to the production of 'endogenous' acetate, 3-OH butyrate and acetoacetate (Baird, 1977). The release of amino acids, from muscle tissue and their deamination in the liver results in a greater need for detoxification and excretion of amino groups and there is an associated increase in urea production and in plasma urea concentration (Bender, 1985).

The changes in the blood constituents that were measured in response to propionate withdrawal suggest that net lipolysis and proteolysis had occurred despite the provision of infusions designed to supply energy in quantities well above the accepted theoretical requirement for maintenance (ARC, 1980). This assertion may be questioned, however, on the basis that the efficiency of utilization of infused acetate and butyrate might be reduced under conditions where glucose is in short supply (MacRae & Lobley, 1982). This would lead to a corresponding increase in the amount of acetate and butyrate needed to meet the animals energy requirements for maintenance. In experiments with fasting sheep, Armstrong and Blaxter (1957a) found that mixtures of acetate, propionate and

butyrate (750:150:100 mmol/mol and 500:300:200 mmol/mol) given intraruminally were utilized with a calorimetric efficiency of between 85.5 and 83.0%. Efficiencies of 86% and 76% were also measured for propionate and butyrate given as single infusions, but when acetate alone was given the efficiency was 59%.

In Experiments 1, 2, 3 and 4 the mixtures of VFA infused were 650:250:100 and 860:0:140 mmol/mol in the initial control and propionate withdrawal periods respectively. In the case of the former it would be safe to assume a k_m value of 75% and on this basis the infusion of 495 kJ ME/kg$W^{0.75}$ per d for a typical 60 kg sheep would provide maintenance plus an excess of 3715 kJ ME/d. Assuming a k_f of say 60% (ARC, 1980) this would allow the retention of 2229 kJ/d. The efficiency of utilization of the mixture of VFA containing acetate and butyrate alone is more difficult to estimate but even if the most pessimistic view is taken i.e. that k_m was a low as 59% the infusates given were sufficient to supply maintenance plus an excess of 1829 kJ ME/d. Furthermore, if the lowest reported value for the efficiency of utilization of infused acetate for fattening of 33% (Armstrong & Blaxter, 1957) is applied to the infused acetate/butyrate mixture still provided energy sufficient for the retention of 603 kJ ME/d.

Thus, if the observed changes in blood composition measured when propionate was isoenergetically withdrawn from the infusions were associated with tissue mobilization, that mobilization seems likely to be related to specific limitations in nutrient supply rather than energy deficiency per se. It is also probable that the metabolic response observed was initiated by the falling insulin:glucagon ratio in blood plasma. It can be argued that, in

turn, this was precipitated by a reduction in propionate supply from the gut or reduced blood glucose concentration which resulted from the withdrawal of propionate from the infusions.

Protein Metabolism

The N required for maintenance. The initial stages of the 4 experiments carried out were designed to equilibrate the animals in a state of zero N accretion. The quantities of casein N given to the animals to bring about this condition were calculated from daily N balance data and it was found that the casein N requirement for maintenance for the cow used in Experiment I was equal to the mean values calculated from the 9 sheep used in the subsequent experiments (Table IV(1).

The mean quantity of casein N required in the infusions to provide the N needed by the animals for maintenance was 463 $\pm$ 14 mg casein N/kgW$^{0.75}$ per d. This quantity of N when given in the form of sodium caseinate would provide approximately 370mg N/kgW$^{0.75}$ per d at the tissues if an efficiency of absorption of 1.0 and an efficiency of utilization of absorbed N of 0.8 are assumed (see Black et al., 1973; Roy et al., 1970).

This N allowance is smaller than the equivalent value of 429mg N/kgW$^{0.75}$ per d calculated from the measurements of endogenous urinary N excretion taken during the infusion experiments of Hovell et al. (1983) at the Rowett Research Institute. Conversely, it is greater than the 108mg N/kgW$^{0.75}$ per d recommended by the ARC (ARC, 1980).

Variance between the values calculated from experiments involving the intragastric infusion of nutrients and those undertaken

Table IV(1): The N required (mg/kgW$^{0.75}$ per d) by the experimental animals for tissue maintenance.

Experiment no.	Species	
	Cow	Sheep
1	370 (n=1)	-
2	-	380±10 (n=4)
3	-	330±0 (n=2)
4	-	390±30 (n=3)

Values are means ± SE or SD. The grand mean (n=10) was 370±14 mgN/kgW$^{0.75}$ per d.

at the Rowett Research Institute may be attributable to differences in the liveweights of the experimental animals used (see ARC, 1980). Work here involved the use of 8 sheep with liveweights ranging from 52-72kg whereas sheep of 26-30kg were used by Hovell and his co-workers (Hovell et al., 1983). It is difficult, however, to reconcile this argument with the findings of Experiment I, where the N requirement of a 500kg LW cow was equal, on a metabolic liveweight basis, to the mean N requirements of sheep used in 3 subsequent experiments (see Table IV(1)). The discrepancy between the measured values discussed above and those originally recommended by the ARC (ARC, 1980) is due to fundamental differences in the methods of derivation. The ARC employed a factorial calculation based on an estimate of endogenous urinary N excretion (UN(e)) with a small additional allowance for dermal loss. Disregard of metabolic faecal N (MFN) loss and the possible diversion of endogenous N to microbial N in certain nutritional circumstances (see Hovell et al., 1983; Ørksov & Macleod, 1982) resulted in underestimates of the requirements of N for maintenance. Accordingly, Ørskov and Macleod (1982) showed the $UN_{(e)}$ of cattle measured using intragastric infusions to be 300-400mg $N/kgW^{0.75}$ per d and that the addition of faecal N to UN(e), as measured in normally fed animals, gave a total N excretion of the same order. Acceptance of the inaccuracies of the ARC (1980) recommendations led to the introduction of a revised allowance of 350 mg $N/kgW^{0.75}$ per d (ARC, 1984) during the course of the experimental work reported in this thesis.

The effects of propionate withdrawal from the infusions on N utilization. The isoenergetic withdrawal of propionate from the infusions in all experiments had a marked and consistent effect on N utilization. The N excreted by sheep in Experiments 2-4 increased from a mean of 450mg $N/kgW^{0.75}$ per d to 620mg $N/kgW^{0.75}$ per d. Reintroduction of propionate in Experiments 3 and 4 or glucose in Experiment 2 at equivalent doses to those administered during the initial control period of each experiment consistently resulted in a reduction in N excretion to levels below those measured in the initial period.

Such responses are indicative of an improvement in the efficiency of utilization of casein N during a period in which glucose-precursor supply was restricted. Furthermore, the adaptation process appeared to have residual characteristics and its effects were measurable following the reintroduction of adequate exogenous glucose precursors (see Experiments 2 and 3).

Attempts to describe the time course of the improvement in the efficiency of utilization of N in terms of changes in excretion with time were unsuccessful. In Experiment 3, subsequent to a short period in which propionate was withdrawn from the infusions, animals were subjected to a prolonged period in which propionate supply was restricted to 50% of the control level (i.e. that in the initial period). However, the results suggested that provision of propionate at 50% of that given in the control period was adequate to meet the animal's requirements. it is quite probable that those requirements had changed in response to the complete withdrawal of propionate from the infusions in the preceeding period.

Experiment 4 was designed to impose the most prolonged and severe restriction on glucose-precursor supply to the animals that appeared to be experimentally attainable and even in this experiment, it was not possible to show a statistically significant relationship between N excretion and time during the period in which the adaptation in N metabolism was thought to occur though this failure, in part, reflected the day to day variation in the N balance data. Further analysis of the N data using the 'cusum' technique however did indicate that there was a change in the N excreted by sheep, implying an improved efficiency of utilization after 4-5d following propionate withdrawal from the infusions (Figure III(6)). Once again, however, the technique of analysis did not provide a stastically significant effect (i.e. $P > 0.05$). In order to increase the amount of data for statistical analysis the N data for the initial periods in which propionate was withdrawn from the infusion (i.e. P2 of Experiments 2, 3 and 4) were pooled. When these results, representing values for, at maximum, 9 animals were subjected to polynomial regression analysis a relationship between N excretion and time was revealed which was described by the the 3rd level polynomial regression equation (Figure IV(1):

$$y = 0.4427 + 0.131x - 0.02383x^2 + 0.001220x^3$$

$$(r = 0.70, P < 0.05)$$

where y = N excreted/kg$W^{0.75}$ per day

x = The time (d) following propionate withdrawal from the infusions.

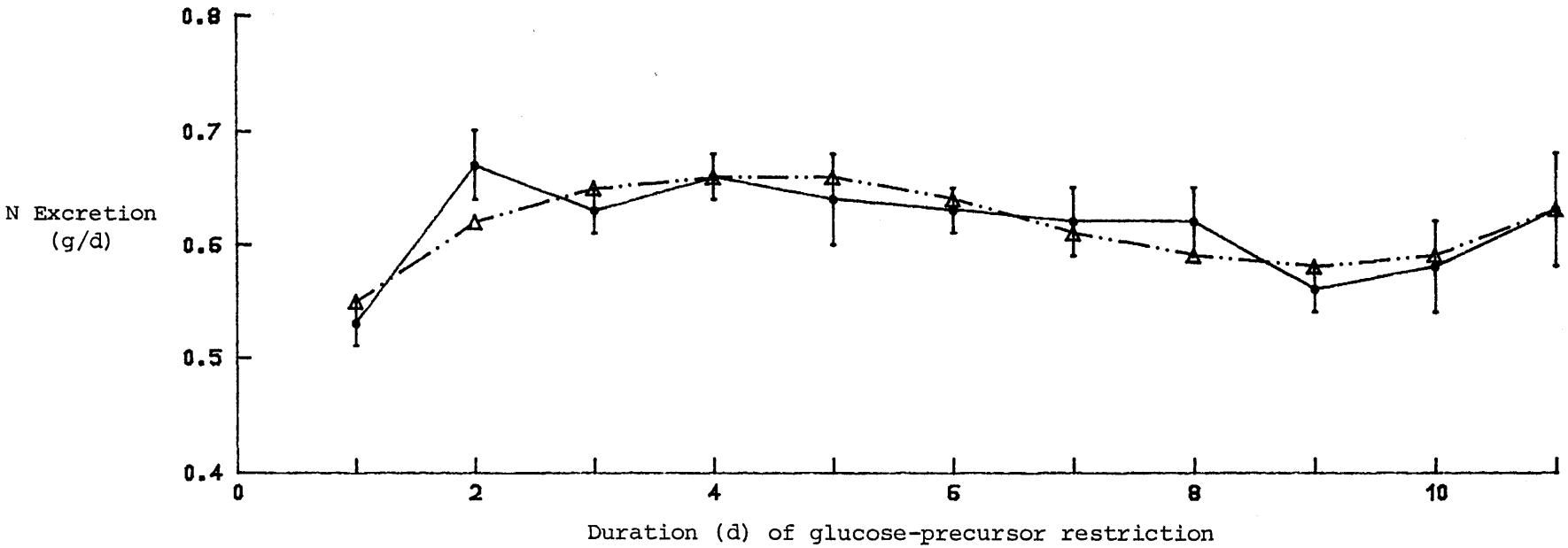

Figure IV(1): The effect of glucose-precursor restriction on the N excreted by sheep. (●—●) Mean values ± SEM. (△···△) Predicted values from the polynomial regression equation $y = 0.4427 + 0.131x - 0.0238x^2 + 0.00122x^3$. ($r = 0.7$, $P < 0.05$).

It is apparent from the analysis that after an initial increase in N excretion during the first 4d of treatment N excretion proceeded to fall. A further increase in the excretion of N then apparently occurred during the last 2d of the treatment, though this effect may reflect the fact that only 3 animals were subjected to the propionate withdrawal treatment for periods as long as 11 days and atypical quantities of N were excreted by 1 of those animals (see Experiment 4).

The net result of the observed improvement in the efficiency of utilization of casein N was that the casein N requirement for maintenance was effectively reduced. Similar improvements in the efficiency of utilization of protein was reported by Hovell et al. (in press) with sheep sustained by the intragastric infusion of nutrients and subjected to periods of protein undernutrition.

Plasma amino acid concentrations. The effects of the experimental treatments on the concentrations and relative proportions of amino acids in blood plasma were generally quite marked. The interpretation of such data from 'normally' fed animals presents difficulties due to the post-prandial variation in energy and protein supply, associated with meal feeding and its effect on amino acid flux through amino acid pools which vary in size in response to transient changes in nutrient supply.

However, the use of intragastric infusion to maintain a constant energy and protein supply should ensure that amino acid supply to the liver is more or less constant and that the utilization of amino acids for oxidation and gluconeogenesis also remains constant. Accordingly, it can be argued that under these

circumstances changes in the concentrations or relative proportions of specific amino acids in response to changes in nutrient supply more accurately reflect changes in the balance between their release into the blood from the tissues and their uptake and use by other tissues for protein synthesis, gluconeogenesis and oxidation in response to the treatments imposed. Similarly, if the total amino acid concentrations are unaffected by treatments changes in the proportions of amino acids will also reflect changes in these plasma concentrations.

When the animals were given 'control' infusions the plasma amino acid 'profiles' were similar to those found in the physiologically normal range (see Table IV(2)) of a conventionally fed sheep. With propionate withdrawn (see Table IV(3)) from the infusions there were marked reductions in the proportions and concentrations of the NEAA's - alanine, citrulline and glycine, serine and aspartate. Smaller reductions in the proportions of glutamate and proline were also observed together with an increase in cystine and little change in the proportion of ornithine.

A concomittant increase in the proportions and concentrations of the EAA's - arginine, histidine, valine, threonine, soleucine, phenylalanine, lysine, leucine was also measured together with a small reduction in the proportion and concentration of methionine in blood plasma.

These findings largely follow the same patterns of change as the reported changes in plasma amino acid concentrations which occur in response to starvation in sheep and cows (see Koenig & Boling, 1980; Baird et al., 1972; Baird et al., 1977; Heitman & Bergman, 1980).

Table IV(2): The concentration of amino acids (μmol/100ml) in the blood plasma of sheep[1] and cows[2].

Amino acid	Sheep	Cow
Threonine	15.3	8.40
Methionine	2.8	2.00
Isoleucine	11.9	17.00
Leucine	16.2	13.00
Phenylalanine	6.2	4.20
Lysine	18.7	8.22
Histidine	9.9	6.41
Arginine	15.6	7.50
Valine	22.7	26.50
Aspartate	4.5	1.96
Serine	10.8	8.57
Glutamate	22.1	6.12
Glycine	47.9	24.00
Alanine	25.9	18.00
Tyrosine	7.4	3.84
EAA	119.3	-
NEAA	118.6	-
TAA	237.0	-
NEAA-Glycine	70.7	-
TAA-Glycine	190.00	-
EAA/NEAA	1.01	-

1. 46kg wethers fed alfalfa hay:concentrate diet (Cross et al., 1975). 2. Vernon & Peaker (1983).

Table IV(3): The effects of isoenergetic propionate withdrawal on the proportions(mol/100mol) of amino acid in blood plasma.

Amino acid	% change in the proportion of amino acid			
	Experiment			
	2	3	4	Mean change (+/- SE)
	Non-essential amino acids			
Alanine	-55*[1]	-67*	-64*	-62 (3.6)
Citrulline & Glycine	-27	-33*	-41*	-34 (4.1)
Serine	-45	-55*	-44*	-48 (3.5)
Cystine	24	100	31	52 (24.0)
Glutamate	-7	-10	-18	-12 (3.3)
Proline	-18	-26	5	-13 (9.3)
Aspartate	-23	-41*	-37	-34 (5.5)
Ornithine	6	38	44	29 (12.0)
	Essential amino acids			
Arginine	56	28	34	39 (8.5)
Histidine	8	10	8	9 (0.7)
Methionine	-22	-9	-9	-13 (4.3)
Valine	128*	99*	95*	107 (10.0)
Threonine	14	24	1	13 (7.0)
Isoleucine	73	105*	89*	89 (9.2)
Phenylalanine	17*	14	29	20 (4.6)
Tyrosine	1	16*	3	7 (5.0)
Leucine	74	120*	107*	100 (14.0)
Lysine	1	50*	7	19 (15.4)
Total amino acid conc. (µmol/100ml)	0	4	-2	0.7 (1.8)

Values refer to the % change in the proportions of the amino acids when P1 and P2 values from each experiment in which amino acids were measured are compared. 1. * denotes that the change was significant ($P < 0.05$) for the particular experiment referred to.

It appears that in both of these circumstances the rate of utilization of NEAA for gluconeogenesis and oxidative purposes exceeds the rate at which they became available through mobilization, and the blood plasma concentration falls. That a reduction in NEAA concentration in blood plasma provides circumstantial evidence for their utilization for gluconeogenesis is also in accord with the view expressed by Egan et al. (1970) and Wolf and Bergman (1972).

The concomittant increase in the concentration and proportions of the EAA suggests they contribute to a smaller degree than the NEAA's to gluconeogenesis or oxidation. This response is broadly in keeping with the view that EAA's are spared for essential protein synthesis whereas NEAA's are more readily used to supplement gluconeogenic requirements. However, there were inconsistent changes in the proportions of methionine, cystine, glutamate, proline and ornithine which should be commented upon.

It can be argued that glutamate and alanine, being major contributors to gluconeogenesis (Bergman & Heitman, 1978), would behave similarly when propionate was withdrawn from the infusions. The fact that glutamate concentration remained relatively unchanged in response to propionate withdrawal may be the result of an unusual modification in its role in metabolism. It is possible that under conditions where gluconeogenic demand for the amino acid is especially high, availability for its critical role in transamination reactions is threatened. To ensure its continued supply it may therefore be spared from gluconeogenesis and effectively assume the role of an EAA. It is also interesting to note that the concentration of proline, the precursor of glutamate (see Bender,

1985) was similarly relatively unresponsive to the withdrawal of propionate from the infusions.

The effect of propionate withdrawal on the sulphur amino acids was characterized by a slight reduction in the proportion of methionine in blood plasma and an increase in the proportion of cystine. The response of methionine is in contrast with the other EAA's and suggests that there was a large requirement for the amino acid. This finding is not inconsistent with its role as the first limiting amino acid in protein synthesis (Schelling et al., 1973; Stokes et al., 1981).

The increase in cystine concentration, which was largely in contrast with the response of the other NEAA's that were measured, may reflect the intimate association between cystine and methionine. A reduction in the quantity of cystine taken up for oxidation or gluconeogenesis may be envisaged to reduce the demand for methionine from which cystine is synthesized.

The proportion of ornithine in the blood plasma also increased despite it being a non-essential and gluconeogenic amino acid. This may reflect an increase in urea cycle activity, in which ornithine is an intermediate in the conversion of the NH_3 released from protein catabolism into urea before excretion.

By inference it can be argued that the concentrations of the other urea cycle intermediates, citrulline and arginine should also be increased in this situation together with a reduction in the substrate, aspartate. However, in the case of citrulline the change may have been obscured by the fact that it was measured in combination with glycine. The concentration of aspartate was reduced but its status as a gluconeogenic NEAA makes it inadvisable to

conclude that the change was solely due to its use in the urea cycle. Interpretation of the change in arginine concentration is also subject to the same limitation because there appears to be a degree of sparing of almost all EAA's in response to the treatment imposed.

In summary the results suggest that when propionate is withdrawn from the infusions the rate of utilization of the NEAA's -, alanine, serine, citrulline and glycine and to a lesser extent, aspartate, became higher than their release from mobilized tissue and as such they were major contributors to gluconeogenesis. This was not the case with the EAA's - valine, leucine, isoleucine and arginine, the proportions of which were increased in response to propionate withdrawal. This is evidence for a protective mechanism by which these amino acids are used sparingly for gluconeogenesis and oxidation in order that the demands made upon them for critical synthetic purposes may be met.

Protein and energy interaction

It may be argued that the changes in the concentrations of blood plasma constituents and N excretion that were described above in response to propionate withdrawal may be intimately associated. It has been demonstrated that when exogenous glucose-precursor supply is restricted, 'glucose exclusion' occurs in which glucose utilization is minimised in order that it may be spared for the animal's critical requirements (see Lindsay, 1970 & 1979; Owen et al., 1967; Leng, 1970a). This adaptation is likely to have occurred in the experimental animals in response to the withdrawal of propionate from the infusions and may also be associated with the increase in efficiency of utilization of N that was measured.

Accepting that the association does exist, a credible explanation for the observed reduction in the amount of protein catabolised for gluconeogenesis in response to successive propionate withdrawal treatments (see Experiment 2, P2 Vs P5) is provided. Furthermore, the characteristic effects of glucose exclusion appear to be residual and the responses in N excretion shown by the animals to this adaptation deserve consideration. During Experiment 2, successive 'control' treatments (i.e. P1 and P5) were interspersed with periods where glucose precursor supply was restricted. The effect of these treatments was a reduction in the N required for maintenance. If this effect is associated with glucose exclusion, which seems likely, it may be argued that an identifiable component of the N requirement in the initial control period (P1) was reserved for oxidation or gluconeogenesis rather than protein synthesis. When the requirement for glucose was reduced as a result of residual effects of glucose exclusion the protein originally destined for gluconeogensis was 'spared' and made available for protein synthesis with the net result that a reduction in the maintenance N requirement was measured.

Similar protein 'sparing' effects were observed in sheep receiving protein free infusions supplemented with glucose precursors (Asplund et al., 1985).

Glucose utilization

An objective of the experiments was to provide an alternative, indirect means of estimating the glucose requirements of ruminants. From the N data obtained during Experiments 2, 3 and 4, excretion was increased from a mean value of 450mgN/kgW$^{0.75}$ per d to 620mgN/kgW$^{0.75}$ per d in response to propionate withdrawal from the isoenergetic and

isonitrogenous infusions. If it is assumed that an average amino acid contains 14.3% N; that deaminated amino acid yields glucose with an efficiency of 0.58 (Krebbs, 1965); and that no potentially gluconeogenic amino acid is directly oxidized, it can be calculated that the synthesis of 2.52g glucose/kg$W^{0.75}$ per d or approximately 54g/d for a 60kg sheep was possible.*

This estimate however, does not take account of other gluconeogenic endogenous substrates. Glycogen, lactate and glycerol are potential contributors to the glucose pool when propionate is withdrawn and it may be argued that their omission from the calculation would result in underestimation.

The concentrations of certain metabolites that were measured in the blood plasma of animals given isoenergetic, propionate-free infusions indicated that a net lipolysis was likely to have occurred despite the provision of an above-maintenance energy supply. Specifically concentrations of acetate, acetoacetate, 3-OH butyrate and NEFA were significantly increased in response to the treatment and there was a reduction in the insulin:glucagon ratio. The hormonal changes that were measured would also be permissive in a release of hepatic glycogen into the blood. However, there is evidence to suggest that when heavy demands are made upon this source of glucose precursor it is exhausted in less than 24hrs (see Vernon & Peaker, 1983). All values of N excretion measured in the experiments reported in this thesis were period means from which measurements made on the first day of the period were omitted. Accordingly, it is unlikely that glycogen was a significant contributor to the glucose pool after the first day of the propionate-withdrawal treatment and its effect on the calculation therefore was equally insignificant.

*Refer to Appendix 56.

A similarly insignificant contribution to the glucose precursor pool may be attributed to lactate when propionate is withdrawn from the infusions. Lactate released into the blood as part of the Cori cycle (see Cori, 1931; Cori & Cori, 1929; Giesecke & Stangassinger, 1980) may be considered as 're-cycled glucose' and as such made no net contribution to glucose precursor supply.

The contribution of triacylglyceride glycerol to the glucose pool is difficult to determine quantitatively from the experimental results. Estimates of the contribution of glycerol to gluconeogenesis in fasted sheep, using isotope dilution procedures, however, have been as high as 40-50% (see Bergman, 1973; Bergman et al., 1968) but these high values may be due to the inherent error associated with such procedures.

An approximation of the upper limit of glycerol's contribution to gluconeogenesis provides evidence to support the view that the above values are too high. If it is assumed that 80% of the energy utilized by fasted sheep is provided from adipose tissue (see Vernon, 1981; Lindsay, 1975) a fasting 60kg sheep would require to mobilize approximately 99g fat/d (this value assumes a fasting metabolism of $0.23 \frac{MJ}{kgW^{0.73}}$ MJ per d and that the gross energy value of animal fat is 39kJ/g). The major fatty acids constitutents of the adipose tissue of sheep are palmitate (C_{16}, 29%); stearate (C_{18}, 25%); and monoenic acids ($C_{18:1}$, 36%) (see Lister et al., 1983). Based on this analysis an average composition of $C_{16.5}H_{34}COOH$ may be assumed for each of the fatty acids in the adipose tissue triglyceride. Lipolysis would result in the release of 1mol glycerol (92g) per mol of triglyceride (869g) catabolised. The lipolysis of 99g fat/d therefore would result in the release of 10.48 glycerol, equivalent to the production

of 1021 g/d glucose. Thus the absolute maximum quantity of glycerol released from the adipose tissue of fasted sheep would be equivalent to approximately 19% of the glucose utilized by the fasted animal. it is improbable that glycerol would actually contribute this quantity of glucose, however, because maximum efficiencies were assumed for the conversion of triglycerides to fatty acids and glycerol and the conversion of glycerol to glucose. Accordingly, a preferred value of 15% could be adopted as the maximum potential contribution of glycerol to the glucose pool. Taking glycerol into account the estimated glucose requirement would be increased from 2.52 to $2.9 g/kgW^{0.75}$ per d. As discussed earlier, the effects of propionate withdrawal on substrate supply were such that they are likely to have brought about adaptive changes in glucose utilization. Accordingly, the utilization rates calculated above may be considered to be equal to the animal's minimal requirements. Similar rates of utilization should, therefore be found in fasted sheep in which adaptations designed to spare glucose would also have occurred. For comparison, the glucose utilization rates measured in fasted, non-pregnant, non-lactating sheep from various sources are shown (Table I(9)). Determination of the rates were all made using continuously infused radio-isotope dilution procedures. The values determined by the isotopical procedures tended to be higher than the values calculated during this series of experiments and the isotopic data showed considerable variation both between and within estimates. Reasons for the variation have been discussed in detail previously (see Introduction) and largely result from procedural inconsistencies between laboratories and physiological differences between the animals used. Recycling of labelled C through the glucose pool also

causes difficulties in the intepretation of the results obtained, inevitably leading to overestimation of the rate of irreversible loss of marked glucose from the pool.

The overestimation of glucose irreversible loss and, therefore, the animal's glucose utilization when measured using isotopic techniques probably contributes significantly to the differences between estimates made previously and those made using the intragastric infusion technique. It is likely that the estimate of 2.9g glucose/kgW$^{0.75}$ per d is, therefore, a more accurate reflection of the minimal glucose requirement of the sheep than the rather higher values derived from radio-isotope dilution procedures. Further evidence supporting this view is provided by Asplund et al. (1985) who proposed a value of 2.71g glucose/kgW$^{0.75}$ per d based on the gluconeogenisity of the glucose precursors theoretically available to a starved sheep.

The intragastric infusion technique as a research tool

The experiments conducted here, in which the intragastric infusion procedure was employed to make indirect estimates of the glucose requirements of ruminants by perturbing glucose-precursor supply demonstrates the potential of the system in the study of nutrient utilization. However, there are foreseeable difficulties in the conduct of comparative studies in which the gluconeogenic potential of exogenous glucose-precursors is to be measured or compared. Such comparisons must take account of changes in the efficiency of utilization of N which result from previous restriction of exogenous glucose-precursors. In order to eliminate these 'within-animal' variances experimental designs which employ

cross-over procedures (Scheffler, 1979) would need to be used, together with regular calibrations of the animals' responsiveness, measured by infusing doses of glucose to account for adaptations in the utilization of N.

Wider implications

The findings of the experimental work described in this thesis support the view that there is an intimate association between energy and protein metabolism in the ruminant (see Macrae & Lobley, 1982). The results also show that amino acids have a dual role, as substrates for gluconeogenesis and as substrates for protein synthesis. Furthermore, it has been shown that the utilization of glucose and protein are both variable and are influenced by previous nutrition.

The amount of glucose used for basal metabolite purposes depends on the balance between the exogenous supply of gluconeogenic-precursors and the demands made upon these precursors by the tissues. When the supply is insufficient to meet the demand, the demand is reduced by substitution of non-gluconeogenic substrates for glucose in order that glucose may be spared for essential requirements.

Glucose sparing also results in marked changes in protein utilization. It has been shown that reducing the glucose requirement of a ruminant or supplementation of its glucose supply (Asplund et al., 1985), both reduce the gluconeogenic demand for amino acids which then become available for protein synthesis.

The net effect of the commutation of amino acids between gluconeogenesis and protein synthesis can be seen as an increase in

the efficiency of utilization of protein. If this view is accepted it may also be argued that ruminants which suffer undernutrition in the course of the normal animal production practices currently employed may show similar adaptations to those described above (see Allden, 1970; Drew & Reid, 1975, 1975a and 1975b; Reid & White, 1977). Housed fattening stock are usually subjected to a winter 'storage' period in which food intake is restricted. Similarly, beef cattle reared on a 'suckler' system often suffer food shortages in the winter as a result of the seasonal growth pattern of herbage. When such animals are reintroduced to adequate nutrition, accelerated tissue repletion which is referred to as 'compensatory growth', occurs.

Compensatory growth has been attributed to the fact that with energy undernutrition there is an associated reduction in microbial protein synthesis in the rumen and consequently a reduction in amino acid supply to the small intestine (see ARC, 1980). In these circumstances it is possible that protein supply to the small intestine is not adequate to meet the animal's maintenance requirement which occasions a net loss of N from the tissues. Associated with this loss is a shortfall in the supply of amino acids potentially available for gluconeogenesis which may bring about the adaptive processes which are designed to spare glucose.

When adequate nutrition is eventually restored the residual effects of glucose sparing remain, and there is a reduction in the quantity of amino acids required for gluconeogenesis. There is, therefore, an effective sparing of amino acids which may either be used for protein synthesis or NADPH and glycerol-3-phosphate production via the TCA or pentose phosphate pathway. The latter

possibility has important implications for energy utilization as NADPH and glycerol-3-phosphate are required for the efficient utilization of acetate for lipogenesis (Macrae & Lobley, 1982).

Glucose sparing may also be of importance in other nutritional circumstances and one that is of significance to the dairy cow in particular is related to the utilization of mobilized adipose tissue. Gluconeogenic TCA intermediates are necessary for the complete oxidation of the C_2 fragments resulting from lipolysis (Rook & Thomas, 1983). A shortfall in the supply of TCA intermediates results in the partial oxidation of these fragments via alternative pathways. The ketone bodies resulting from these pathways, in particular acetoacetate and 3-OH-butyrate, when found in the blood in high concentrations (> 0.8mmol/l), are toxic and cause metabolic inefficiency.

The ability of ruminants to utilize energy more efficiently when adequate protein is available was reported by Whitelaw et al. (1985) in sheep sustained by intragastric infusions of VFA and sodium caseinate and by Macrae et al. (1985) with sheep given diets which resulted in the supply of different quantities of amino acid to the small intestine. Similarly, Armstrong (1980) reported increased N retention in sheep given readily fermentable carbohydrate supplements which result in the formation of a rumen VFA mixture biased towards propionate, which is gluconeogenic rather than acetate which is ketogenic.

Thus it may be argued that the efficiency of utilization of both energy and protein are interrelated and that the relationship between them depends on the balance between the quantities of ketogenic or gluconeogenic substrates supplied in the diet. The

interchange of substrates through the TCA cycle and its satelite pathways is therefore an essential component of the mechanism behind the responses described above.

The changes in efficiency of utilization of N discussed above also have important implications for current rationing systems (ARC, 1984). The factorial calculations employed in the formulation of rations for ruminants assume a fixed maintenance requirement for N to which are added incremental allowances for the foetus, milk, wool and tissue synthesis. Conclusions drawn from the work conducted for this thesis together with the findings of Hovell et al. (in press) which are in accord, suggest this method of protein rationing may be subject to significant error and that the level of inaccuracy resulting dependents on the animal's previous nutrition.

Furthermore, it would appear at present that traditional rationing systems will be replaced by, more complex, computer models of ruminant metabolism. Models that are currently available, however, make no allowance for a variable maintenance requirement for N (see Gill et al., 1984). The implications here are that such models will be of limited predictive value in circumstances where glucose sparing responses have been induced.

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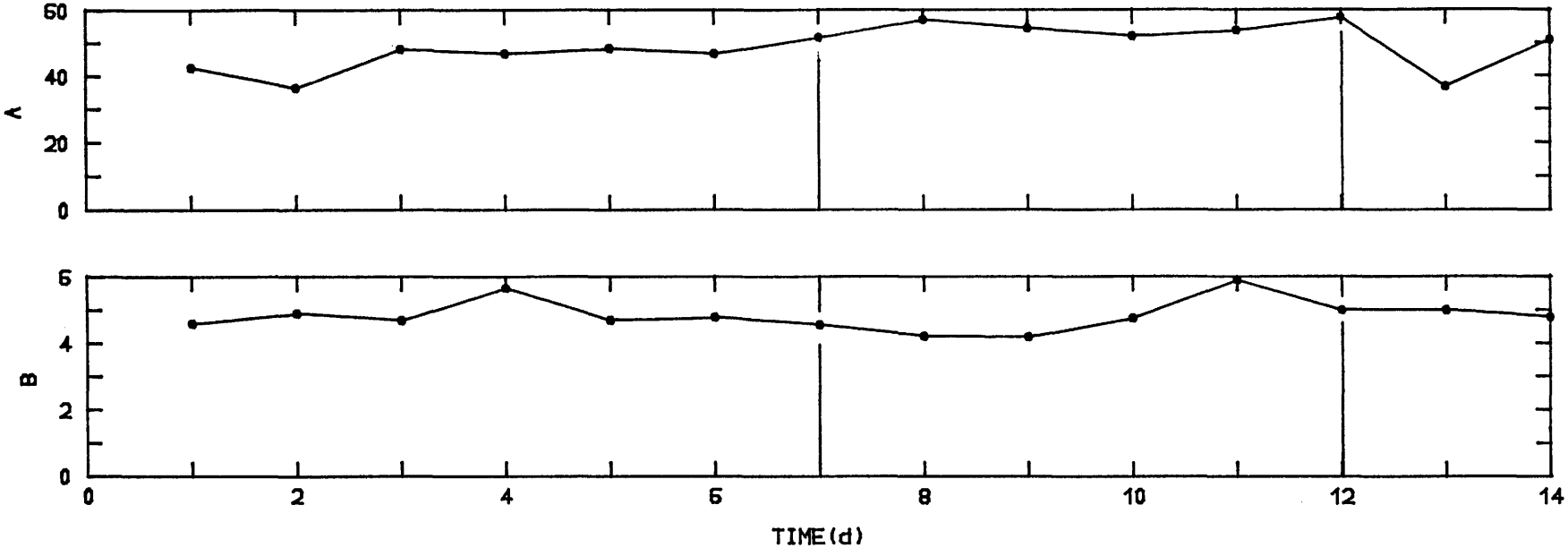
APPENDICES

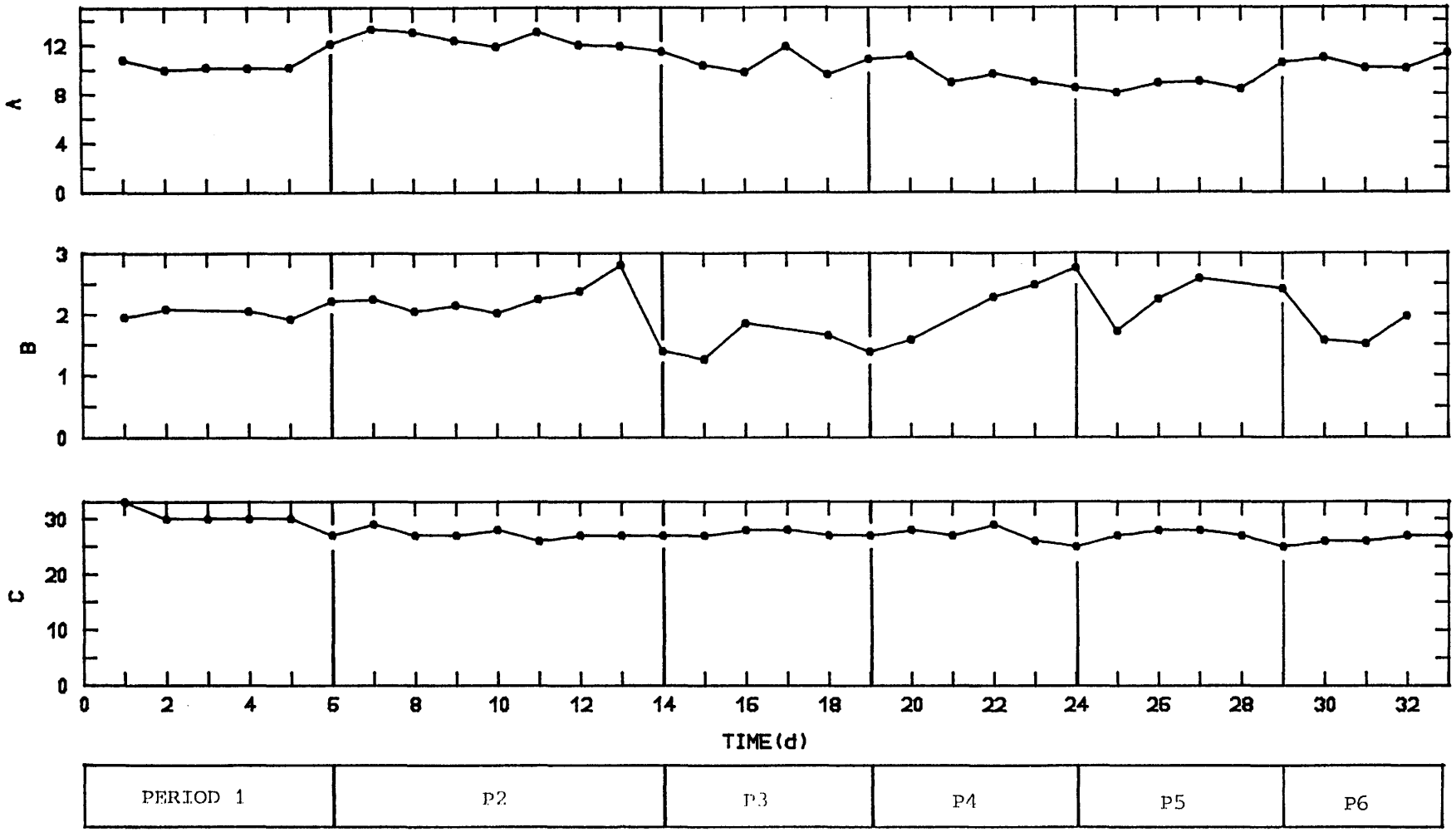
APPENDICES 1 to 54

Raw experimental data: Period divisions in the graphical representation of data correspond to the first day of treatment. Urine collection was carried out at 1000h each day, introduction of fresh infusates occurred daily at 1100h and blood sampling was carried out at 1400h on each sampling day.

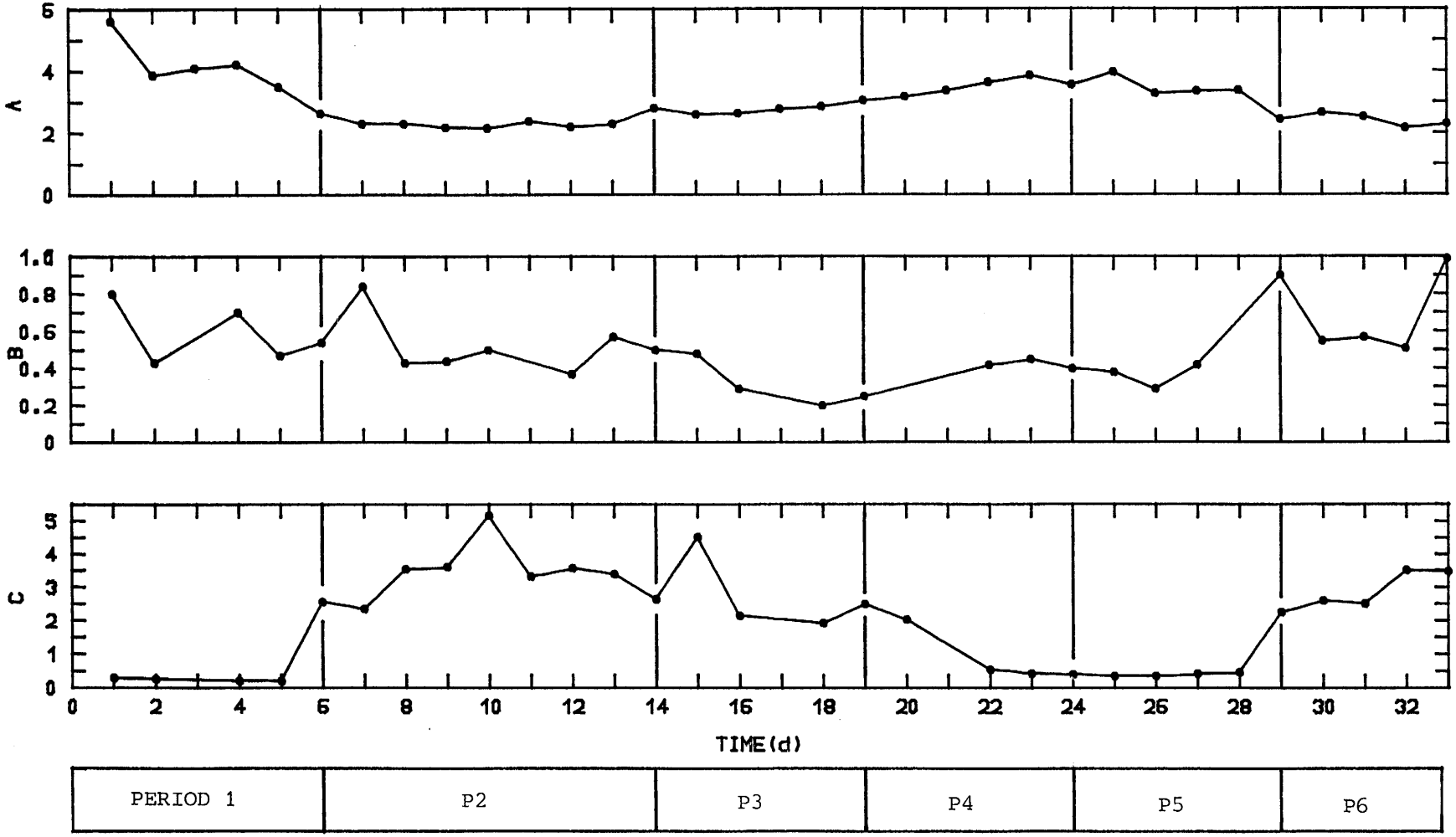


PERIOD 1	P2	P3

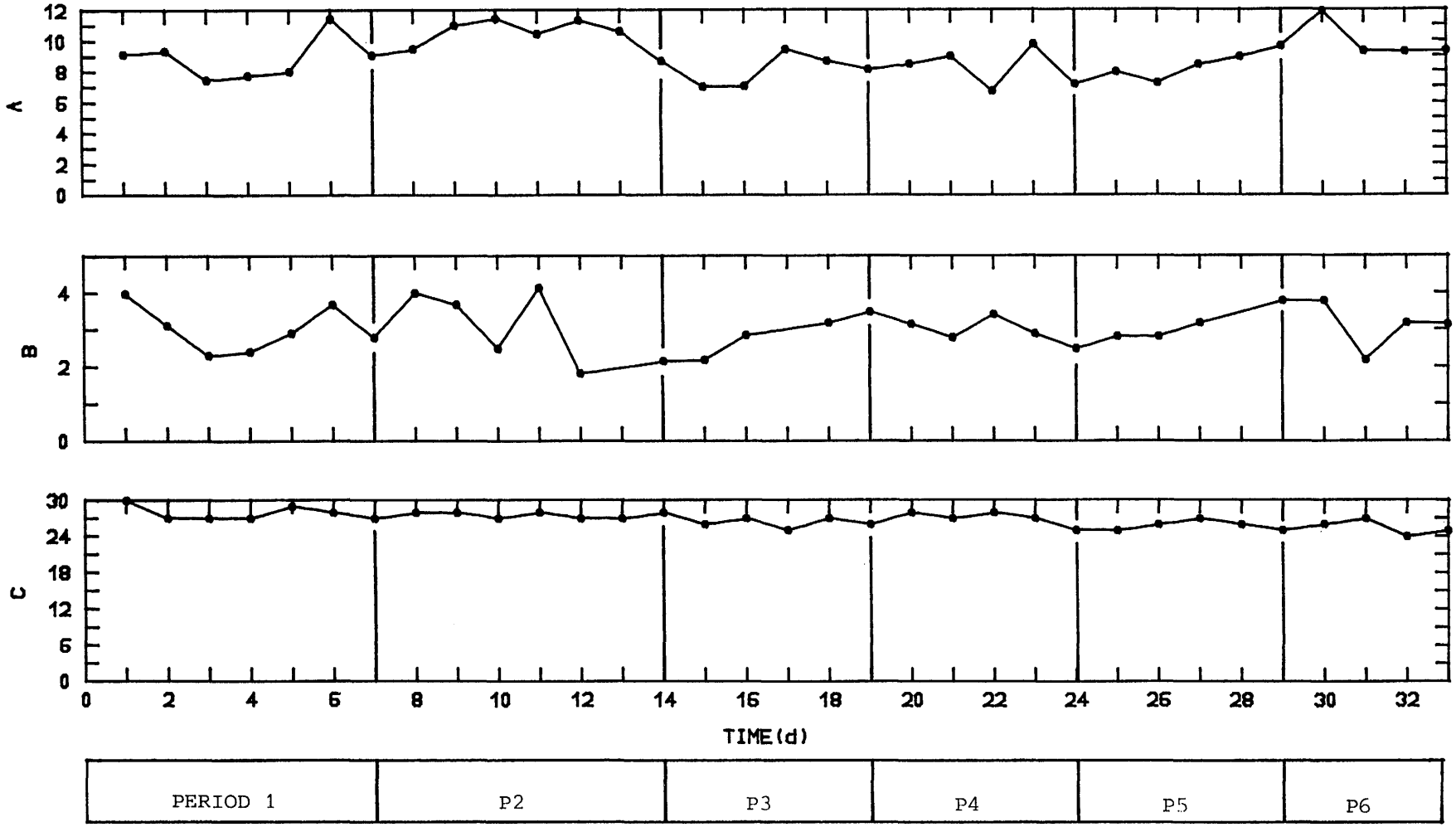
Appendix 1: Experiment 1b (Cow 257). A. N excretion (g/d). B. The concentration of glucose (mmol/l) in blood plasma.



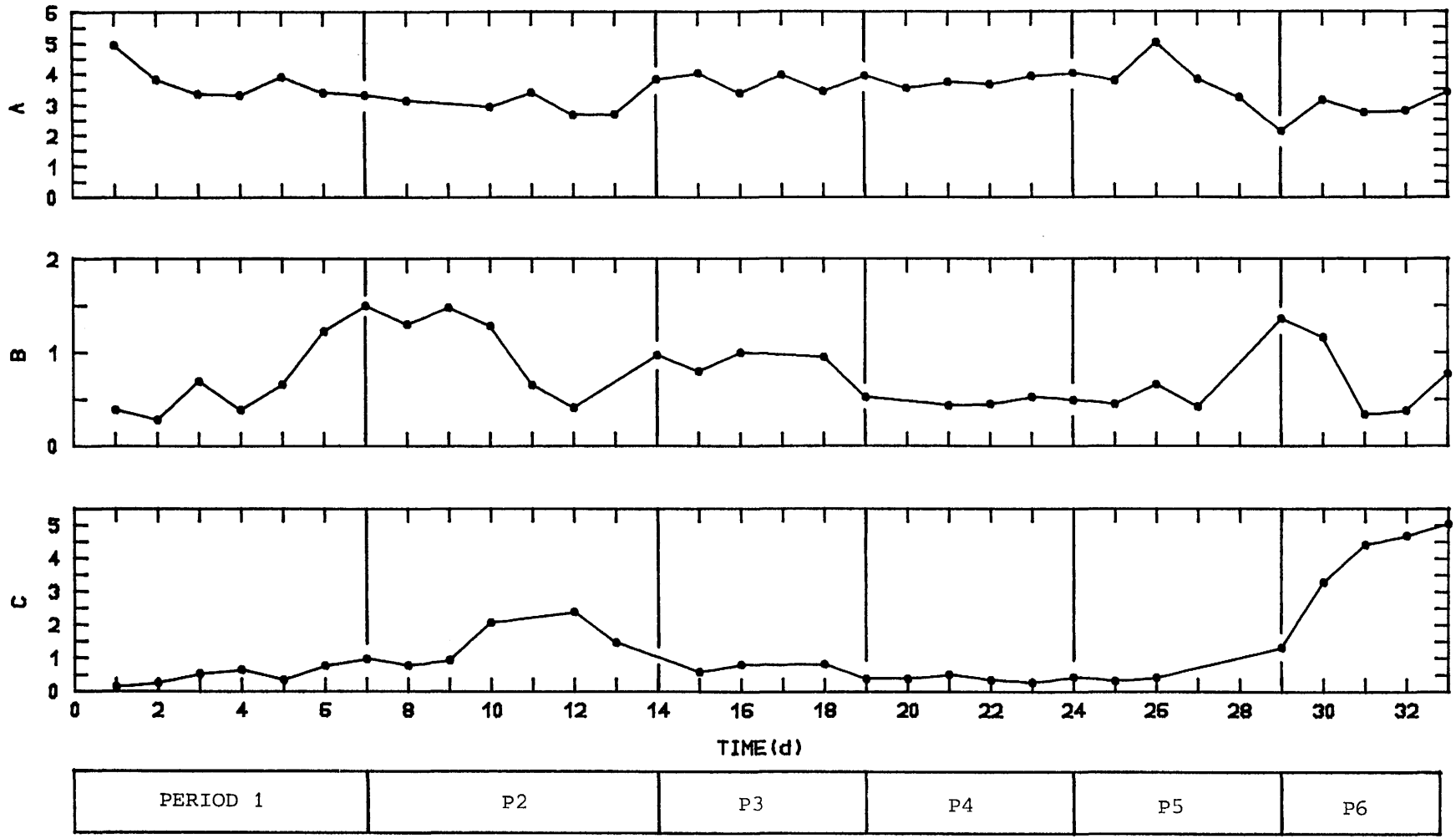
Appendix 2: Experiment 2 (Sheep 463). A. N excretion (g/d). B. The concentration of urea (mmol/l) in blood plasma. C. Blood packed cell volume (%).



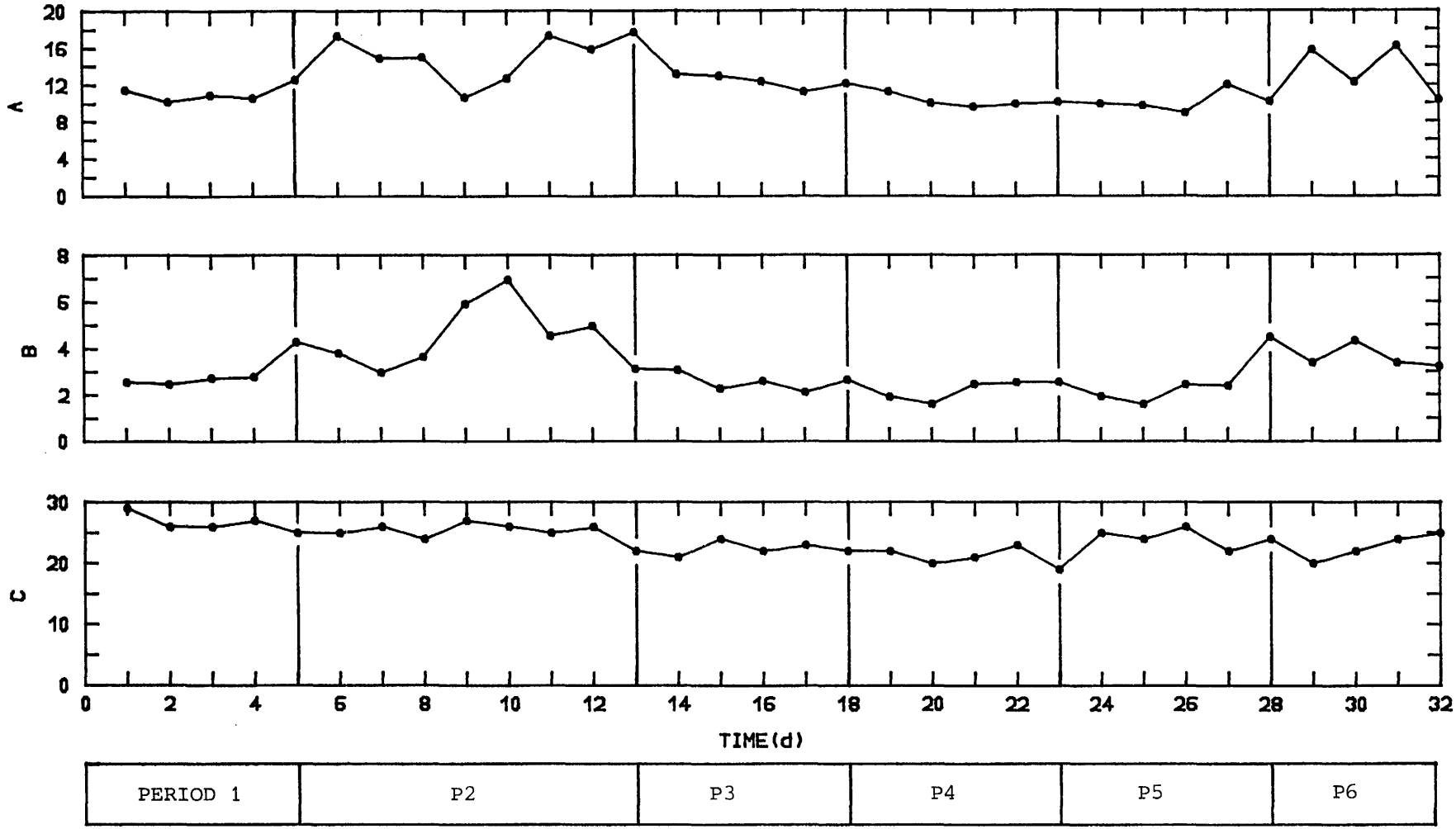
Appendix 3: Experiment 2 (Sheep 463). The concentrations of A. Glucose (mmol/l), B. NEFA (mmol/l) and C. 3-OH butyrate.



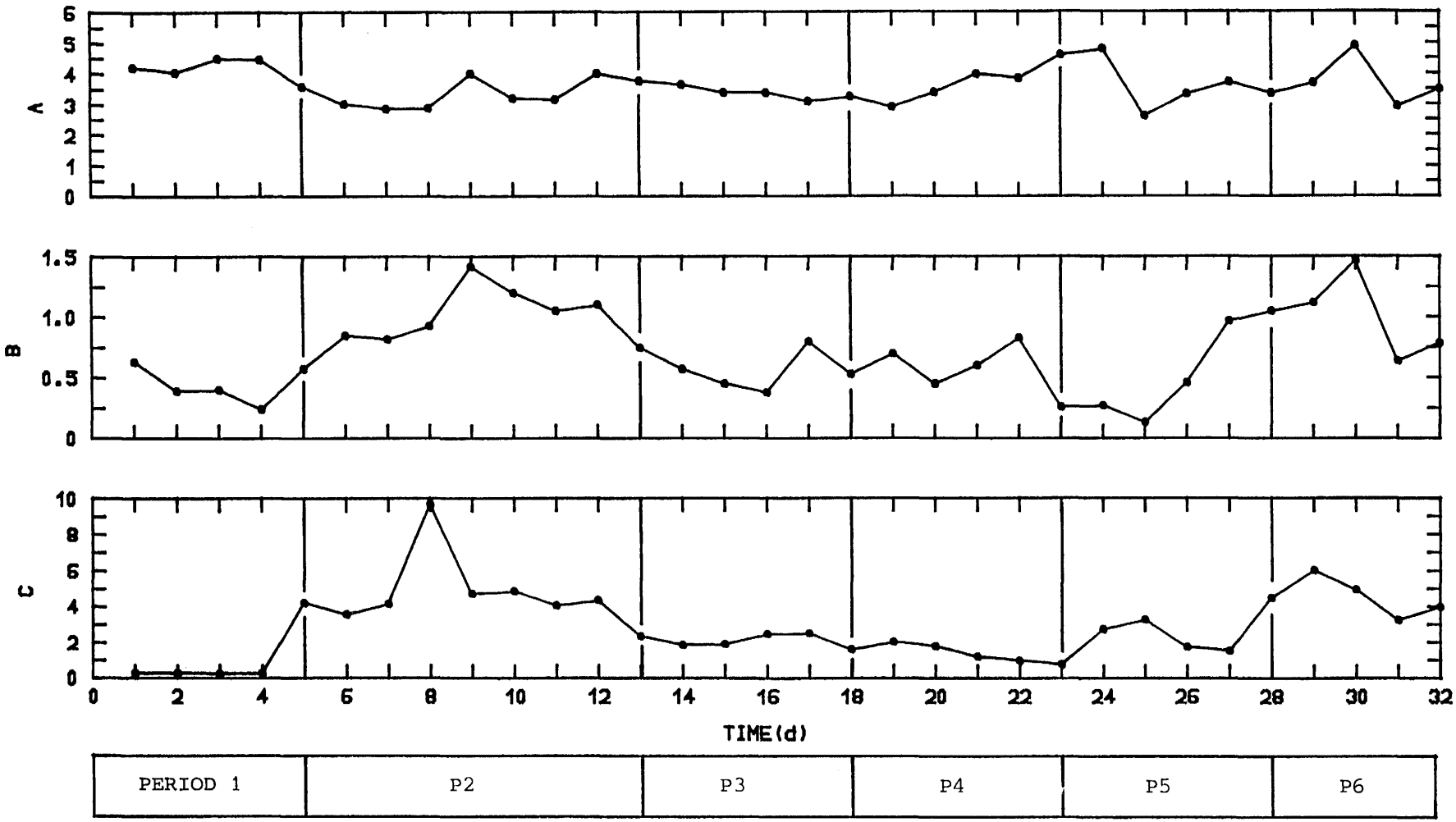
Appendix 4: Experiment 2 (Sheep 466). A. N excretion (g/d). B. Urea concentration (mmol/l) measured in blood plasma. C. Blood packed cell volume (%).



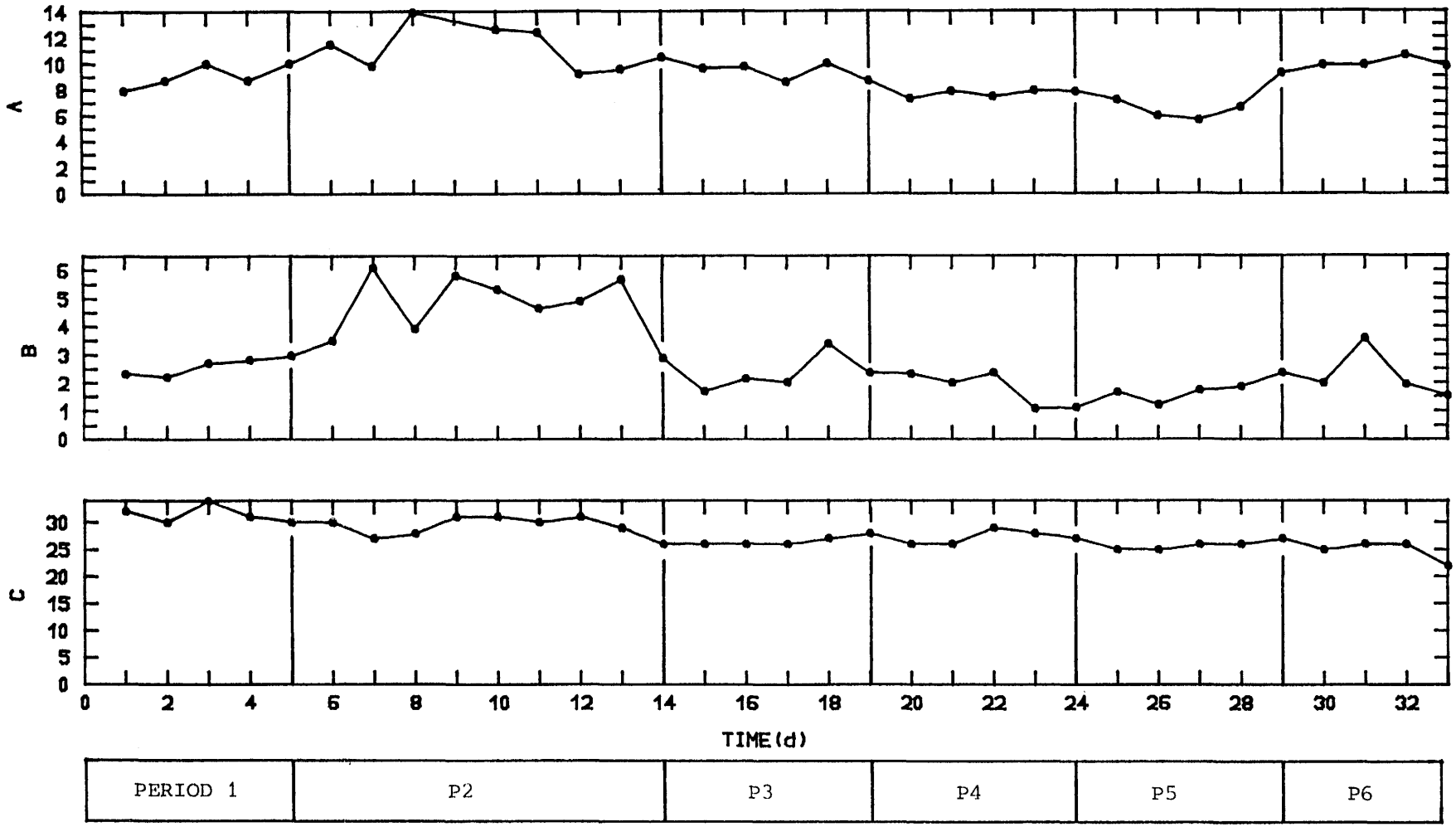
Appendix 5: Experiment 2 (Sheep 466). A. Glucose concentration (mmol/l). B. NEFA concentration (mmol/l). C. 3-OH butyrate concentration (mmol/l) measured in blood plasma.



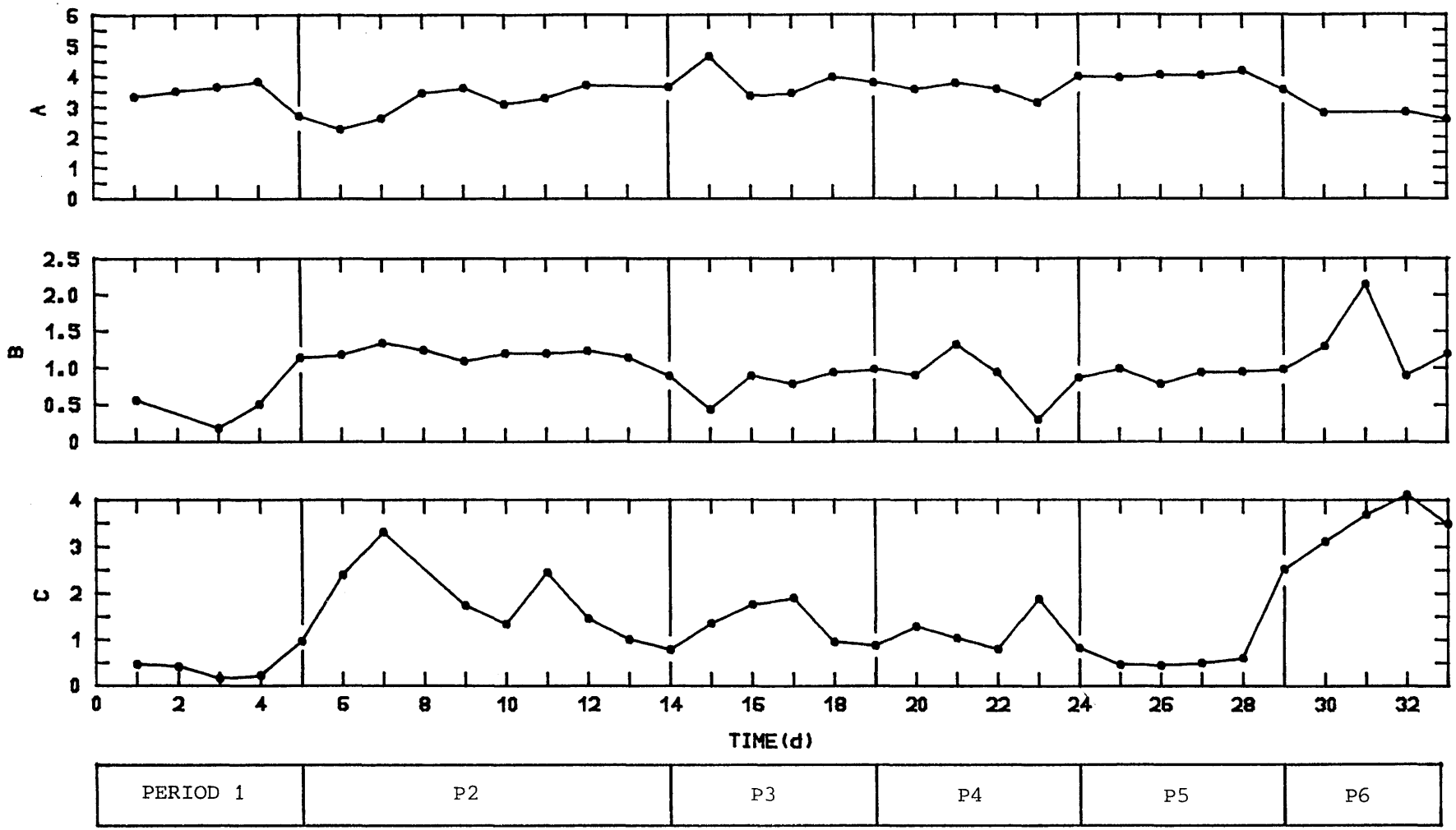
Appendix 6: Experiment 2 (Sheep 467). A. N excretion (g/d). B. Urea concentration (mmol/l) measured in blood plasma. C. Blood packed cell volume (%).



Appendix 7: Experiment 2 (Sheep 467). A. Glucose (mmol/l). B. NEFA (mmol/l). C. 3-OH butyrate (mmol/l) concentrations measured in blood plasma.



Appendix 8: Experiment 2 (Sheep 468). A. N excretion (g/d). B. Urea concentration (mmol/l) measured in blood plasma. C. Blood packed cell volume (%).



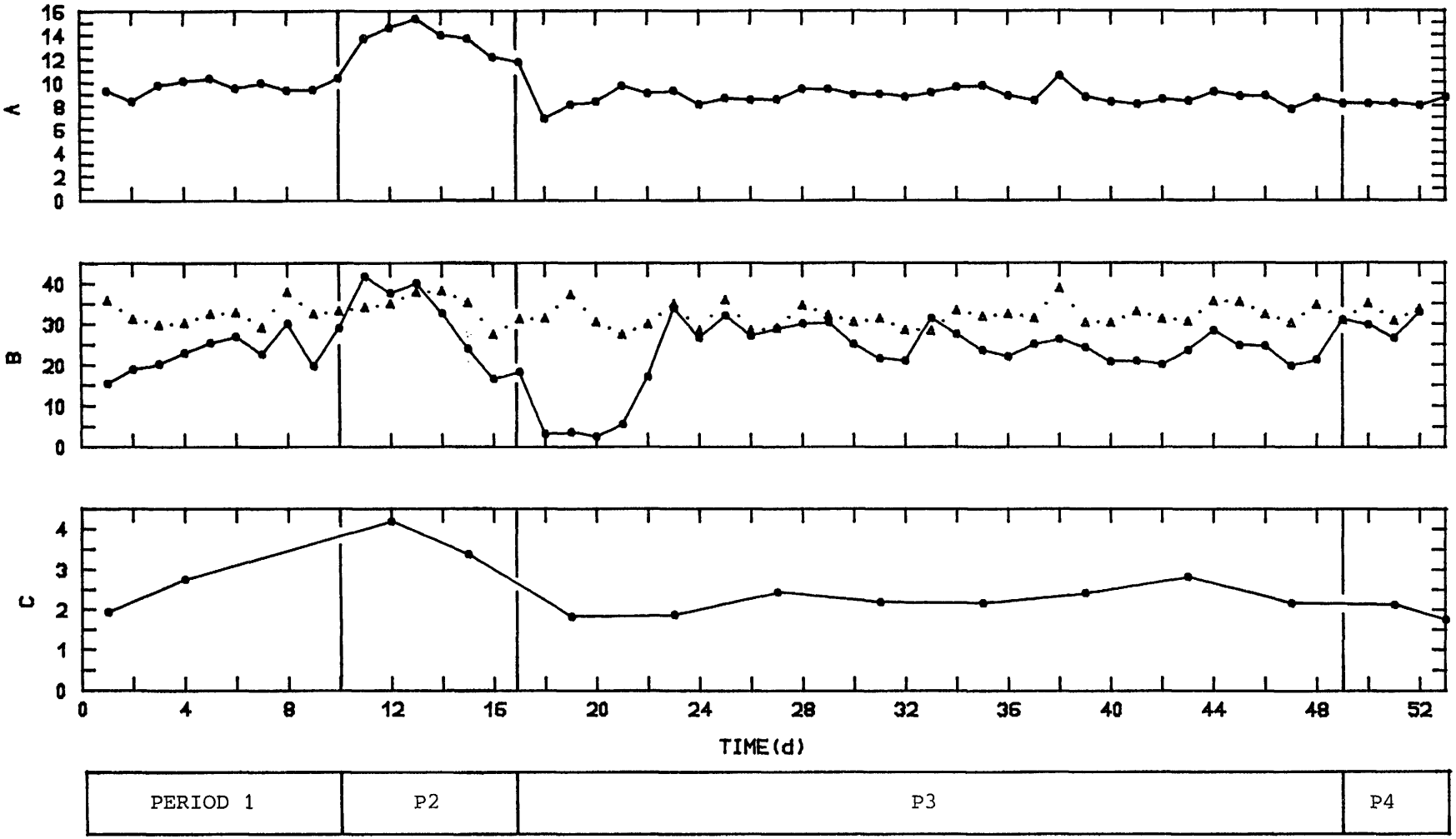
Appendix 9: Experiment 2 (Sheep 468). A. Glucose (mmol/l). B. NEFA (mmol/l). C. 3-OH butyrate concentration (mmol/l) measured in blood plasma.

Amino acid	Sheep 463 (Period/Day)				Sheep 466 (Period/Day)			
	[1]1/4[2]	2/13	5/28	6/33	1/6	2/12	5/28	6/33
Aspartate	0.53	0.41	0.61	0.38	0.63	0.47	0.60	0.40
Threonine	2.64	2.75	1.25	3.20	1.51	2.54	3.46	2.66
Serine	4.77	1.57	6.81	2.18	3.75	2.17	2.74	1.89
Glutamate	14.12	11.48	7.80	11.63	12.72	12.27	12.57	12.46
Proline	3.39	2.88	4.12	4.01	3.32	3.36	4.14	6.24
Citrulline& Glycine	34.42	18.26	44.24	23.10	32.47	26.55	31.78	29.95
Alanine	7.63	2.82	6.00	3.15	9.54	4.10	5.06	3.37
Cystine	0.50	0.41	0.59	0.38	0.23	0.30	0.27	0.41
Valine	5.70	18.90	7.29	17.13	6.51	12.43	13.08	11.98
Methionine	0.74	0.54	0.39	0.55	0.67	0.58	0.70	0.35
Isoleucine	3.07	5.39	2.30	4.41	2.52	4.38	3.95	4.00
Leucine	5.44	11.00	4.76	9.04	6.22	8.44	3.13	7.28
Tyrosine	1.59	1.98	0.97	1.24	1.78	1.73	1.42	1.15
Phenylalanine	2.13	2.90	1.43	1.67	2.48	2.82	2.70	1.95
Histidine	3.44	4.40	2.73	4.62	3.91	4.06	4.21	4.34
Ornithine	3.86	5.15	5.16	5.13	4.77	5.40	3.55	4.96
Lysine	4.27	4.58	2.59	3.91	4.55	5.54	3.79	3.30
Arginine	1.76	4.59	1.01	4.27	2.41	2.87	2.83	3.30
Total amino acids (µmol/100ml)	231.90	190.50	268.90	218.80	215.23	250.40	229.10	201.00

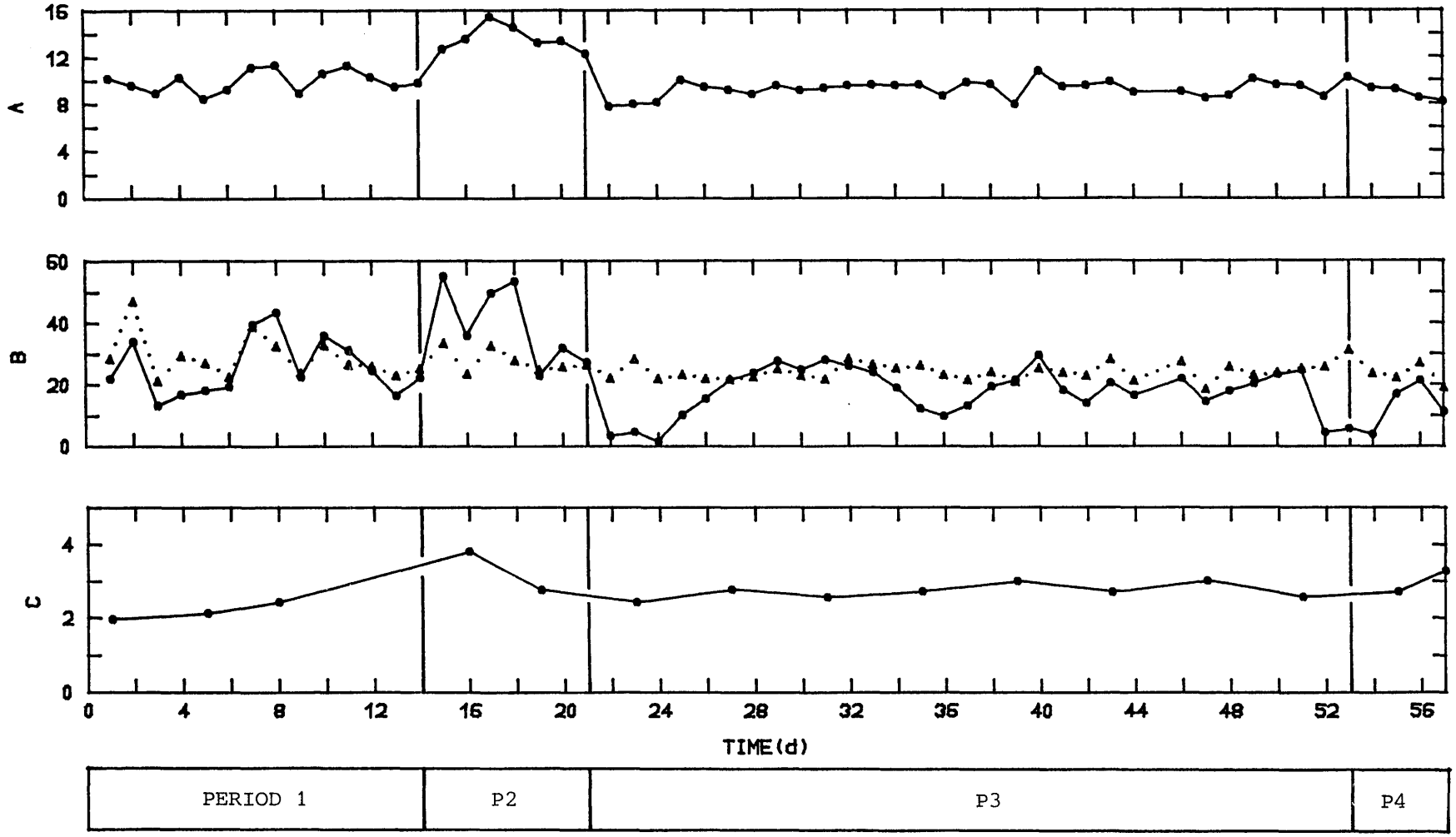
Appendix 10: Experiment 2. Amino acid composition (mol/100mol) measured in the blood plasma of sheep 463 and 466. 1. Refer to Table III(4) for treatments. 2. The day of the experiment on which the blood sample was taken.

Amino acid	Sheep 467 (Period/Day)				Sheep 468 (Period Day)			
	[1]1/4[2]	2/12	5/28	6/33	1/4	2/12	5/28	6/33
Aspartate	0.80	0.69	0.80	0.54	0.82	0.60	0.40	0.84
Threonine	3.06	2.61	1.33	2.95	1.68	2.17	2.33	0.86
Serine	3.68	1.69	9.46	1.87	1.95	2.39	1.43	6.75
Glutamate	12.28	13.92	8.38	7.86	12.21	9.95	11.90	8.32
Proline	3.99	2.68	5.34	4.57	4.21	3.24	3.15	2.80
Citrulline & Glycine	27.93	22.20	31.47	23.69	31.95	25.21	21.83	39.90
Alanine	9.90	3.88	7.75	2.92	13.82	7.39	4.02	6.13
Cystine	0.29	0.29	0.17	0.40	0.34	0.69	0.34	0.37
Valine	6.81	15.88	9.12	16.47	7.53	13.38	15.32	10.87
Methionine	0.99	0.55	0.72	0.72	0.70	0.75	0.58	0.35
Isoleucine	2.49	4.13	2.23	5.14	2.45	4.29	5.95	2.63
Leucine	4.74	8.69	4.86	8.97	4.31	7.98	9.77	5.50
Tyrosine	1.56	1.36	1.26	1.38	1.38	1.31	1.19	0.36
Phenylalanine	2.00	2.17	1.41	1.62	1.60	1.68	1.85	1.17
Histidine	3.66	5.41	2.53	4.22	3.09	4.06	3.05	3.00
Ornithine	5.17	6.06	4.56	5.46	5.46	3.83	7.09	3.90
Lysine	6.10	3.85	4.57	6.77	4.39	5.57	5.31	4.08
Arginine	4.54	3.93	4.02	4.42	2.12	5.52	4.48	2.11
Total amino acids (µmol/100ml)	219.20	214.90	236.10	210.90	209.30	217.60	247.30	208.30

Appendix 11: Experiment 2. Amino acid composition (mol/100mol) measured in the blood plasma of sheep 467 and 468. 1. Refer to Table III(4) for period treatments. 2. The day of the experiment on which the blood sample was taken.



Appendix 12: Experiment 3 (Sheep 466). A. N excretion (g/d). B. (•) creatine excretion (g/d) and (▲) creatinine excretion (g/d). C. Urea concentration (mmol/l) measured in blood plasma.



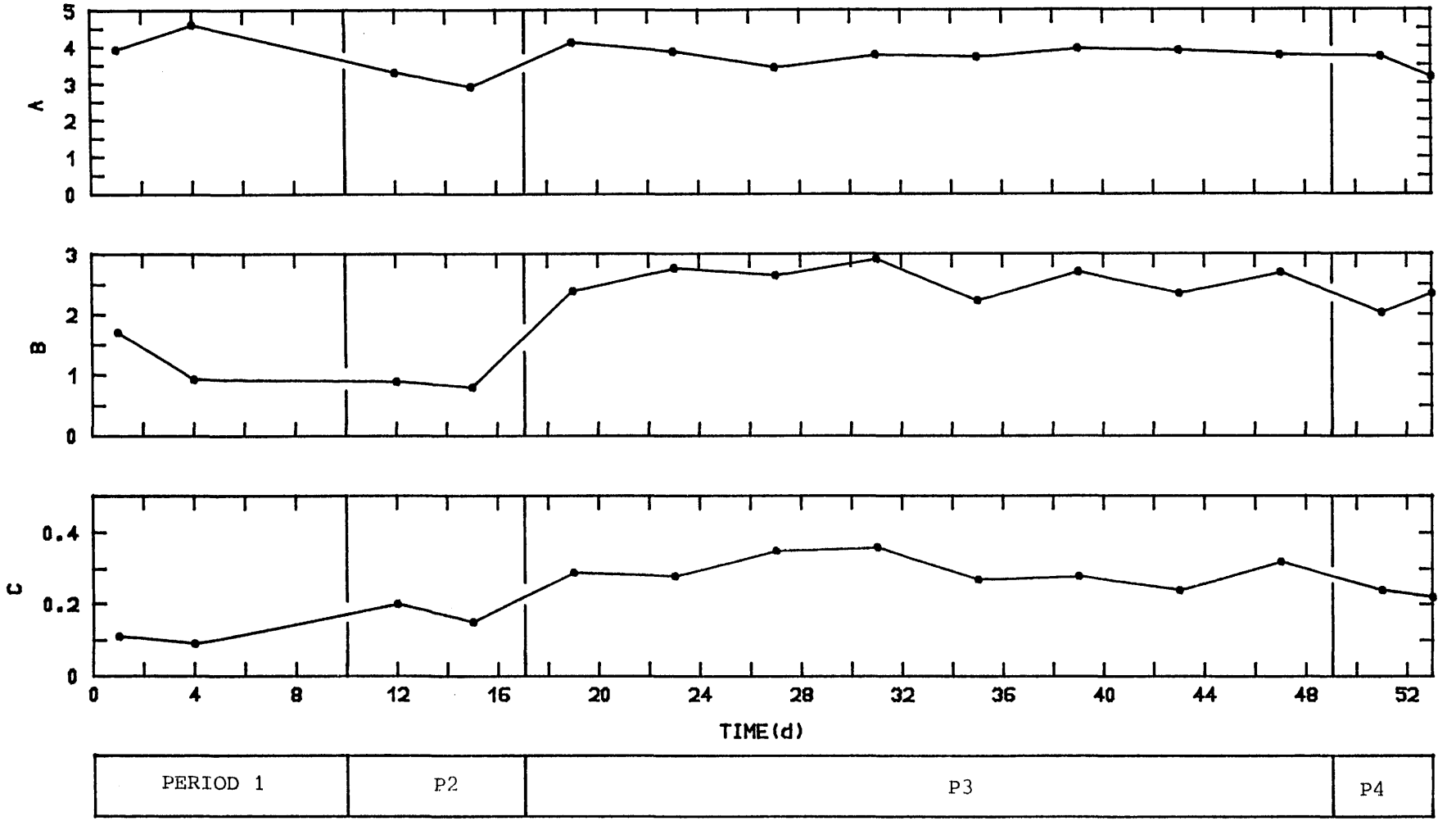
Appendix 13: Experiment 3 (Sheep 468). A. N excretion (g/d). B. (•) creatine and (▲) creatinine excretion (g/d). C. Urea concentration (mmol/l) in blood plasma.

Amino acid	Period 1			Period 2		Period 3								Period 4	
	Day 1	5	8	16	19	23	27	31	35	39	43	47	51	55	57
Aspartate	0.76	0.70	0.64	0.31	0.49	0.49	0.43	0.46	0.63	0.56	0.85	0.45	0.39	0.67	0.62
Threonine	4.04	3.01	3.85	3.95	4.08	2.07	1.90	2.31	2.33	2.71	3.03	2.05	2.17	2.00	2.49
Serine	4.44	4.20	5.69	1.55	2.65	5.60	4.90	5.46	6.76	5.67	5.97	5.60	5.27	6.32	5.68
Glutamate	9.00	9.86	7.75	9.60	7.22	5.67	6.18	6.27	7.98	7.69	7.82	5.54	5.88	5.73	6.04
Proline	7.00	6.30	6.63	2.36	4.68	5.95	7.26	6.15	7.49	7.85	7.58	4.58	6.17	5.93	6.29
Cit/Glycine	26.43	28.41	24.88	15.09	20.29	37.04	41.66	40.90	31.80	28.94	23.92	46.36	35.32	39.75	34.20
Alanine	9.93	9.20	9.89	3.78	3.90	9.33	9.56	10.13	11.64	11.63	11.04	8.93	8.53	8.92	8.90
Cystine	0.16	0.10	0.22	0.36	0.36	0.24	0.21	0.30	0.35	0.53	0.48	0.46	0.41	0.27	0.62
Valine	7.01	7.70	8.00	17.86	13.79	7.16	6.46	6.31	7.22	7.44	8.36	5.69	7.87	6.06	7.11
Methionine	1.04	0.95	1.11	0.97	0.90	0.83	0.51	0.69	0.71	0.78	1.02	0.55	0.58	0.72	0.89
Isoleucine	2.65	3.28	3.40	7.54	6.04	2.93	2.71	2.06	2.81	3.24	4.05	2.11	2.54	2.25	3.19
Leucine	5.03	5.43	5.30	13.17	9.59	4.99	3.57	3.46	4.23	4.96	6.45	3.81	4.75	3.75	5.21
Tyrosine	3.13	2.95	2.73	2.34	2.54	2.29	1.61	1.78	1.96	2.10	2.72	1.70	2.32	1.57	2.22
Phenylalanine	2.45	2.54	2.39	2.76	2.44	1.91	1.34	1.40	1.60	1.94	2.25	1.39	1.53	1.29	1.67
Histidine	3.71	3.17	3.23	3.57	3.97	2.62	2.79	2.71	2.90	3.19	3.06	2.17	4.69	4.51	4.26
Tryptophan	2.15	1.98	1.73	1.78	1.32	1.68	1.26	1.25	1.15	1.61	1.73	1.35	1.07	0.80	1.02
Ornithine	3.07	2.91	3.50	3.88	4.22	2.17	2.31	2.51	3.00	2.72	2.26	2.08	3.61	3.02	2.51
Lysine	4.09	4.28	5.47	5.32	6.92	4.38	3.26	3.59	3.36	3.84	4.32	3.06	4.51	3.93	4.41
Arginine	3.90	3.04	3.59	3.81	4.61	2.65	2.10	2.27	2.03	2.59	3.11	2.10	2.39	2.52	2.66
Total (µmol/100ml)	246.50	270.00	315.60	223.00	272.70	382.00	333.60	337.70	287.60	273.60	325.60	393.50	379.80	406.20	483.80

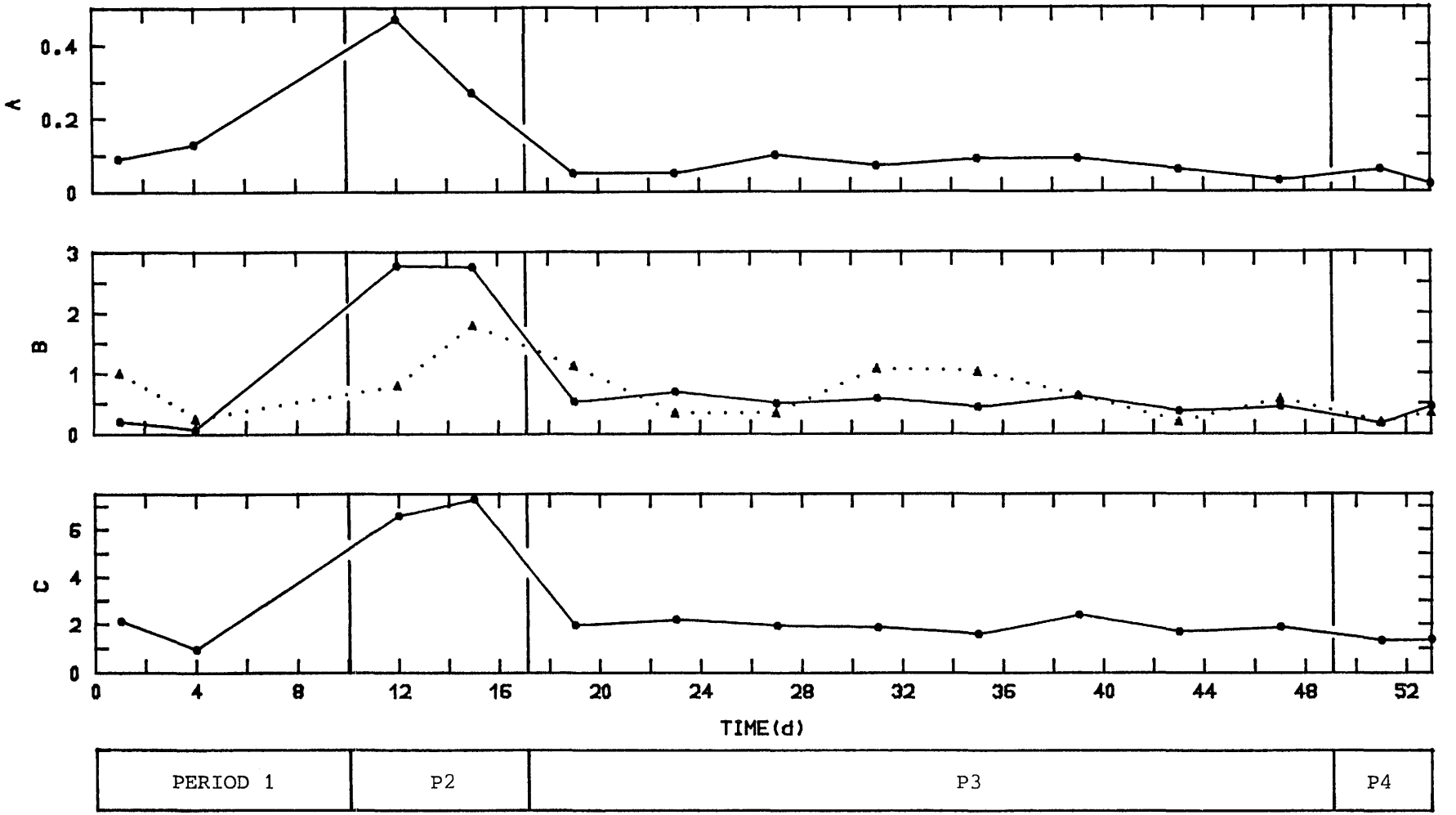
Appendix 14: Experiment 3. Amino acid composition (mol/100mol) measured in blood plasma from sheep 466.

Amino acid	Period 1			Period 2		Period 3								Period 4	
	Day 1	5	8	16	19	23	27	31	35	39	43	47	51	55	57
Aspartate	0.71	0.60	0.74	0.48	0.36	0.52	0.43	0.64	0.54	0.58	0.52	0.57	0.56	0.49	0.57
Threonine	3.26	2.60	3.48	4.38	4.28	1.95	2.03	2.48	2.50	1.96	2.13	1.72	2.41	1.74	1.90
Serine	3.82	4.64	4.76	1.82	2.22	5.89	5.04	7.12	5.50	7.61	6.31	9.64	6.39	6.88	6.53
Glutamate	6.73	7.62	8.29	5.78	7.09	6.21	4.81	7.54	6.63	6.94	5.25	5.97	6.75	5.67	5.86
Proline	6.69	7.25	6.96	6.16	6.72	5.39	5.40	6.97	4.90	5.66	6.35	6.00	8.79	7.16	7.28
Cit/Glycine	24.24	28.20	31.67	19.32	18.14	33.74	48.55	30.79	47.98	37.52	42.79	42.51	39.00	43.06	38.93
Alanine	14.56	11.47	9.15	3.32	3.07	10.25	7.95	9.73	7.09	10.38	8.34	8.45	6.97	9.92	8.96
Cystine	0.34	0.29	0.28	0.39	0.43	0.28	0.77	0.44	0.38	0.39	0.45	0.60	0.31	0.36	0.47
Valine	7.21	6.92	7.15	13.78	12.91	6.76	5.60	7.25	5.66	5.68	5.03	4.81	5.97	4.90	5.83
Methionine	1.46	1.13	0.99	1.07	1.22	0.88	0.66	0.61	0.52	0.67	0.67	0.48	0.69	0.54	0.61
Isoleucine	2.96	3.56	3.29	6.84	5.76	3.24	2.06	2.98	2.33	2.30	1.89	1.88	2.43	2.40	2.68
Leucine	4.81	5.38	4.73	12.36	9.79	4.97	3.18	4.94	3.70	3.95	3.32	3.26	3.92	3.43	4.27
Tyrosine	3.19	2.90	2.36	2.37	2.41	2.38	1.61	2.11	1.38	1.37	1.61	1.29	1.63	1.63	1.99
Phenylalanine	2.14	1.83	2.00	2.59	2.41	1.64	1.33	1.58	1.26	1.08	1.09	0.86	0.95	1.00	1.25
Histidine	3.25	3.04	2.39	2.66	3.36	3.02	0.22	2.98	2.63	2.72	3.09	2.89	3.00	2.35	2.80
Tryptophan	2.16	1.42	1.59	1.14	1.30	1.54	0.92	1.39	0.88	0.85	0.47	0.86	0.48	0.65	0.55
Ornithine	3.24	3.72	2.77	3.71	5.60	3.59	3.26	5.24	3.00	4.14	3.98	3.26	4.04	3.15	3.63
Lysine	5.11	4.46	4.53	7.22	8.43	4.94	4.59	4.15	2.08	4.75	4.68	3.82	4.48	3.54	4.37
Arginine	4.13	2.96	2.84	4.60	4.52	2.81	2.10	1.07	1.39	1.45	2.03	1.12	1.21	1.10	1.53
Total amino acids (µmol/100ml)	218.50	280.80	291.30	310.40	316.00	319.70	429.30	318.60	416.90	398.20	600.70	488.40	585.60	492.90	501.70

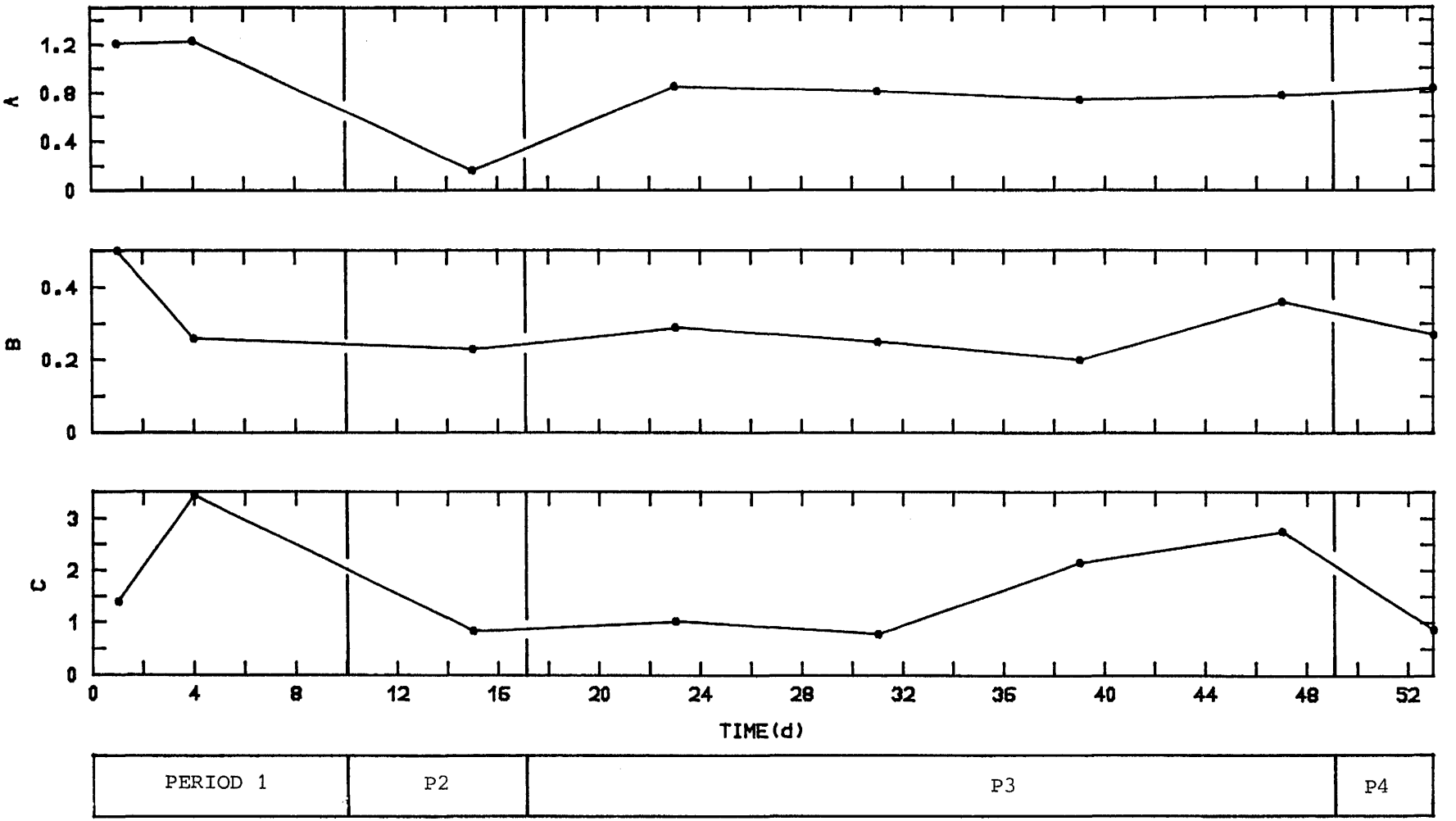
Appendix 15: Experiment 3. Amino acid composition (mol/100mol) measured in blood plasma from sheep 468.



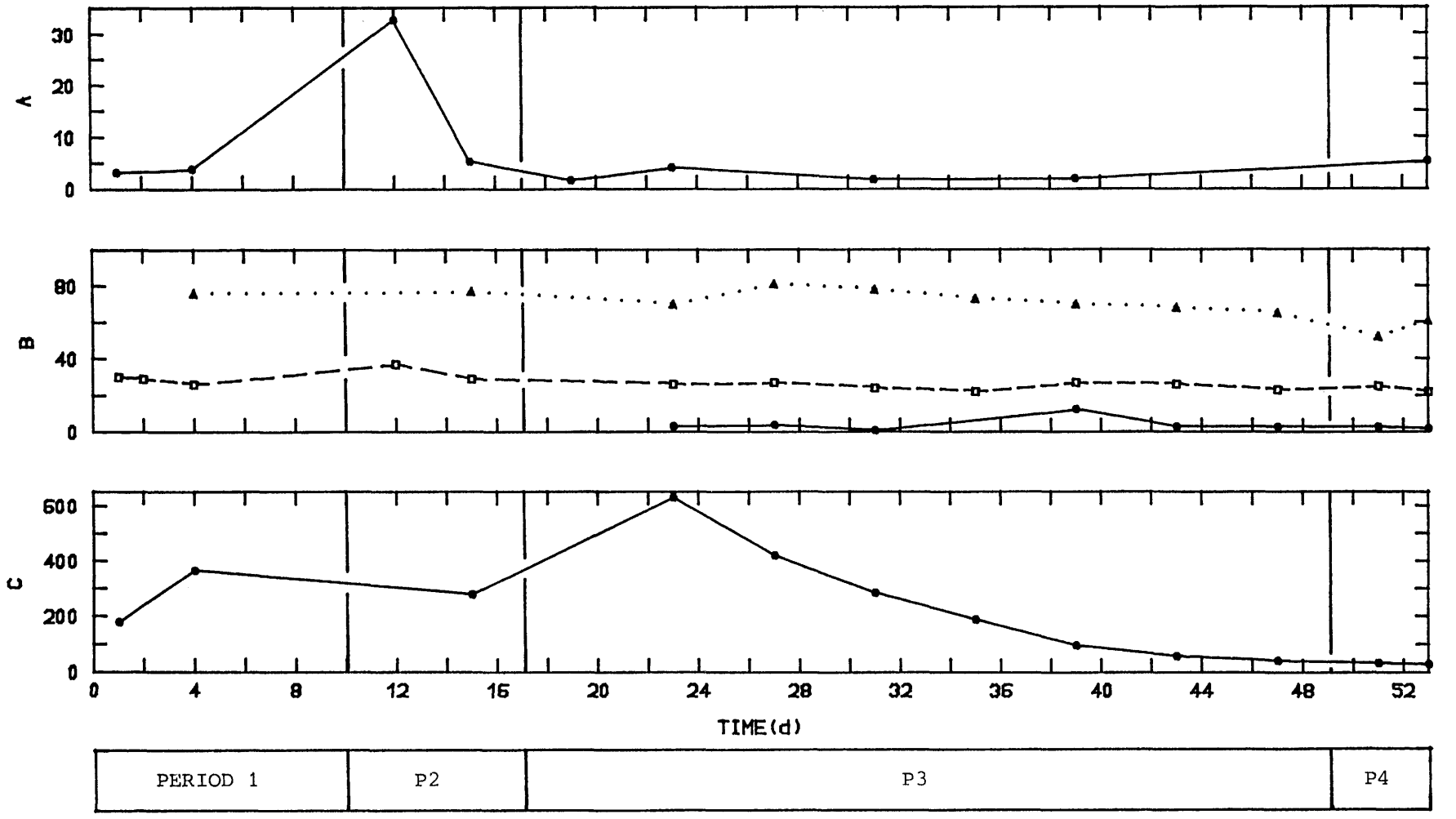
Appendix 16: Experiment 3 (Sheep 466). Concentrations of A. glucose (mmol/l), B. lactate (mmol/l) and C. glycerol (mmol/l) measured in blood plasma.



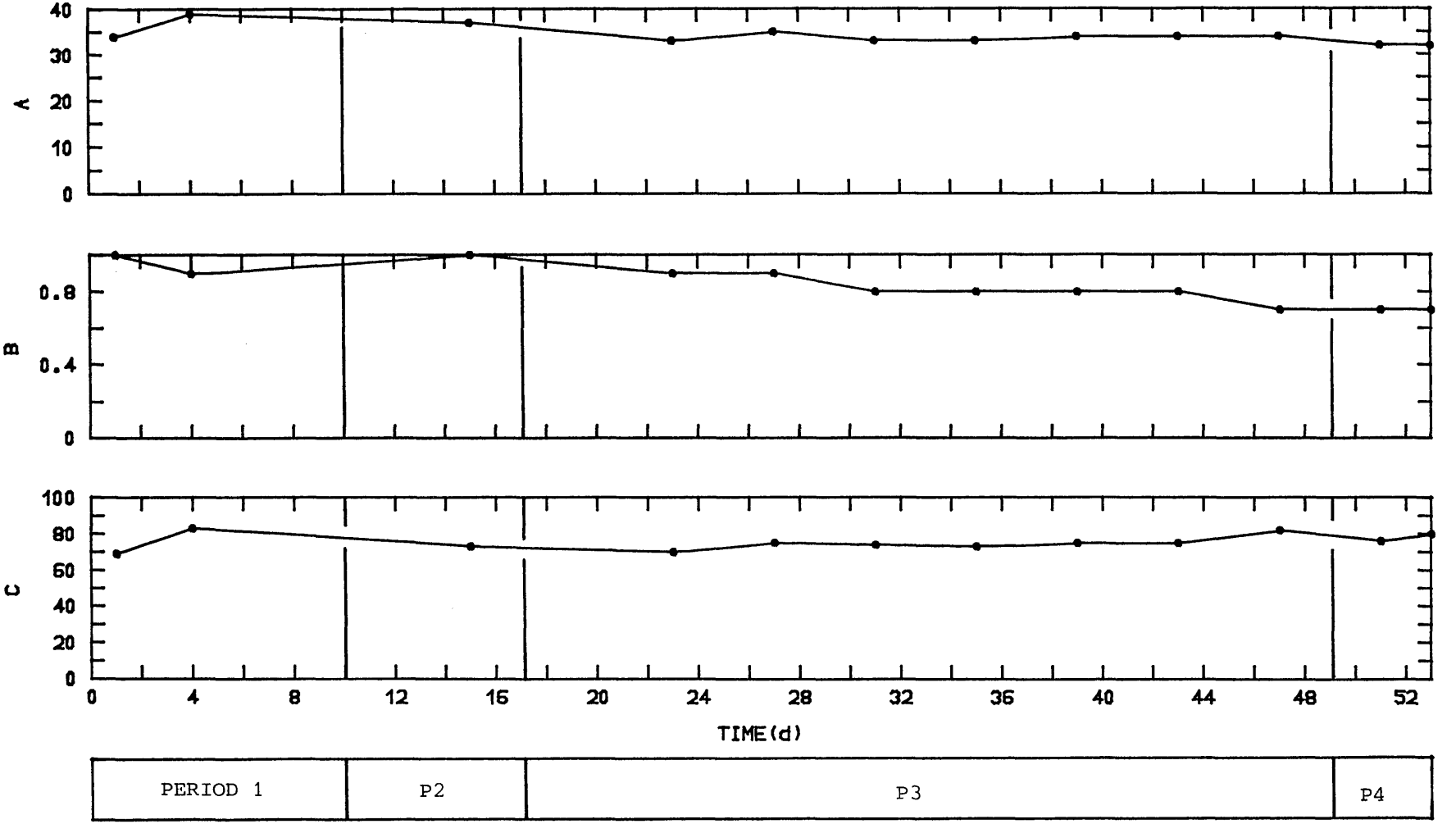
Appendix 17: Experiment 3 (Sheep 466). Concentrations of A. NEFA (mmol/l), B. (•) 3-OH butyrate (mmol/l) and (▲) acetoacetate (mmol/l) and C. acetate (mmol/l) measured in blood plasma.



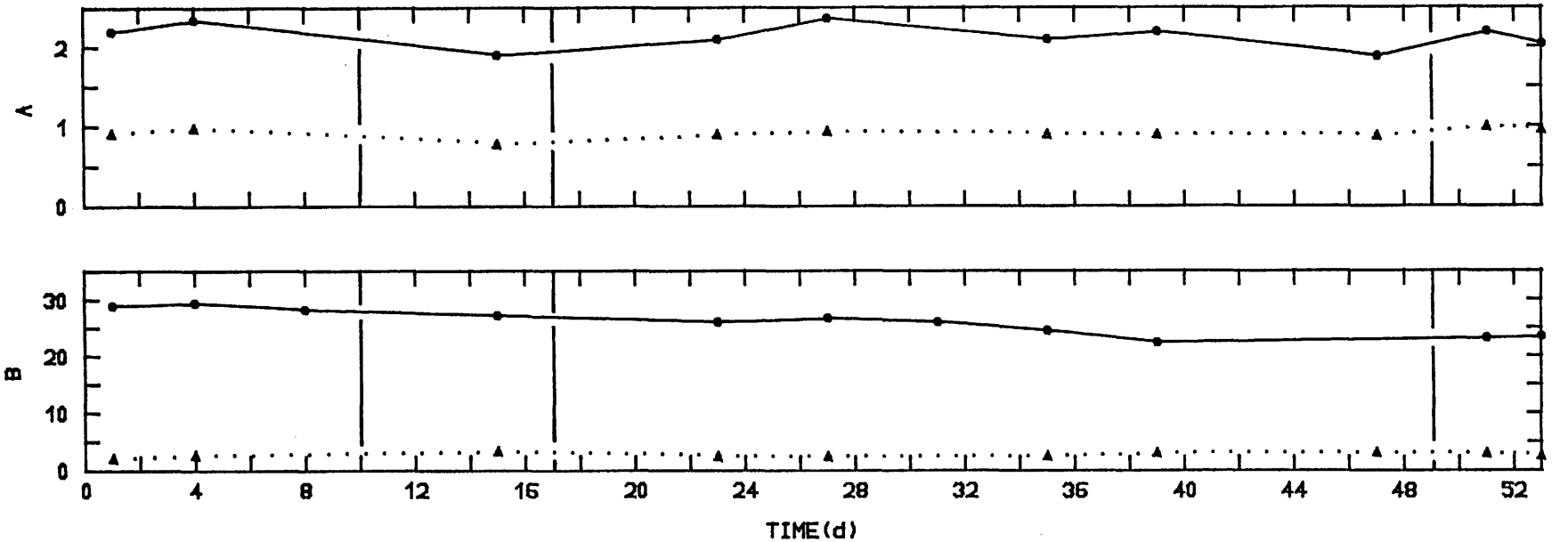
Appendix 18: Experiment 3 (Sheep 466). Concentration of A. Insulin (ng/ml), B. Glucagon (ng/ml) and C. Growth hormone (ng/ml) measured in blood plasma.



Appendix 19: Experiment 3 (Sheep 466). Concentration of A. Cortisol (ng/ml), the activity of B. (•) sorbitol dehydrogenase (iu/l), (▲) L-γ-glutamyltransferase (iu/l) measured in blood plasma and, (□) whole blood packed cell volume (%). C. glutamic oxaloacetic transaminase (iu/l) activity measured in blood plasma.

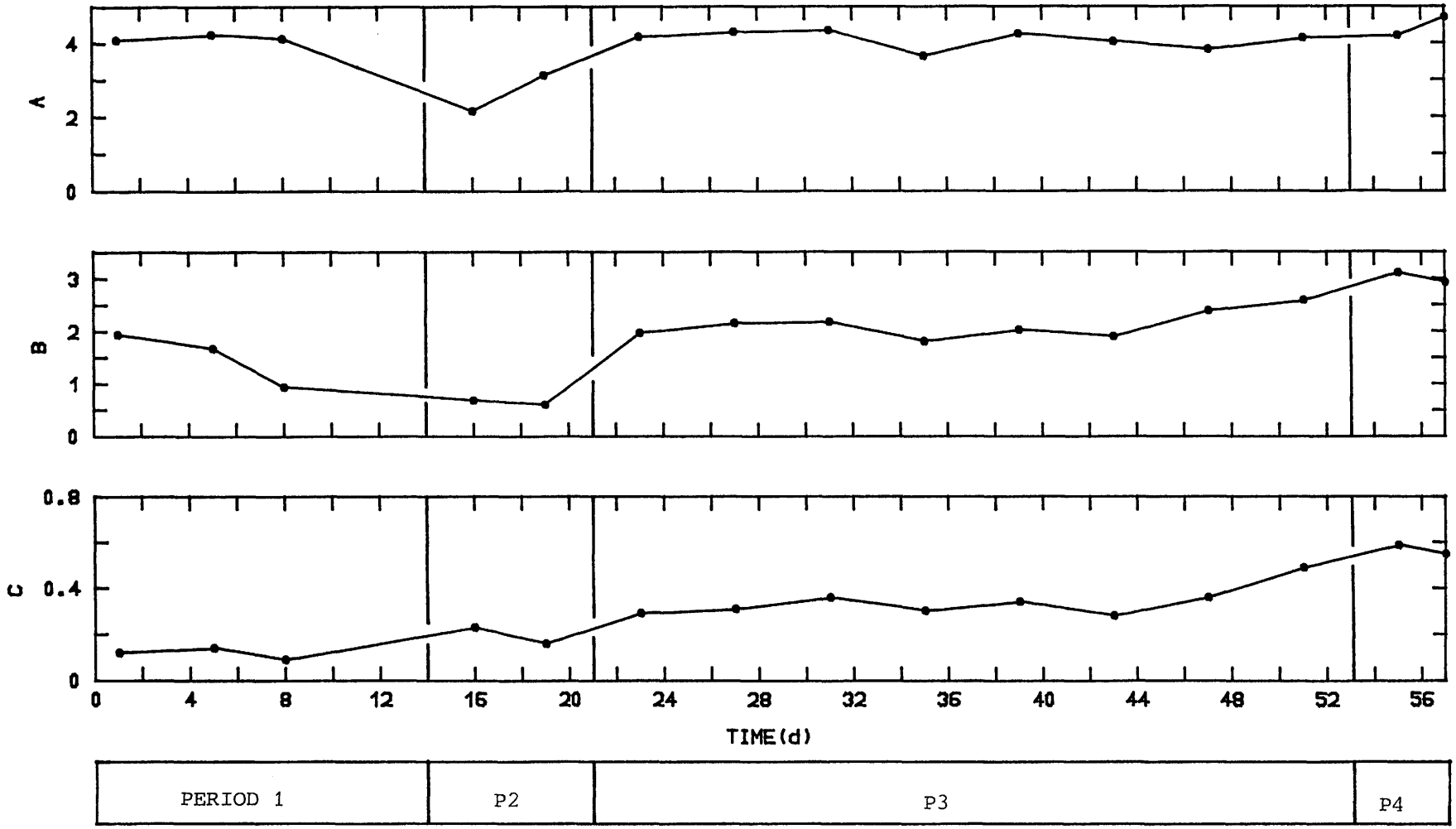


Appendix 20: Experiment 3 (Sheep 466). A. Albumin (g/l). B. Albumin:globulin ratio and C. Total protein content (g/l) measured in blood plasma.

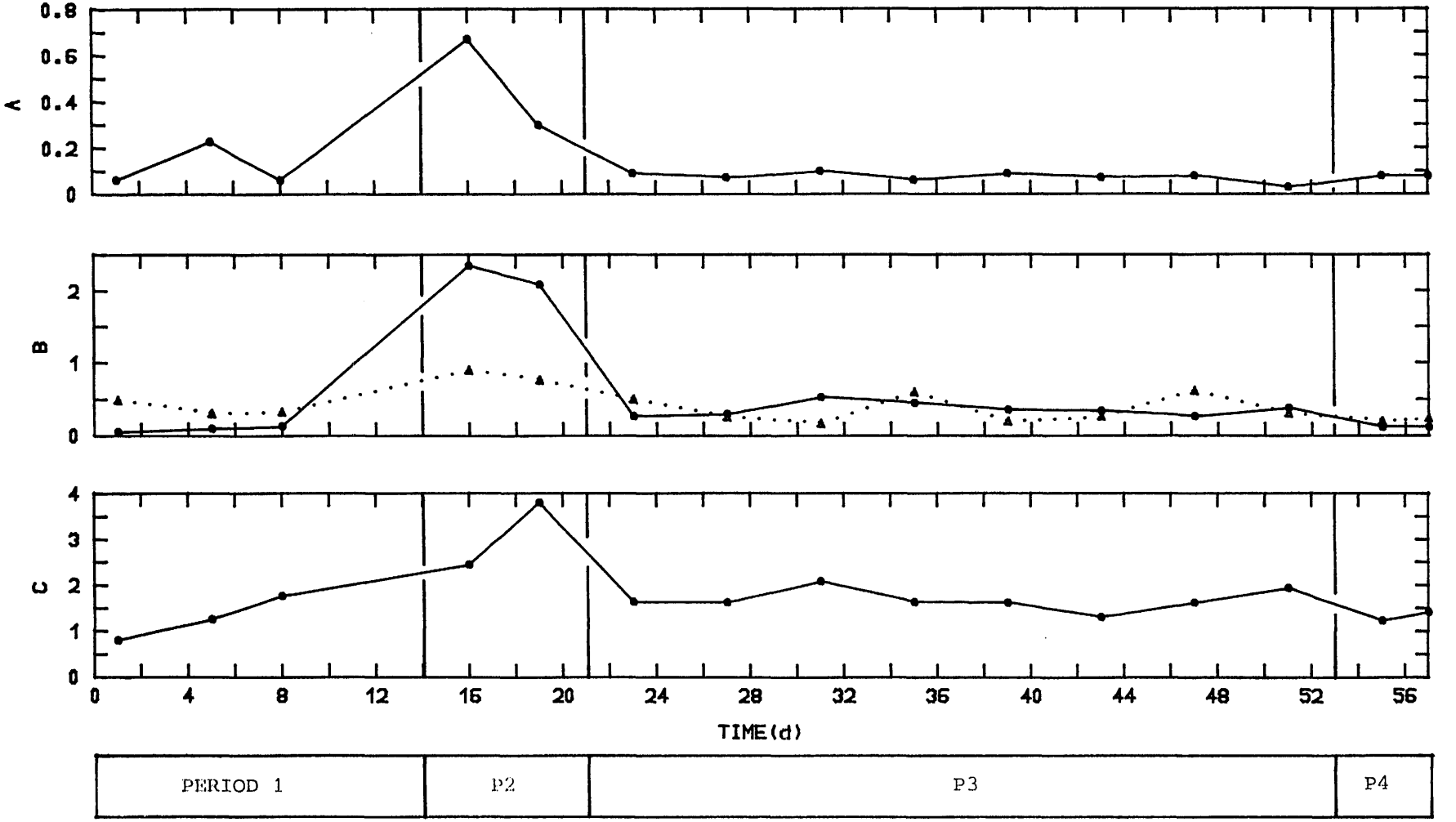


PERIOD 1	P2	P3	P4

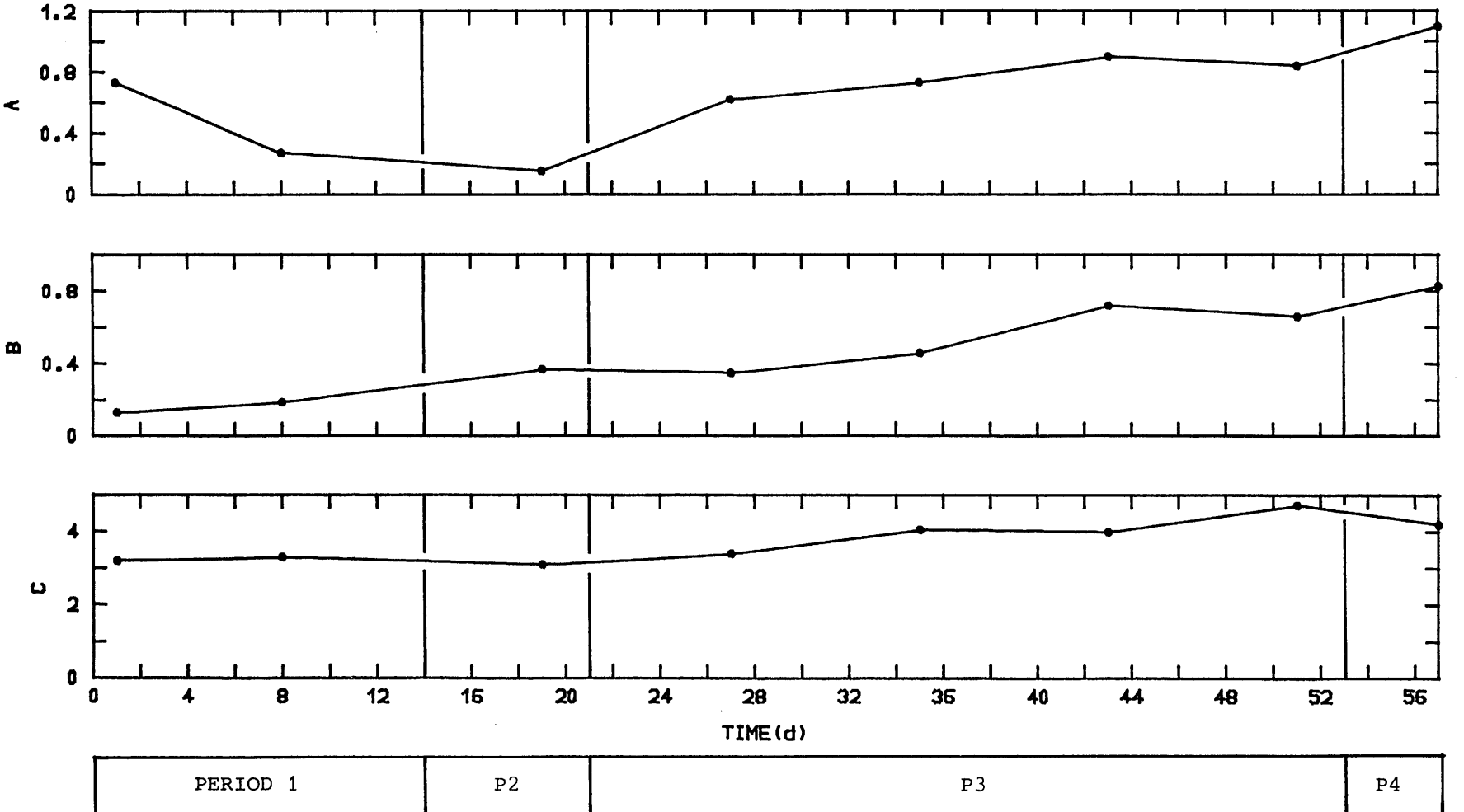
Appendix 21: Experiment 3 (Sheep 466). The concentration of A. (•) calcium (mmol/l), (▲) magnesium (mmol/l). B. (•) copper (mmol/l) and (▲) phosphorus (mmol/l) measured in blood plasma.



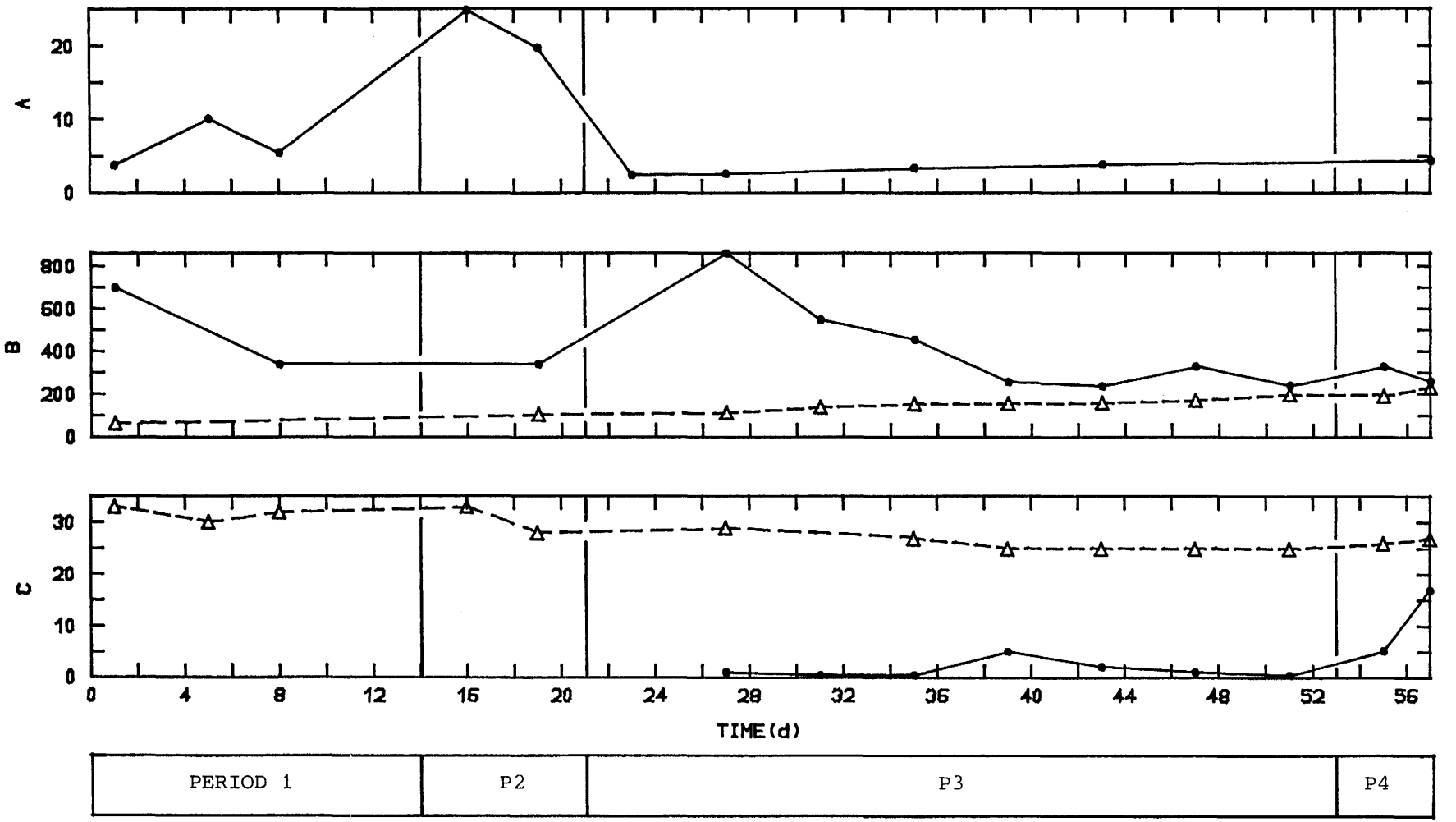
Appendix 22: Experiment 3 (Sheep 468). The concentration in blood plasma of A. Glucose (mmol/l). B. Lactate (mmol/l). C. Glycerol (mmol/l).



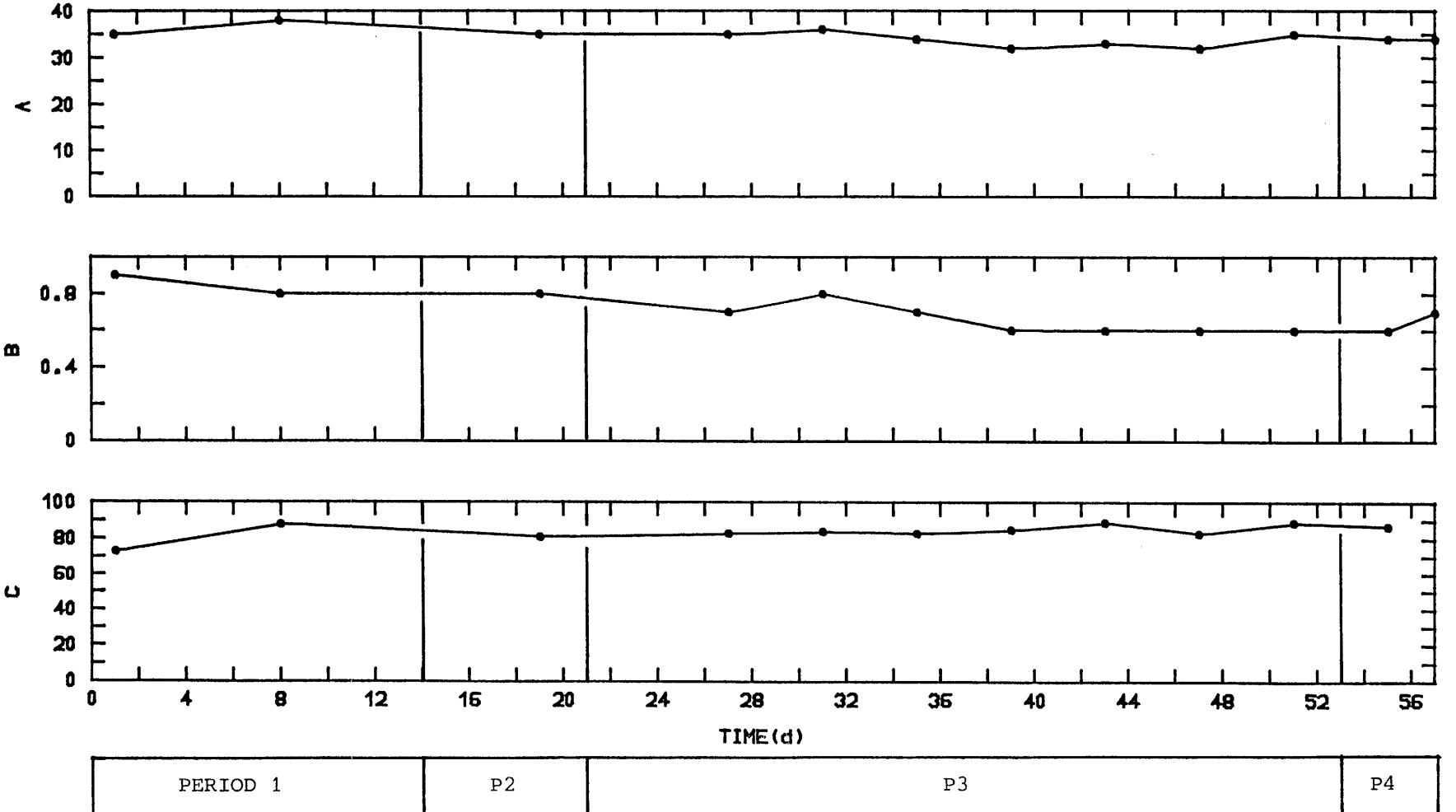
Appendix 23: Experiment 3 (Sheep 468). The concentration in blood plasma of A. NEFA (mmol/l). B. (•) 3-OH butyrate (mmol/l) and (▲) acetoacetate (mmol/l). C. Acetate (mmol/l).



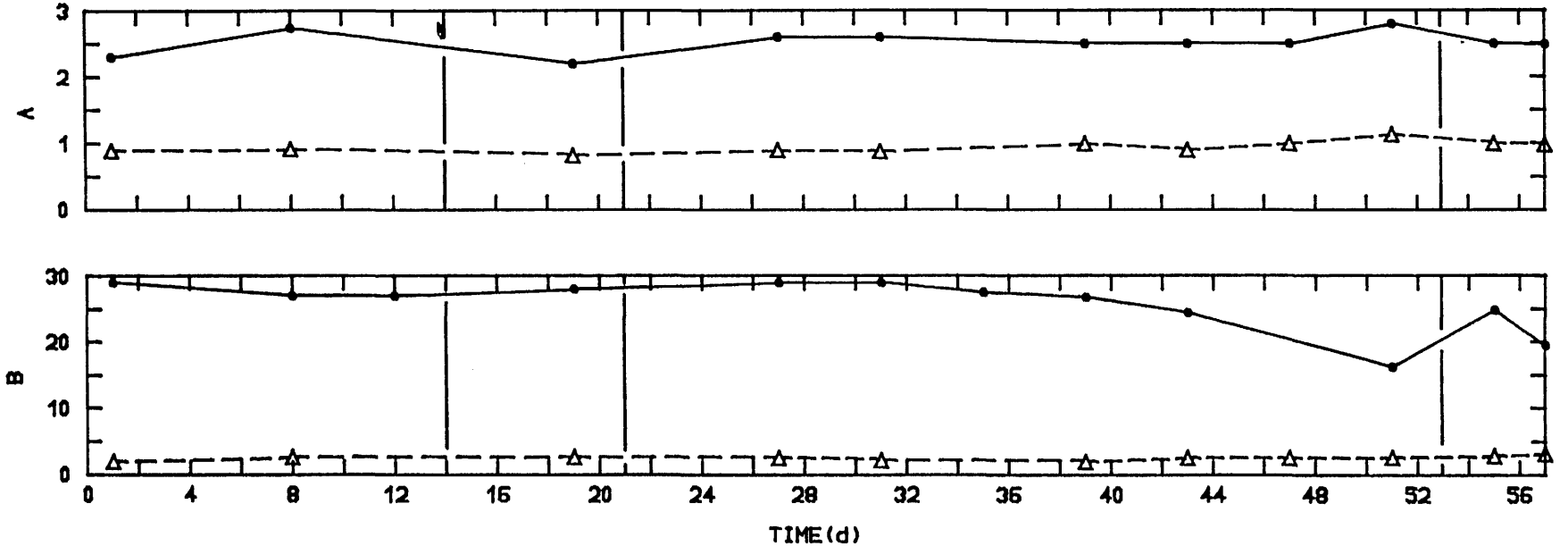
Appendix 24: Experiment 3 (Sheep 468). The concentration in blood plasma of A. Insulin (ng/ml), B. Glucagon (ng/ml) and C. Growth hormone (ng/ml).



Appendix 25: Experiment 3 (Sheep 468). The concentration in blood plasma of A. cortisol (ng/ml), the activity in blood plasma of glutamic oxaloacetic transaminase B. (●) (iu/l) and (▲) L-γ- glutamyl-transferase (iu/l). C. (•) sorbitol dehydrogenase (iu/l) and (Δ) blood packed cell volume (%).

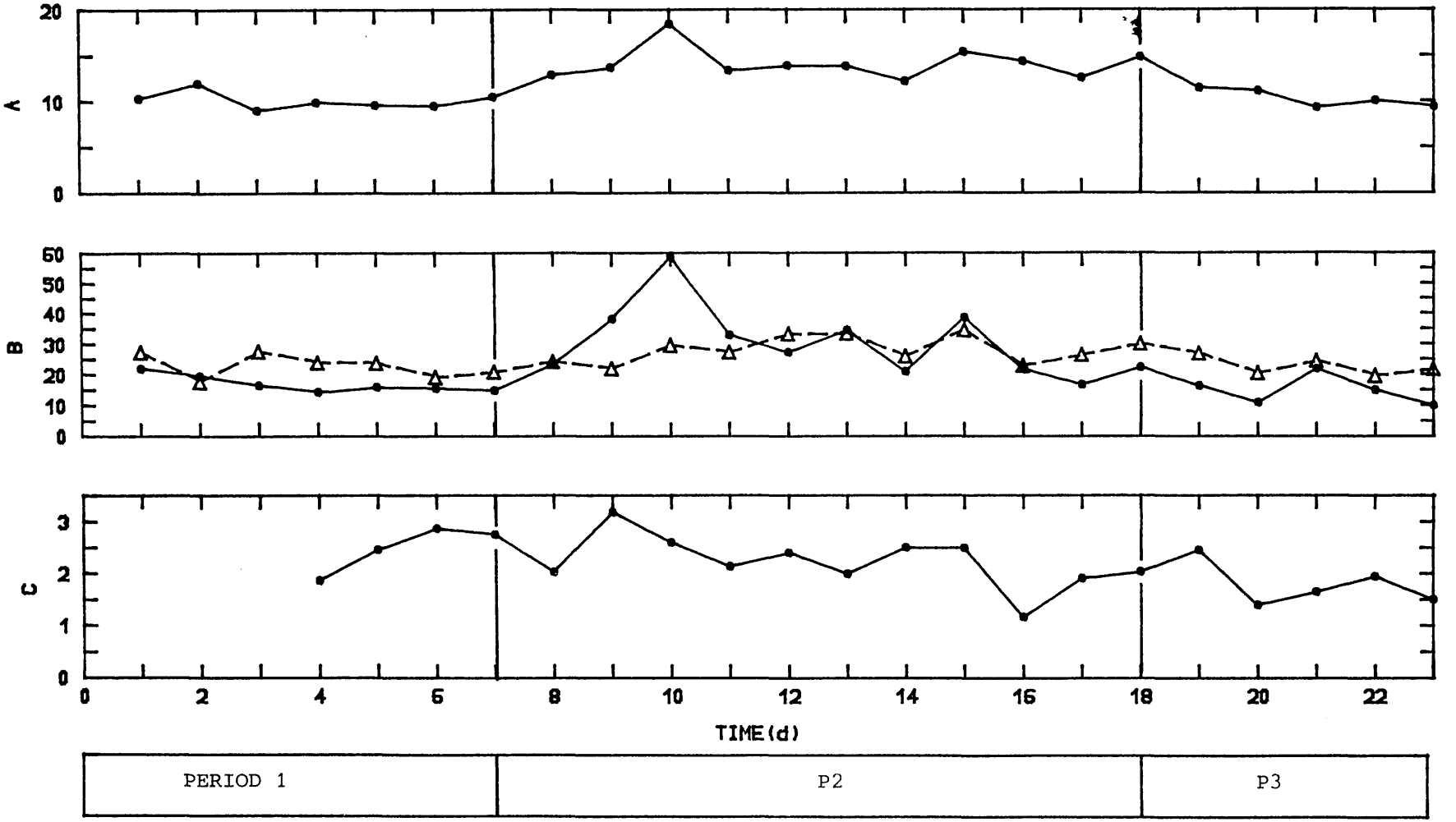


Appendix 26: Experiment 3 (Sheep 468). A. Albumin concentration (g/l). B. Albumin:globulin ratio and C. Total protein concentration (g/l) in blood plasma.

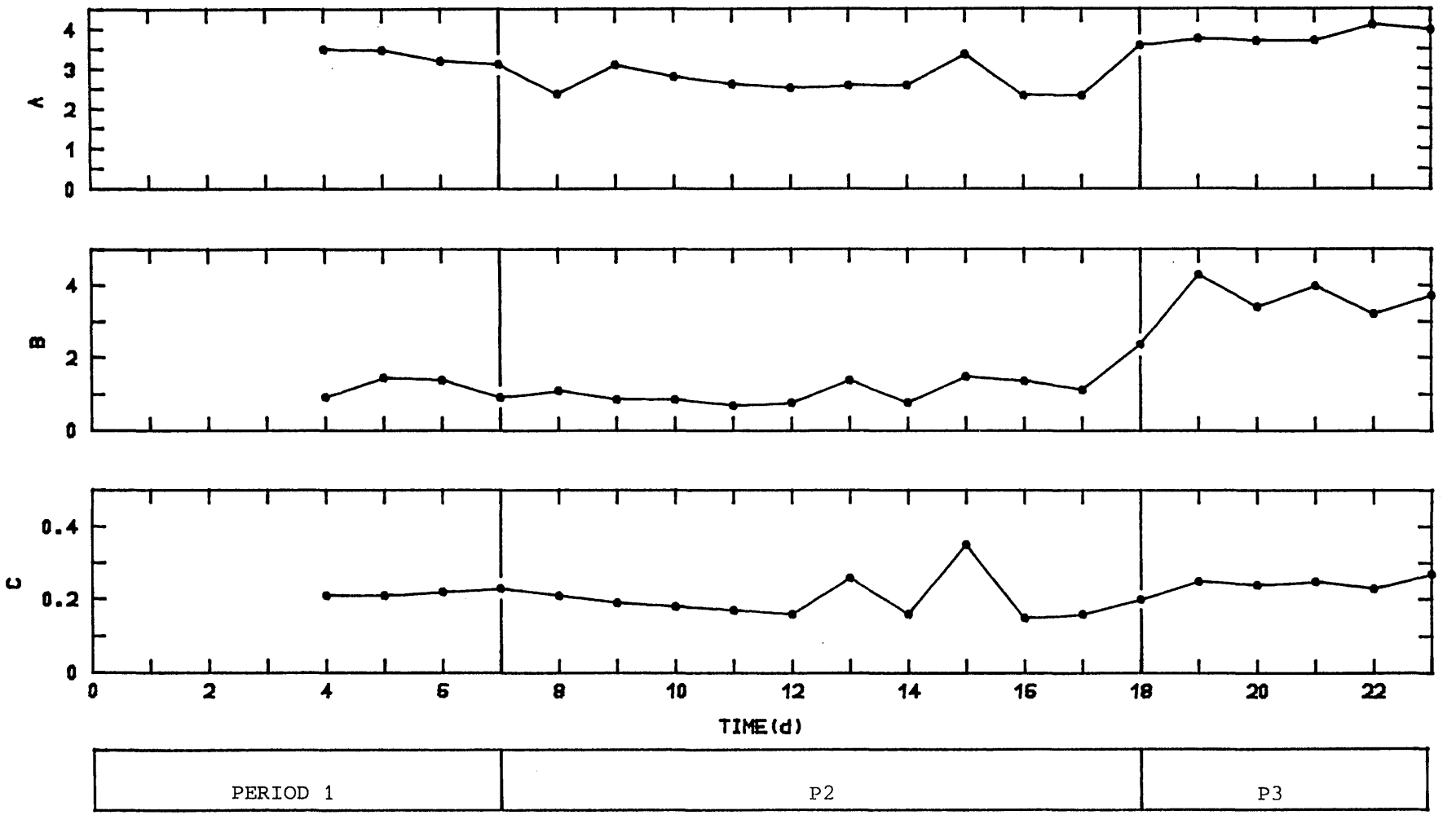


PERIOD 1	P2	P3	P4

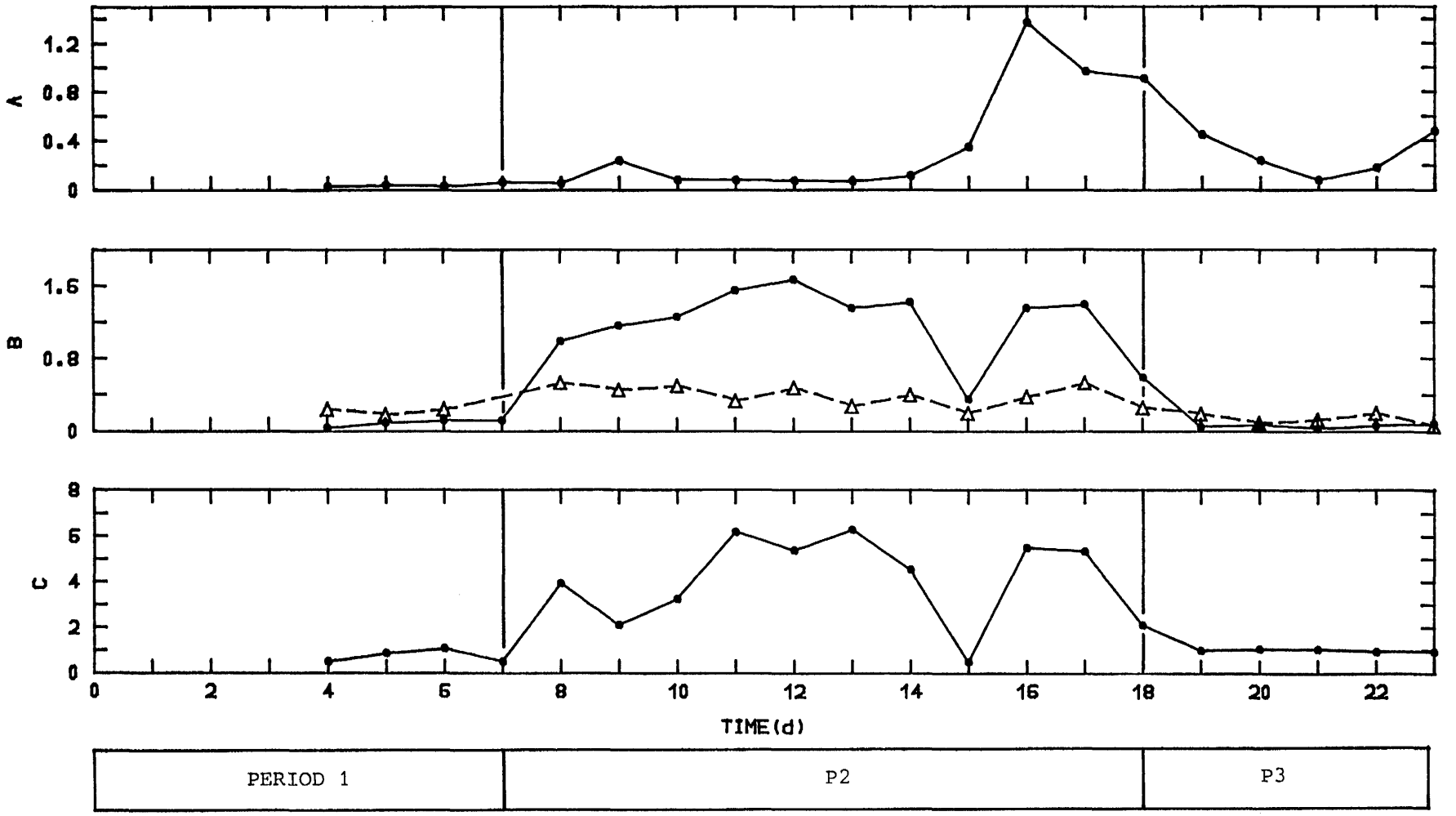
Appendix 27: Experiment 3 (Sheep 468). The concentration in blood plasma of A. (•) calcium (mmol/l), (▲) magnesium (mmol/l). B. (•) Copper (mmol/l) and (△) phosphorus (mmol/l).



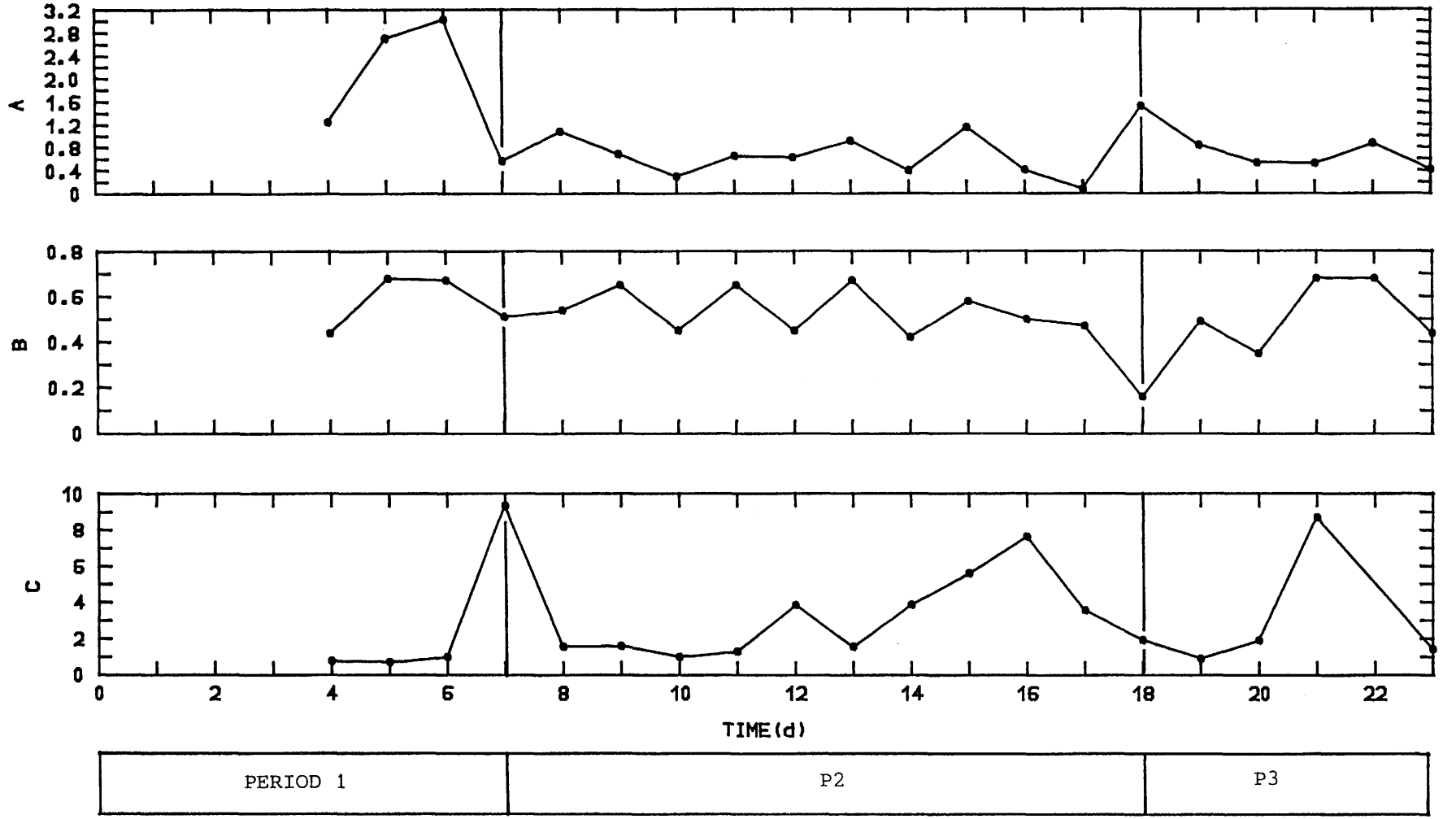
Appendix 28: Experiment 4 (Sheep 462). A. N excretion (g/d). B. (•) creatine and (Δ) creatinine excretion (g/d). C. Urea concentration (mmol/l) in blood plasma.



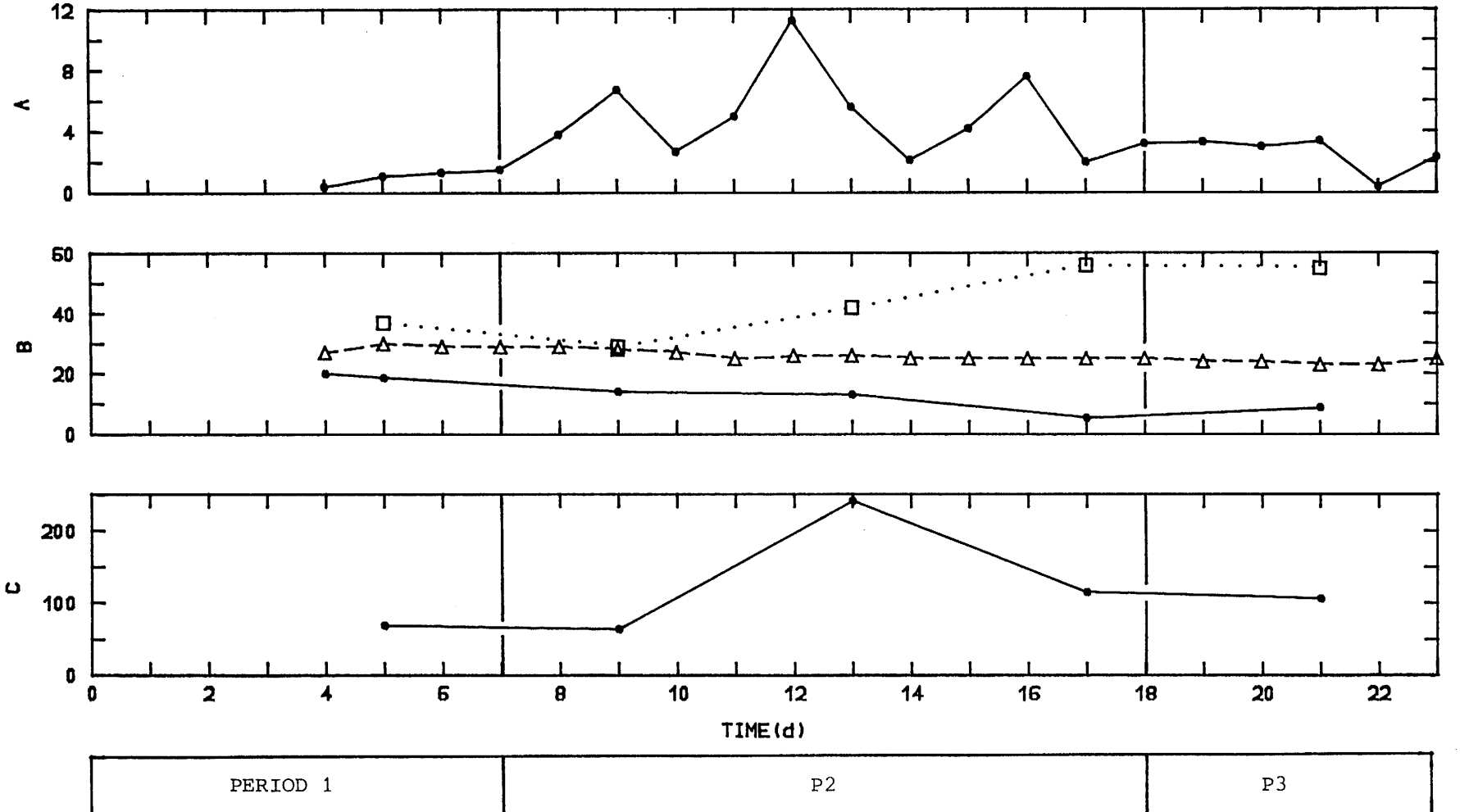
Appendix 29: Experiment 4 (Sheep 462). The concentration of A. Glucose (mmol/l), B. Lactate (mmol/l) and C. Glycerol (mmol/l) in blood plasma.



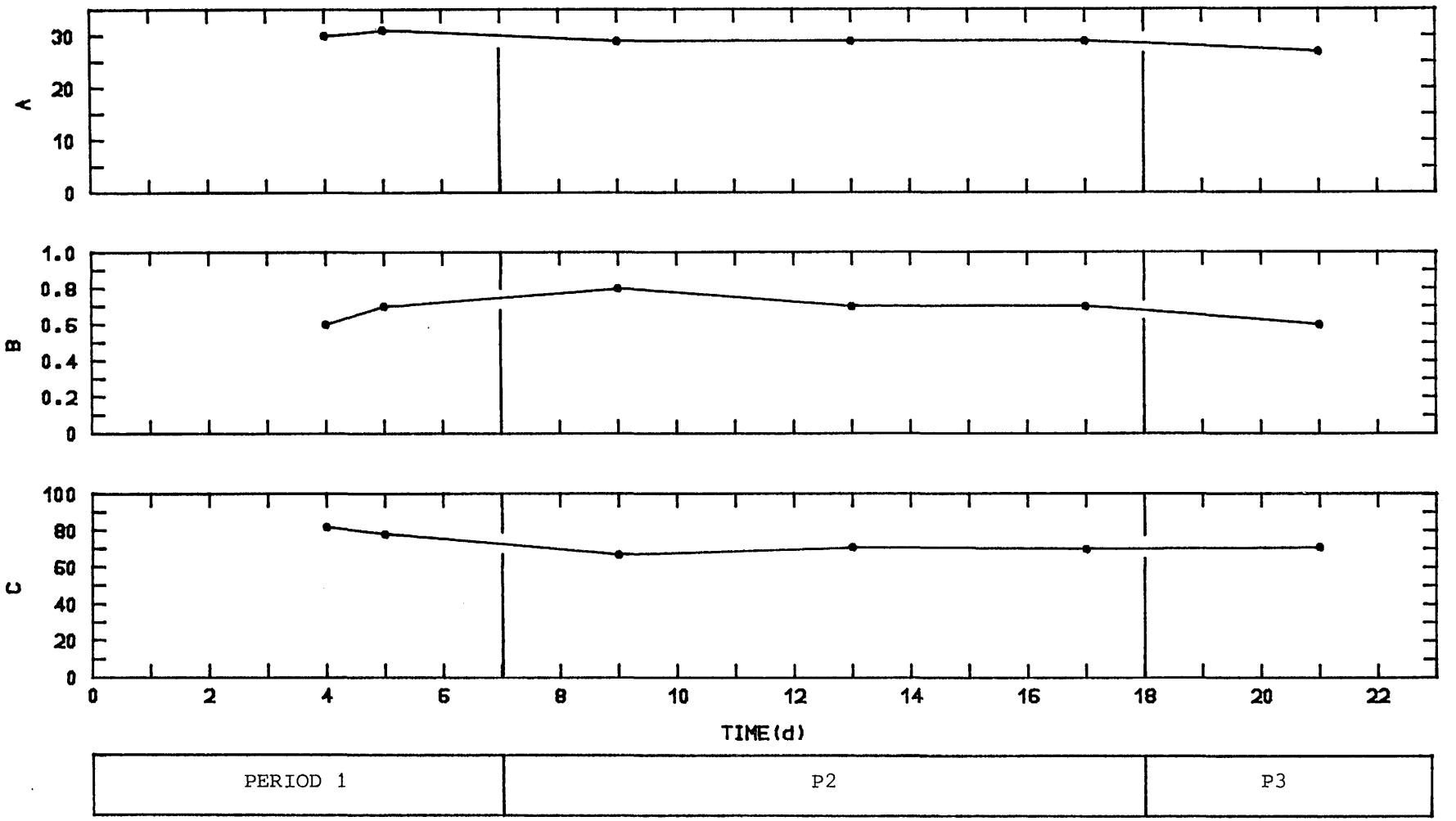
Appendix 30: Experiment 4 (Sheep 462). The concentration of A. NEFA (mmol/l), B. (•) 3-OH butyrate (mmol/l) and (Δ) acetoacetate (mmol/l) and C. Acetate (mmol/l) in blood plasma.



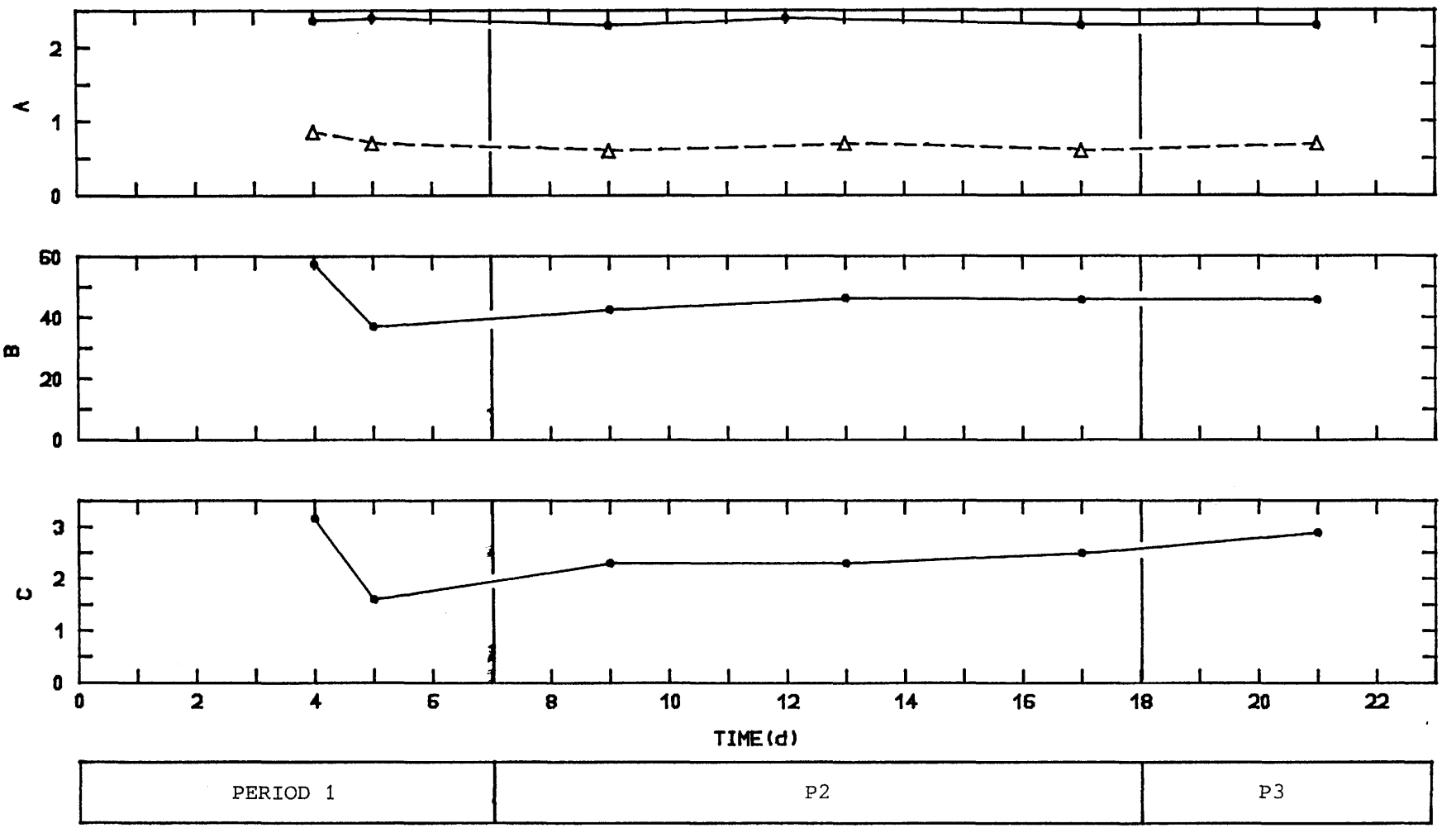
Appendix 31: Experiment 4 (Sheep 462). The concentration of A. Insulin (ng/ml), B. Glucagon (ng/ml) and C. Growth hormone (ng/ml) in blood plasma.



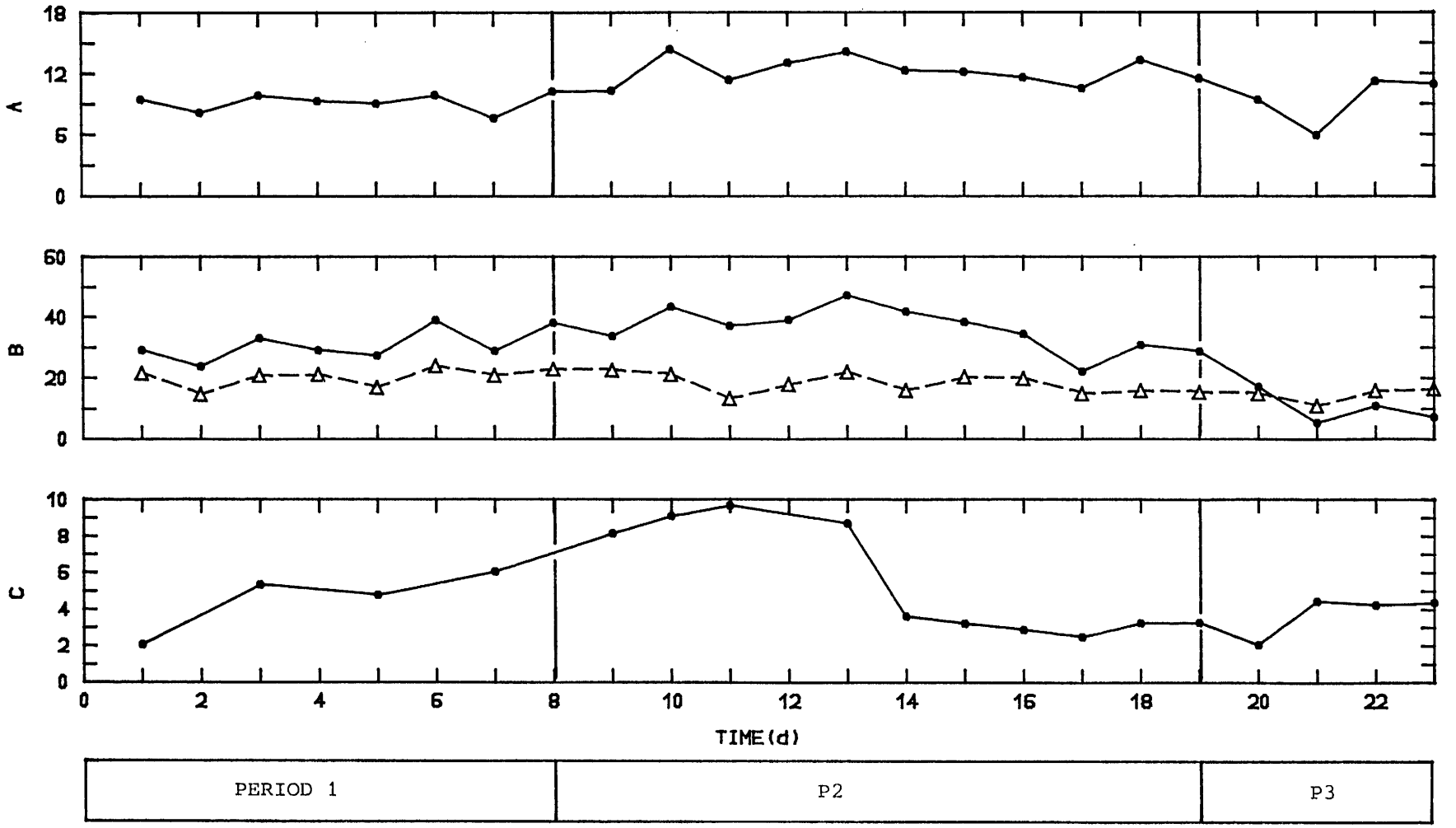
Appendix 32: Experiment 4 (Sheep 462). A. The concentration of Cortisol (ng/ml) in blood plasma. B. (•) sorbitol dehydrogenase (iu/l), (□) L-γ-Glutamyl transferase (iu/l) activity in plasma and (Δ) packed cell volume (%) of blood. C. Glutamic oxaloacetic transaminase activity (iu/l) in blood plasma.



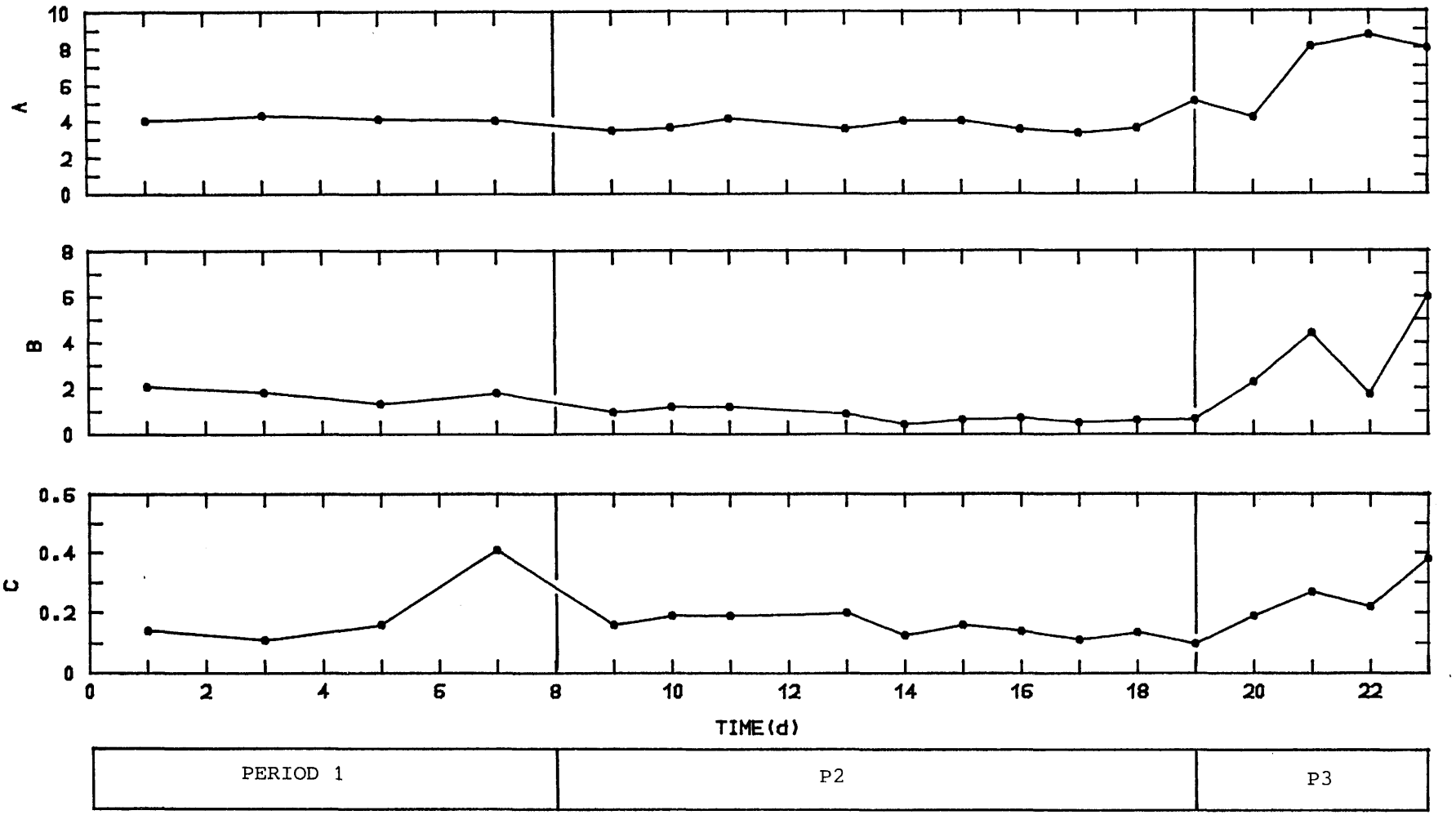
Appendix 33: Experiment 4 (Sheep 462). A. Albumin concentration (g/l). B. Albumin:Globulin ratio. C. Total protein concentration (g/l) of blood plasma.



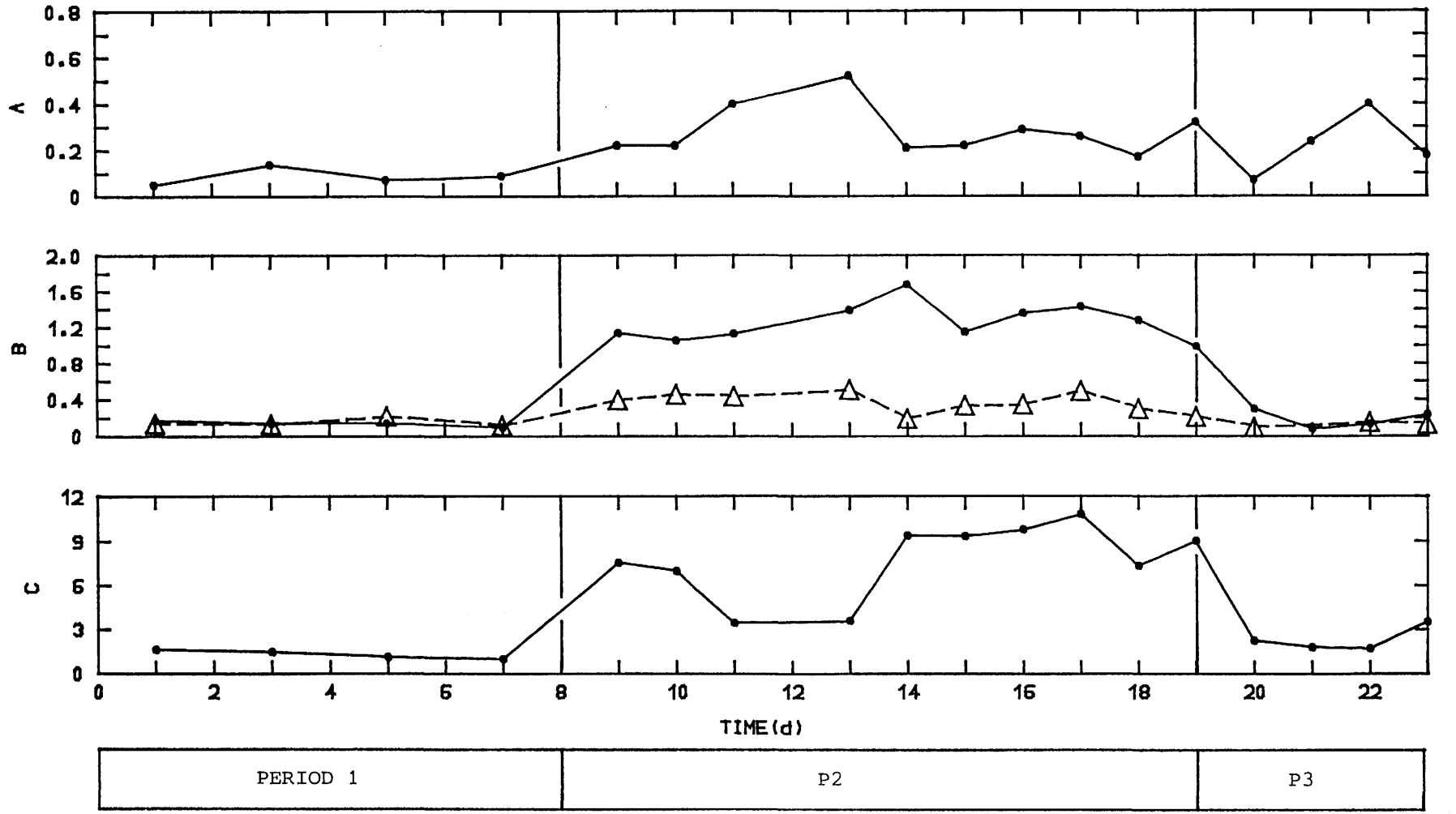
Appendix 34: Experiment 4 (Sheep 462). The concentration of A. (•) Calcium (mmol/l), (Δ) Magnesium (mmol/l), B. Copper (mmol/l) and C. Phosphorus (mmol/l) in blood plasma.



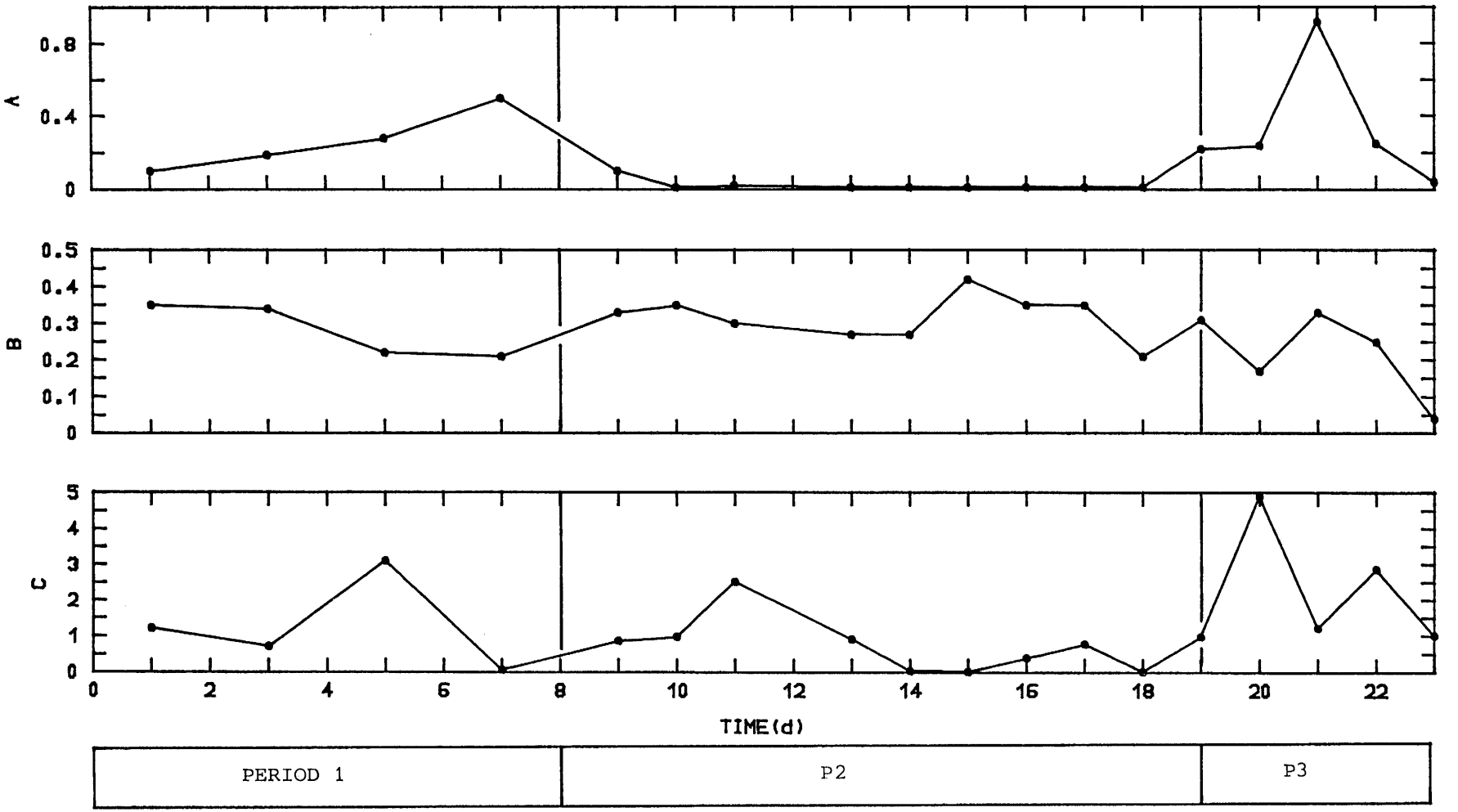
Appendix 35: Experiment 4 (Sheep 6693). A. N excretion (g/d). B. (•) creatine and (Δ) creatinine excretion (g/d). C. Urea concentration (mmol/l) in blood plasma.



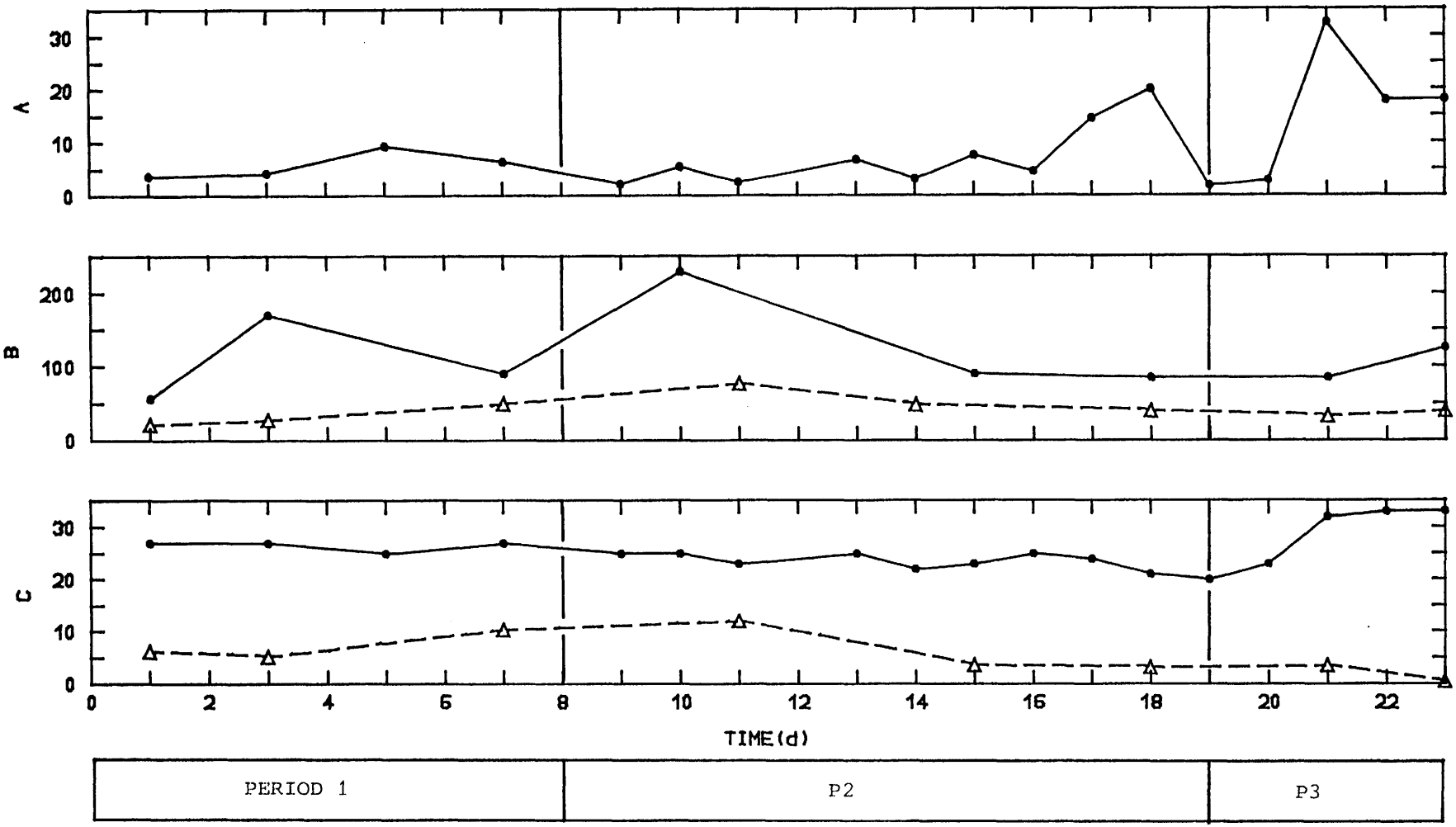
Appendix 36: Experiment 4 (Sheep 6693). The concentration of A. Glucose (mmol/l). B. Lactate (mmol/l) and C. Glycerol (mmol/l) in blood plasma.



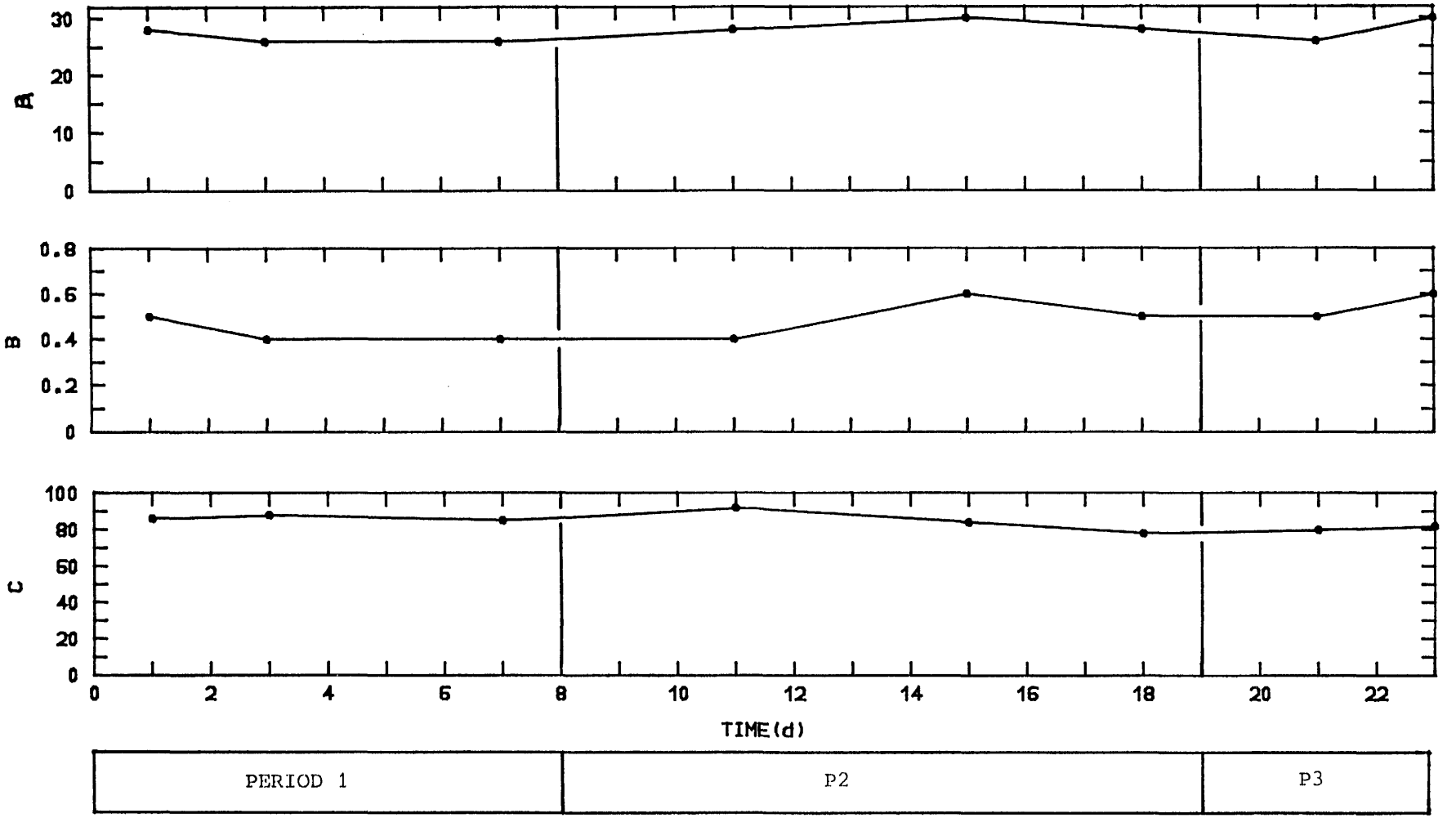
Appendix 37: Experiment 4 (Sheep 6693). The concentration of A. NEFA (mmol/l), B. (•) 3-OH butyrate (mmol/l) and (Δ) acetoacetate (mmol/l) and C. Acetate (mmol/l) in blood plasma.



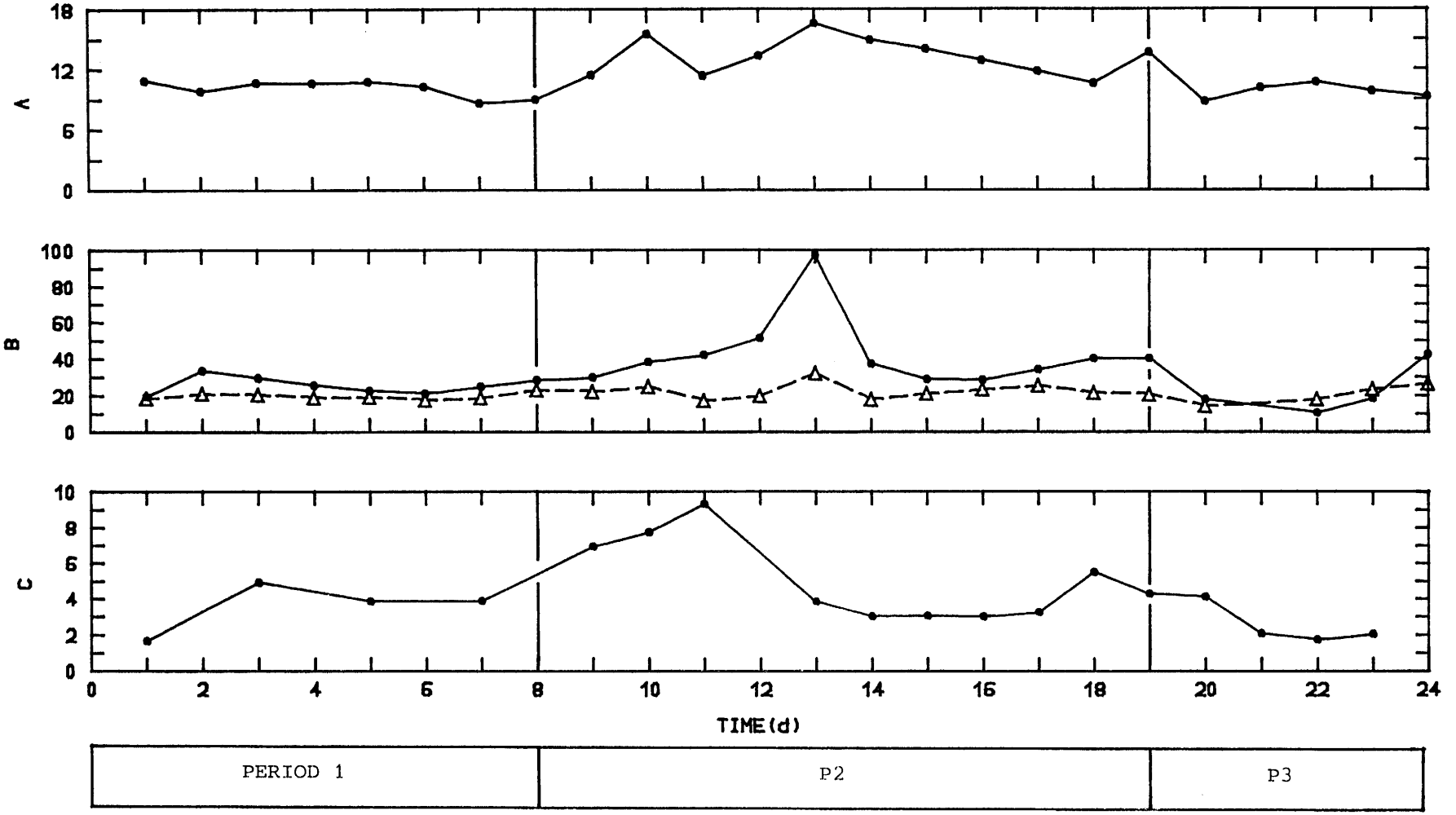
Appendix 38: Experiment 4 (Sheep 6693). The concentration of A. Insulin (ng/ml), B. Glucagon (ng/ml) and C. Growth hormone (ng/ml) in blood plasma.



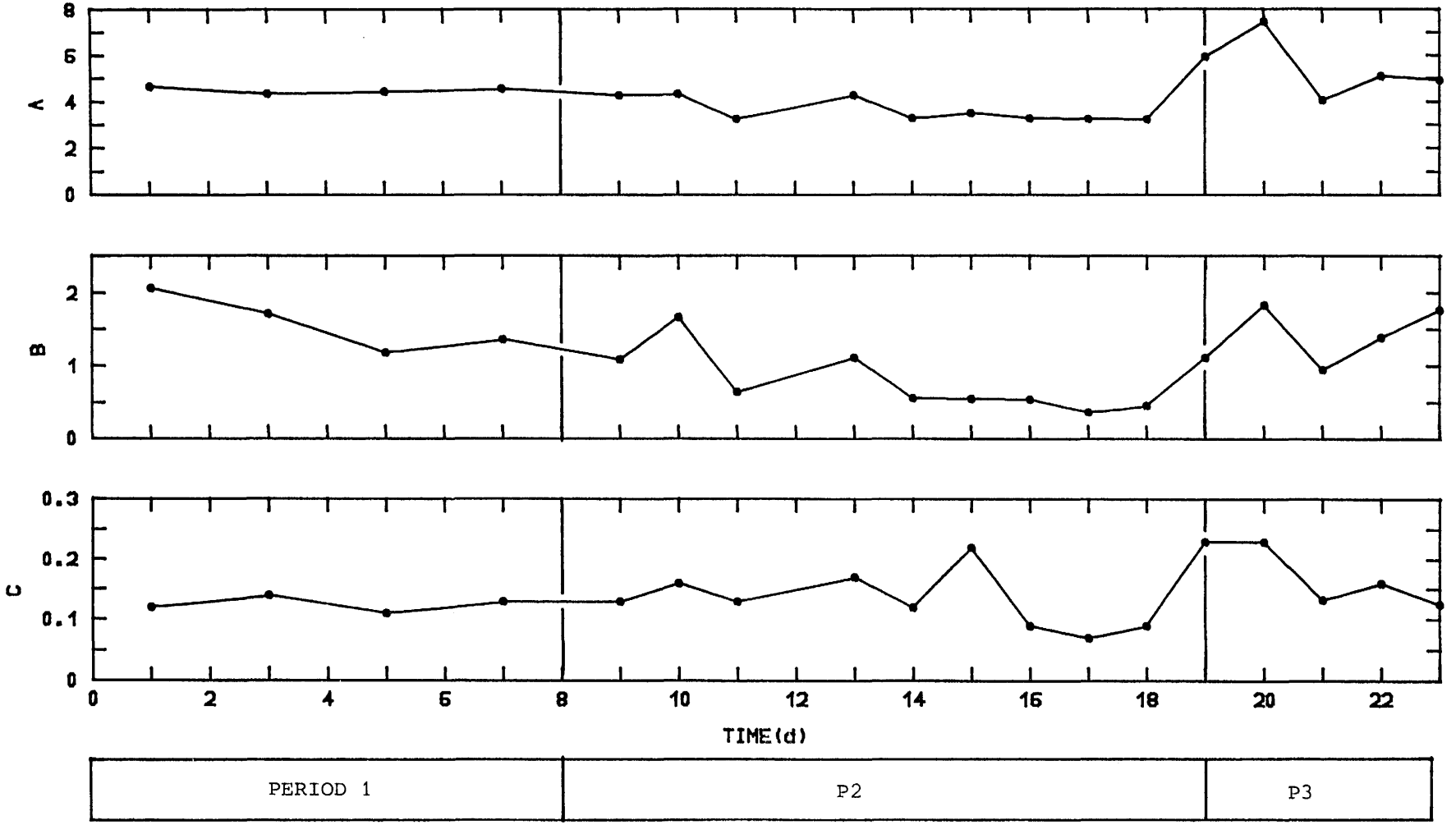
Appendix 39: Experiment 4 (Sheep 6693). The concentration of A. Cortisol (ng/ml). The activity of Glutamic oxaloacetic transaminase B. (●) (iu/l) and (Δ) L-γ-Glutamyl transferase (iu/l) in blood plasma. C. Blood packed cell volume (%) and (Δ) Sorbitol dehydrogenase activity (iu/l) in plasma.



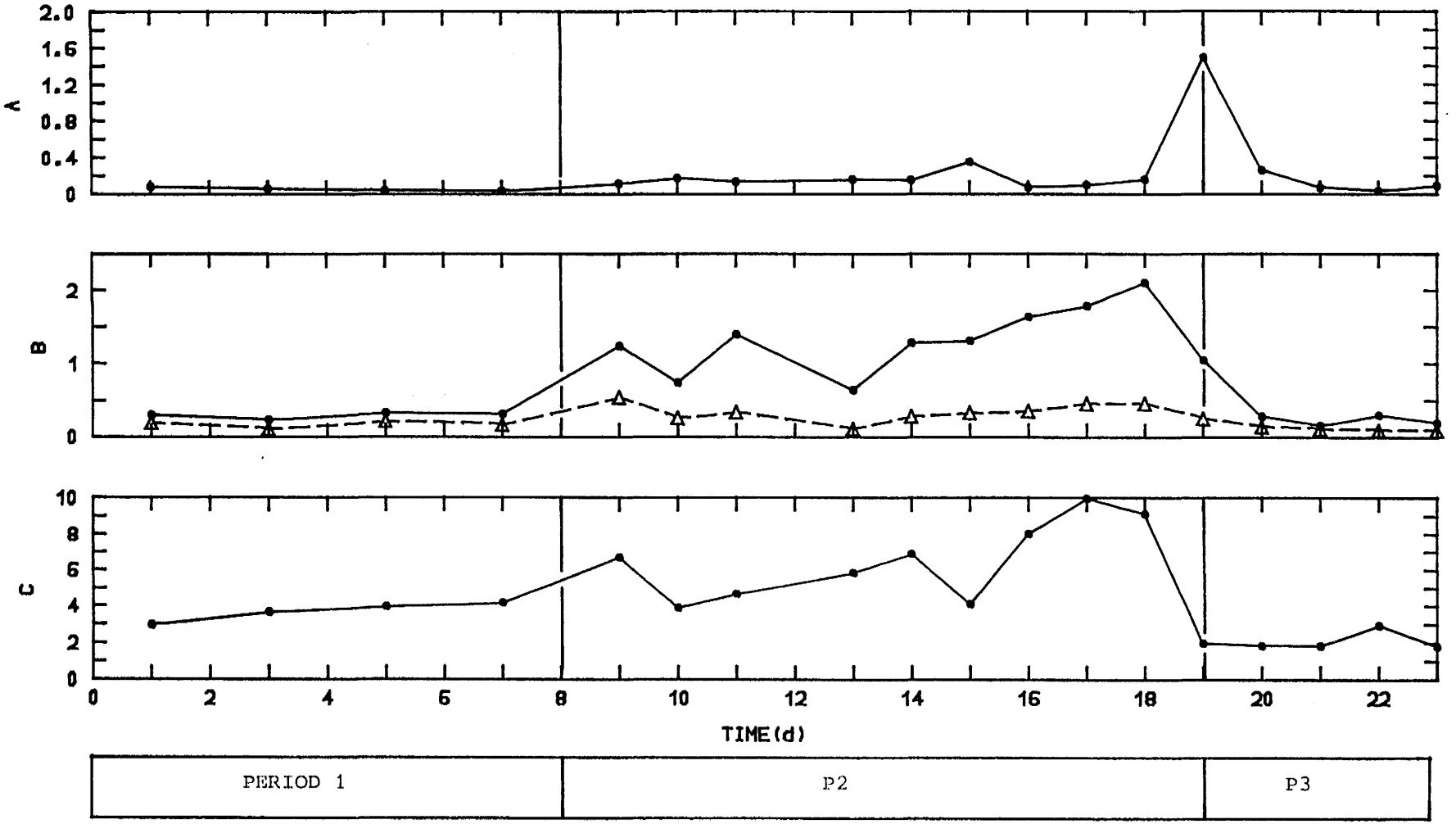
Appendix 40: Experiment 4 (Sheep 6693). A. Albumin (g/l). B. Albumin:Globulin ratio and C. Total protein (g/l) concentration in blood plasma.



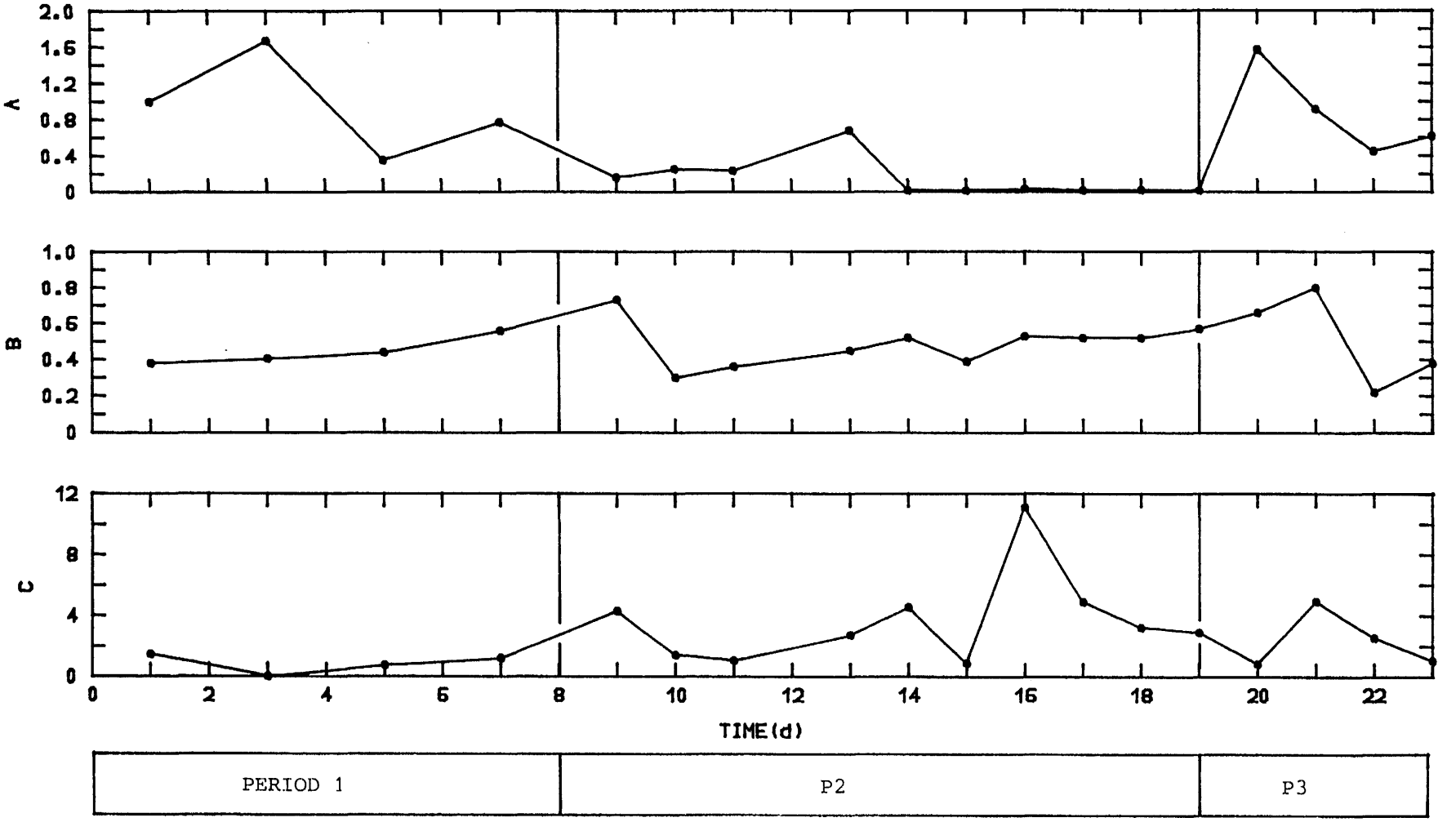
Appendix 42: Experiment 4 (Sheep 6696). A. N excretion (g/d). B. (•) Creatine and (Δ) Creatinine concentration (g/d). C. Concentration of Urea (mmol/l) in blood plasma.



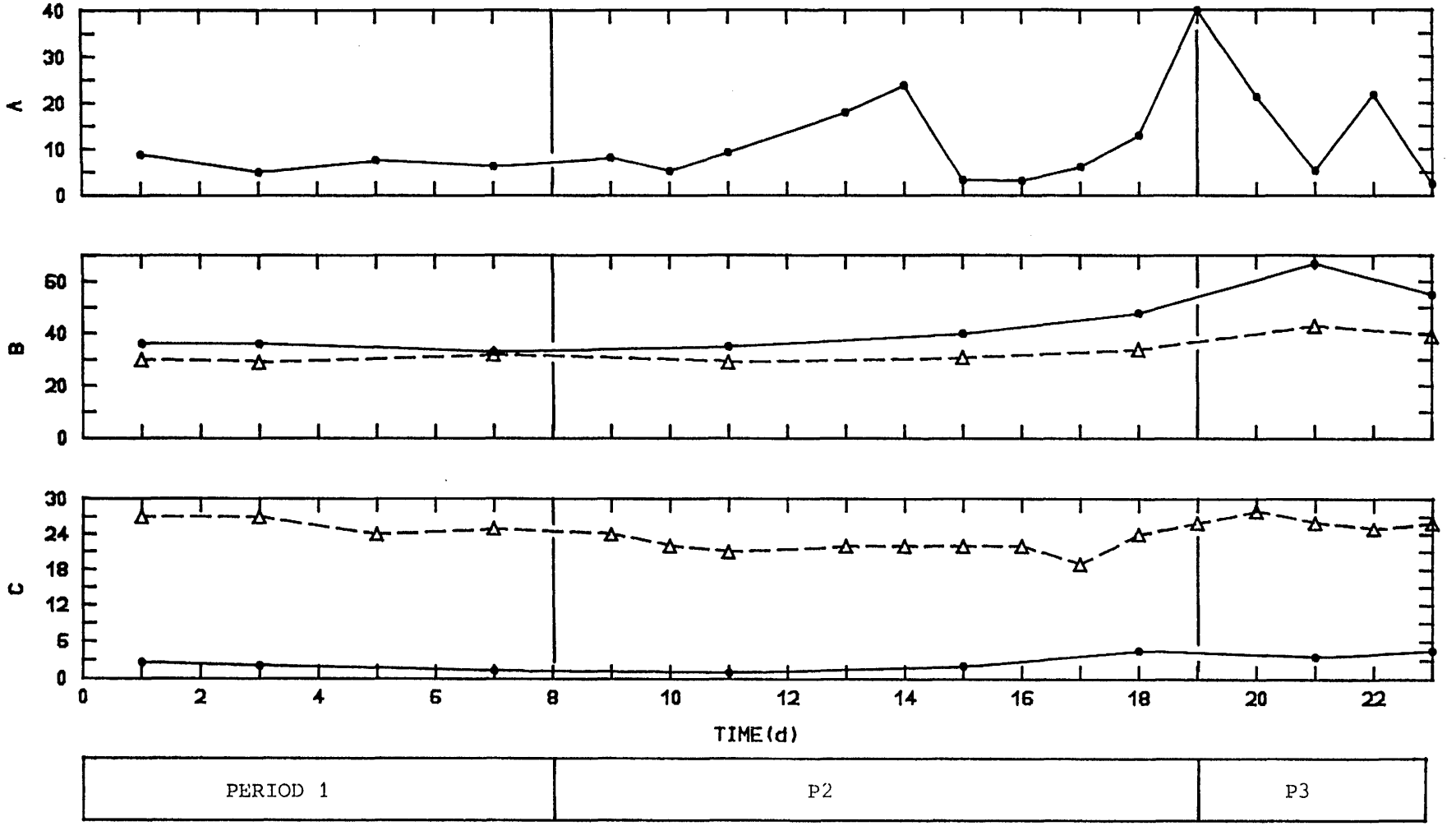
Appendix 43: Experiment 4 (Sheep 6696). The concentration of A. Glucose (mmol/l), B. Lactate (mmol/l) and C. Glycerol (mmol/l) in blood plasma.



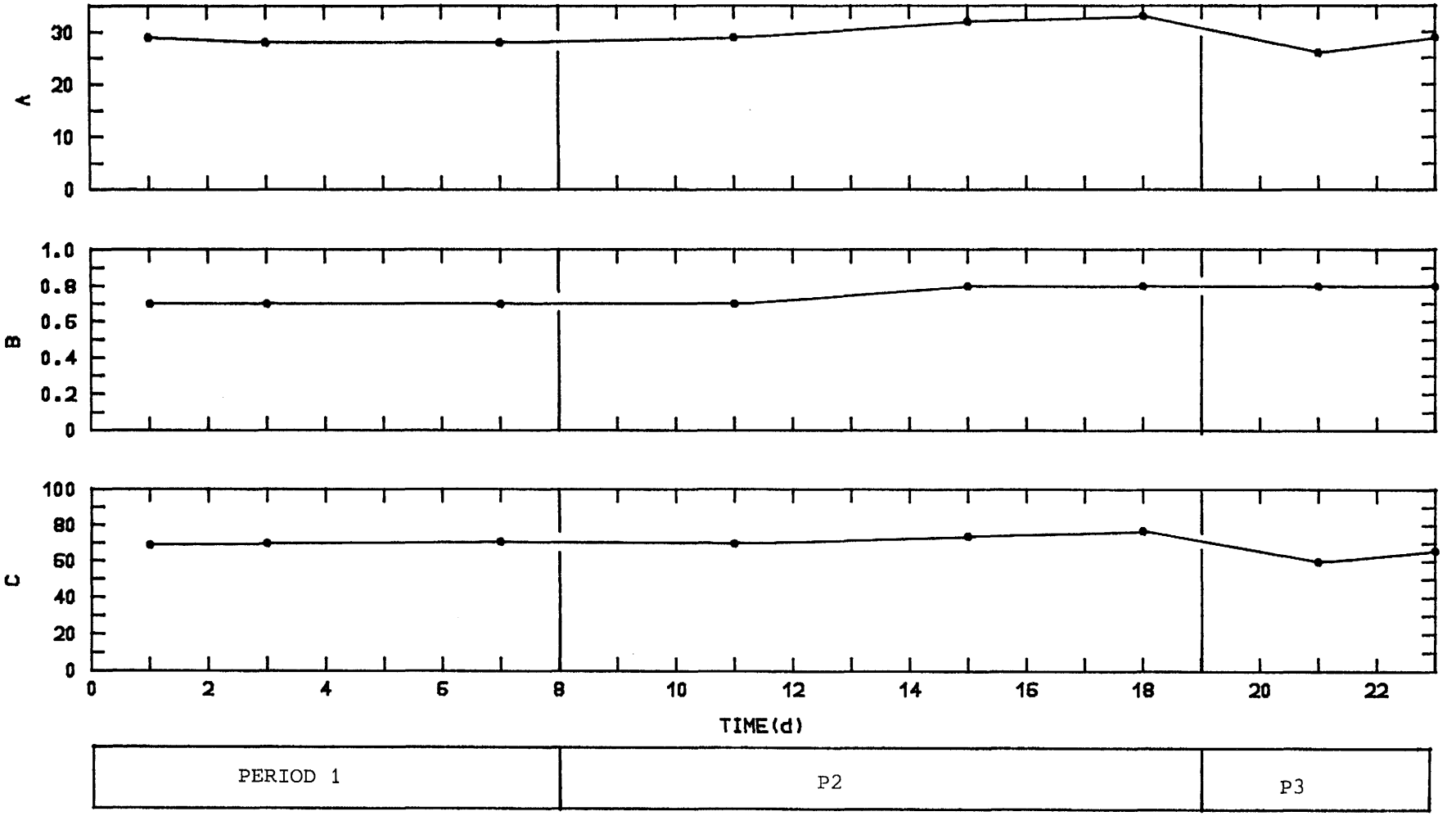
Appendix 44: Experiment 4 (Sheep 6696). The concentration of A. NEFA (mmol/l), B. (•) 3-OH butyrate (mmol/l) and (Δ) acetoacetate (mmol/l) and C. Acetate (mmol/l) measured in blood plasma.



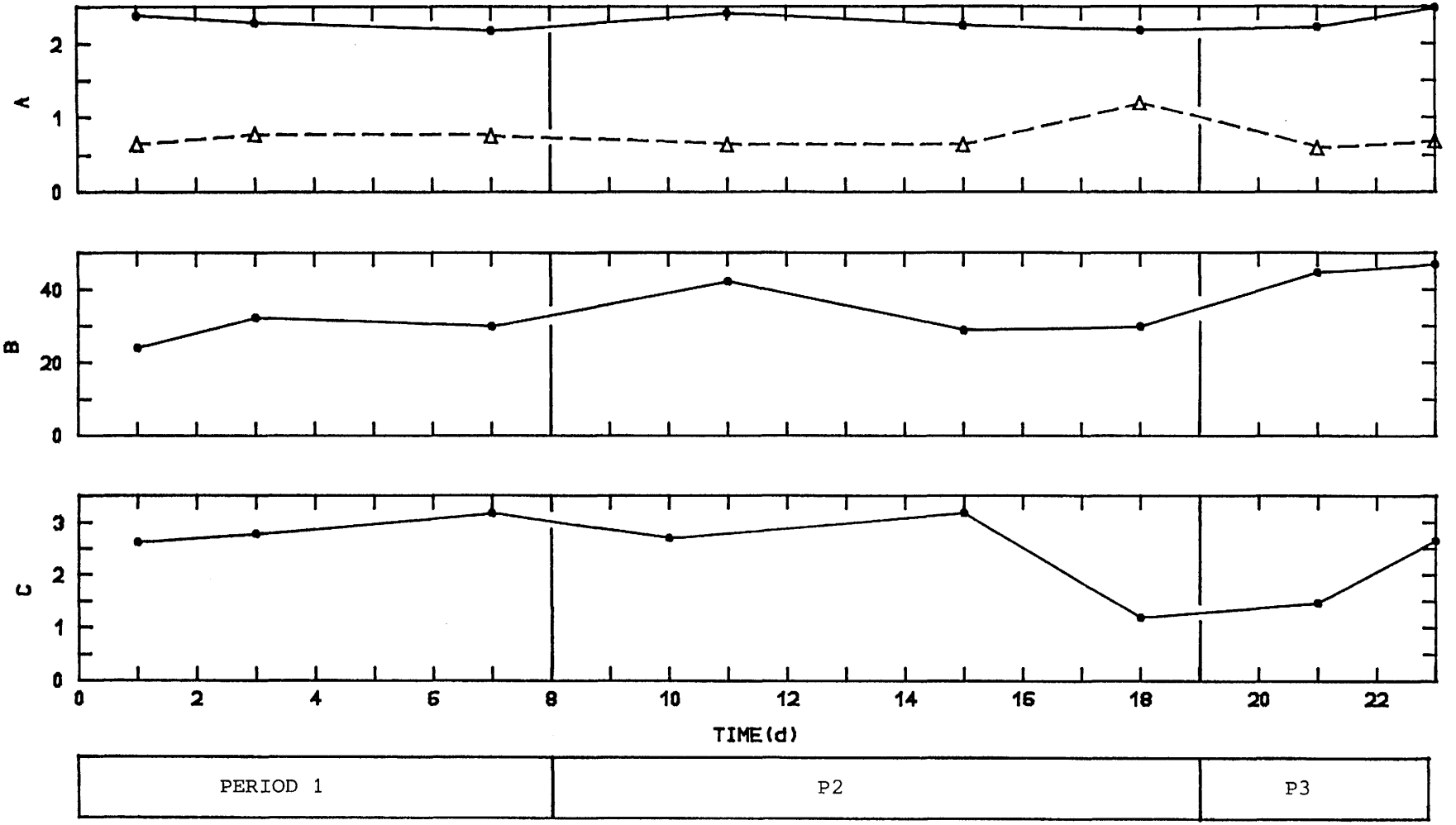
Appendix 45: Experiment 4 (Sheep 6696). The concentration of A. Insulin (ng/ml), B. Glucagon (ng/ml) and C. Growth hormone (ng/ml) measured in blood plasma.



Appendix 46: Experiment 4 (Sheep 6696). The concentration of A. Cortisol (ng/ml). The activity of glutamic oxaloacetic transaminase B.(•)(iu/l) and (Δ) L-γ-Glutamyl transferase (iu/l) measured in blood plasma. C. (•) The activity of Sorbitol dehydrogenase (iu/l) in blood plasma (Δ) Blood packed cell volume (%).



Appendix 47: Experiment 4 (Sheep 6696). A. Albumin (g/l). B. Albumin:Globulin ratio and C. Total protein concentration (g/l) of blood plasma.



Appendix 48: Experiment 4 (Sheep 6696). The concentration of A. (•) Calcium (mmol/l), (Δ) Magnesium (mmol/l), B. Copper (mmol/l) and C. Phosphorus (mmol/l) measured in blood plasma.

Amino acids	Sheep 6693								
	Period 1			Period 2				Period 3	
	Day 1	3	7	9	11	15	18	21	23
Aspartate	0.61	0.58	0.58	0.42	0.40	0.36	0.43	1.02	0.94
Threonine	2.75	1.79	1.71	3.13	3.31	2.50	3.15	0.67	1.77
Serine	4.23	3.97	3.40	2.16	2.24	2.03	2.49	4.83	4.41
Glutamate	5.64	6.85	6.56	5.63	5.34	6.19	5.37	7.50	10.61
Proline	5.65	5.22	4.81	5.50	7.72	7.97	7.89	3.74	2.17
Cit/Glycine	29.17	33.73	30.10	15.17	15.15	17.73	21.60	36.99	35.06
Alanine	9.50	8.69	9.01	3.48	3.72	2.94	2.53	9.06	4.51
Cystene	0.33	0.24	0.46	0.34	0.48	0.39	0.25	0.42	0.30
Valine	10.07	8.73	9.80	18.33	18.97	20.07	17.07	9.39	14.90
Methionine	1.01	0.89	1.10	0.96	0.96	0.58	0.74	0.55	0.59
Isoleucine	3.58	3.58	3.96	7.40	6.67	5.13	2.63	2.20	2.60
Leucine	6.08	5.83	6.93	13.98	12.58	11.29	10.45	6.44	5.57
Tyrosine	2.72	2.66	3.16	2.94	2.47	2.30	3.58	2.13	1.47
Phenylalanine	2.14	2.14	2.45	2.88	2.79	3.15	2.91	2.79	2.22
Histidine	4.00	3.49	3.61	3.53	3.68	4.35	4.70	5.51	4.08
Tryptophan	0.75	0.60	0.92	0.47	0.47	0.55	0.76	0.38	0.43
Ornithine	2.60	2.91	2.48	4.06	3.66	3.33	3.48	1.17	2.54
Lysine	6.55	5.48	5.97	5.93	5.74	5.54	6.15	3.64	4.66
Arginine	2.64	2.64	3.00	3.71	3.67	3.59	3.82	1.90	1.10
Total Aino Acids (µmol/100ml)	314.00	274.30	252.20	315.70	325.10	196.50	252.30	194.80	110.20

Appendix 49: Experiment 4. Amino acid composition (mol/100mol) measured the blood plasma of sheep 6693. See Table III(22) for period treatments.

Amino acids	Sheep 6696								
	Period 1			Period 2				Period 3	
	Day 1	3	7	9	11	15	18	21	23
Aspartate	0.56	0.57	0.69	0.49	0.41	0.56	0.35	0.51	0.50
Threonine	1.89	1.55	1.62	2.03	2.54	2.01	2.39	1.46	1.17
Serine	3.25	3.88	4.13	1.69	2.30	2.85	1.77	5.90	4.09
Glutamate	7.86	7.06	7.64	6.82	5.37	7.10	6.62	5.91	6.61
Proline	6.79	7.24	5.92	4.74	4.95	4.73	4.09	5.43	6.14
Cit/Glycine	31.20	32.14	33.93	20.01	17.08	23.62	18.17	38.40	37.85
Alanine	6.74	9.24	6.99	2.80	2.59	3.48	2.39	5.03	8.18
Cystene	0.21	0.22	0.22	0.31	0.28	0.28	0.33	0.16	0.17
Valine	10.55	18.92	18.94	20.86	19.08	14.70	18.48	8.11	8.45
Methionine	0.59	0.58	0.50	0.35	0.84	0.42	0.46	0.57	0.68
Isoleucine	3.58	3.43	3.36	6.93	7.20	5.25	6.97	3.14	3.16
Leucine	6.00	5.84	5.65	12.54	12.89	10.06	12.15	6.42	6.12
Tyrosine	2.98	2.29	2.47	2.50	2.79	2.35	2.44	2.56	2.26
Phenylalanine	2.19	1.71	1.75	2.26	2.47	2.34	2.23	2.86	2.00
Histidine	3.43	2.94	3.36	4.87	4.01	4.12	4.20	4.04	3.08
Tryptophan	0.55	0.40	0.26	0.85	0.42	0.53	0.50	-	0.40
Ornithine	2.63	2.93	3.30	3.77	4.11	3.96	4.97	2.11	1.96
Lysine	5.00	4.98	5.13	3.59	5.64	5.59	6.75	4.58	3.50
Arginine	4.00	4.08	4.13	2.58	5.03	6.06	4.73	2.82	3.69
Total Amino Acids (µmol/100ml)	268.30	361.70	323.60	210.20	338.10	241.40	245.10	280.50	271.40

Appendix 50: Experiment 4. Amino acid composition (mol/100mol) measured in the blood plasma of sheep 6696. See Table II(22) for period treatments.

Item	Period 1	Period 2			Period 3
	4	9	13	17	21
Aspartate	0.62	0.38	0.30	0.34	0.46
Threonine	4.86	4.21	3.99	3.74	3.24
Serine	3.46	1.66	1.97	1.50	3.95
Glutamate	4.83	2.93	3.37	3.80	4.53
Proline	4.01	5.49	5.87	4.00	11.37
Cit/Glycine	34.44	15.73	20.36	20.65	37.36
Alanine	9.43	3.16	3.43	2.76	8.47
Cystine	0.42	0.55	0.58	0.67	0.47
Valine	8.32	20.03	15.42	17.43	6.82
Methionine	1.13	1.41	1.04	0.85	0.76
Isoleucine	2.94	6.96	5.86	6.90	2.45
Leucine	5.28	12.55	10.72	11.89	3.97
Tyrosine	2.72	3.31	4.11	2.98	2.41
Phenylalanine	2.71	3.57	3.90	3.85	1.95
Histidine	3.29	3.83	3.16	3.10	2.67
Typtophan	1.72	1.24	1.78	2.06	0.89
Ornithine	3.09	4.21	5.17	5.75	2.11
Lysine	4.01	5.19	5.16	4.02	3.11
Arginine	2.71	3.60	3.88	3.69	2.99
Total amino acids (µmol/100ml)	216.20	286.40	231.10	193.50	326.50

Appendix 51: Experiment 4. Amino acid composition (mol/100mol) measured in blood plasma of sheep 462. Se Table III(22) for period treatments.

APPENDIX 52: Listing for Infusion Formulation Program for BBC "B" microcomputer

```
4MODE3
10REM DIET FORMULATION FOR INTRAGASTRIC INFUSION
20REM C.P.GIRDLER (04/06/82)
21PRINT:PRINT:PRINT"THIS PROGRAM IS DESIGNED TO BE USED WITH THE
'MATERIALS & METHODS' SECTION OF 'INTRAGASTRIC INFUSION AND ITS USE
IN THE INVESTIGATION OF RUMINANT NITROGEN AND GLUCOSE METABOLISM'
(GIRDLER, 1986)"
24PRINT:PRINT:PRINT"###INTRAGASTRIC INFUSION FORMULATION###"
26PRINT:PRINT:INPUT"DO YOU WANT A LIST OF THE INDIVIDUAL NUTRIENTS
INFUSED ( Y OR N) ";Z$
27VDU2
30PRINT"INTRAGASTRIC INFUSION FORMULATION":PRINT:VDU3
40PRINT:PRINT"##### ANIMAL DATA #####":PRINT:PRINT
50PRINT:INPUT"ANIMAL IDENTIFICATION    ";A1
60PRINT:INPUT"LIVEWEIGHT (Kg)          ";A2:PRINT
70FORK9=1TO20:PRINT:NEXT:Z=A2^.75:VDU2
80PRINT:PRINT"INFUSIONS FOR BEAST "A1
90PRINT:PRINT"1.---LIPID INFUSION---":PRINT
95 VDU3
100PRINT:INPUT"QUANTITY OF LIPID TO BE INFUSED (gm/d)    ";A
110PRINT:INPUT"QUANTITY OF GLUCOSE TO BE ADDED  (gm/d)   ";J
115IFJ=0THENK=0:IFJ=0GOTO130
120PRINT:PRINT:INPUT"GROSS ENERGY VALUE OF GLUCOSE (kJ/gm)     ";K
130VDU2:PRINT:PRINT"      LIPID EMULSION DATA     ":PRINT
140PRINT"DAILY ALLOWANCE (gms)":PRINT
150PRINT"LIPID                  "A
160PRINT"Na CASEINATE           "A*.4
170PRINT"EMULSIFIER             "A*.15
180PRINT"GLUCOSE                "J
190PRINT"WATER                  "(A*18.45)-J
195PRINT:PRINT"CONSTITUENTS IN THESE PROPORTIONS ARE USED TO PRODUCE
A STOCK LIPID SOLUTION"
196VDU3:PRINT:PRINT:INPUT"PRESS 'RETURN'TO CONTINUE ";T$ :VDU2
200B=A*.4:C=A*.15:PRINT
210PRINT:PRINT"CONTRIBUTION TO THE ME OF THE INFUSIONS (kJ/d)"
220PRINT:PRINT
230PRINT"LIPID                  "A*.95*39
240PRINT"Na CASEINATE           "B*19
250PRINT"EMULSIFIER             "C*.95*39
260PRINT"GLUCOSE                "J*K
270PRINT:PRINT
280PRINT"TOTAL CONTRIBUTION TO ME FRACTION OF THE INFUSIONS (kJ/d)
IS "A(*39*.95)+(B*19)+(C*39*.95)+(J*K)
290E=(A*39*.95)+(B*19)+(C*39*.95)+(J*K)
300PRINT:PRINT
310PRINT" CONTRIBUTION TO PROTEIN FRACTION     ":PRINT:PRINT:VDU3
315PRINT:INPUT"N CONTENT OF Na CASEINATE(mgN/GM DM)";B1
316F=B*B1:VDU2
320PRINT:PRINT"Na CASEINATE (mg N/d)  "F:PRINT:PRINT:VDU3
330FORK9=1TO10:PRINT:NEXT:VDU2
340PRINT"2.---PROTEIN INFUSION---":PRINT:VDU3
350PRINT:INPUT"DESIRED LEVEL OF PROTEIN NUTRITION (*M)";F1:PRINT
```

```
360PRINT:INPUT"TISSUE N REQUIREMENT AT MAINTENANCE (mg N/kg^0.75/d";G
370PRINT:INPUT"N CONTENT OF Na CASEINATE (mg N/gm.DM)";H
380PRINT:INPUT"GROSS ENERGY VALUE OF Na CASEINATE (kJ/gm.DM)";H1
390PRINT:INPUT"EFFICIENCY OF UTILIZATION OF Na CASEINATE (0-1)";H3
400VDU2
410PRINT:PRINT
420PRINT"TISSUE N REQUIREMENT (mg N/d) IS "Z*G*F1:PRINT
430PRINT"TISSUE N TO BE SUPPLIED BY Na CASEINATE INFUSION (mg/d) IS
"(Z*G*F1)-(F*H3)
440PRINT:PRINT
450PRINT"THE REQUIREMENT FOR Na CASEINATE (gm/d) IS THEREFORE
"(((Z*G*F1)-(F*H3))*1/H3)/H
46005=(((Z*G*F1)-(F*H3))*1/H3)/H:06=05*10
470PRINT:PRINT"OR "06" gm OF THE 10% Na CASEINATE SOLUTION"
480PRINT:PRINT:Z9=05
490PRINT"THE CONTRIBUTION OF THE  Na CASEINATE SOLUTION TO ME REQUI
REQUIREMENTS IS (kJ) "H1*Z9
500I=H1*Z9:PRINT:PRINT
510VDU3:L=J*K:PRINT:PRINT:INPUT"PRESS 'RETURN' TO CONTINUE";T$:VDU2
520PRINT"   TOTAL NON-VFA ENERGY CONTRIBUTION TO ME (kJ) IS  "(I+E)
530VDU3:M=I+E:FORK9=1TO10:PRINT:NEXT:VDU2
540PRINT:PRINT"3.---VFA INFUSION---":PRINT:PRINT:VDU3
550PRINT:INPUT"DESIRED LEVEL OF ENERGY NUTRITION (*M)  ";N:PRINT
560INPUT"ME REQUIREMENT AT MAINTENANCE (kJ/kgW^0.75/d)";O:PRINT:PRINT
570PRINT"TOTAL ME REQUIRED AT THIS LEVEL OF PRODUCTION IS (kJ/d)
"N*Z*O:PRINT
580 PRINT"ME SUPPLIED BY NON-VFA SOURCES (kJ/d)            "M:PRINT
590PRINT"ME SUPPLIED BY VFA INFUSION (kJ/d)
"N*Z*O)-M:PRINT:PRINT
600VDU3:P=(N*Z*O)-M:PRINT:PRINT:PRINT
610PRINT"ENTER THE VFA MOLAR RATIO TO BE INFUSED (mol/100mol)"
620PRINT:INPUT"ENTER ACETATE    ";Q
630PRINT:INPUT"ENTER PROPIONATE ";R
640PRINT:INPUT"ENTER BUTYRATE   ";S
650IF(Q+R+S)<100THENGOTO700
660IF(Q+R+S)>100THENGOTO700
670GOTO710
700PRINT:PRINT:PRINT"HEY! THIS VFA MOLAR RATIO IS INCORRECT - INPUT
IT AGAIN":PRINT:GOTO610
710T=Q*75:U=R*74:V=S*88:W=T+U+V:X=(T/W)*1164.8:Y=(U/W)*2074:B1=(V/W)
*2490:P=( N*Z*O)-M
720VDU2:PRINT:PRINT:PRINT"THE QUANTITY OF VFA REQUIRED IS (gm)  "P/
(.01*(X+Y+B1))
730C1=P/(.01*(X+Y+B1))
740PRINT:PRINT
750PRINT"i.e     "(C1*(T/W))" gms ACETATE"
760PRINT"        "C1*(U/W)"      PROPIONATE"
770PRINT"        "C1*(V/W)"      BUTYRATE"
780D1=(C1*(T/W))+(C1*(U/W))+(C1*(V/W))
790PRINT:PRINT"ALSO REQUIRED IS "C1*.027" gms CALCIUM CARBONATE"
800PRINT:PRINT"                 "C1*.488" gms WATER"
810PRINT:PRINT:PRINT"TOTAL QUANTITY OF VFA STOCK SOLUTION REQUIRED
IS "C1+(C1*.027)+(C1*.488)
820C2=C1+(C1*.027)+(C1*.488)
830PRINT:PRINT:PRINT"VFA STOCK SOLUTION ALLOWANCE (gms)  "P/(P/C2)
835VDU3:INPUT"PRESS 'RETURN' TO CONTINUE";T$:VDU2
```

```
840PRINT:PRINT:PRINT"4.---BUFFER INFUSION---":PRINT:PRINT
850VDU3:INPUT"ENTER THE BUFFER REQUIREMENT (gm/Kg^.75/d)    ";E1:PR
INT
860 VDU2:PRINT"BUFFER ALLOWANCE (gm/d) is  "(E1*Z):PRINT:PRINT:VDU3
870FORK9=1TO20:PRINT:NEXT
880VDU2:PRINT"5.---MAJOR MINERAL INFUSION---":PRINT:PRINT:VDU3
890INPUT"ENTER DESIRED LEVEL OF MAJOR MINERAL SUPPLEMENTATION
(gm/KgW^.75/MULTIPLE  OF  MAINTENANCE/d)   ";G1
895VDU2:PRINT:PRINT"MAJOR MINERAL ALLOWANCE (gm/d) is " G1*Z*N
900VDU3:FORK9=1TO10:PRINT:NEXT:VDU2
910PRINT:PRINT"6.---TRACE    MINERAL    INFUSION---":PRINT:PRINT:VDU3
920PRINT:INPUT"ENTER  DESIRED LEVEL OF  SUPPLEMENTATION(gm/Kg^.75/d)"
;Z3:PRINT:VDU2
930PRINT:PRINT"TRACE MINERAL  ALLOWANCE  (gm/d)  IS "Z3*Z:PRINT:PRINT
VDU3
940FORK9=1TO10:PRINT:NEXT
945IFZ$="Y"GOTO950
946GOTO1510 950PRINT:PRINT"QUANTITIES OF THE INDIVIDUAL NUTRIENTS
INFUSED  ARE NOW BEING PRINTED"
960VDU2:PRINT:PRINT
970PRINT"THE QUANTITIES
(g/d) OF INDIVIDUAL NUTRIENTS PROVIDED BY THIS FORMULATION"
980PRINT:PRINT"--- LIPID INFUSION ---":PRINT
1000PRINT"LIPID              "A
1010PRINT"Na CASEINATE       "A*.4
1020PRINT"EMULSIFIER         "A*.15
1030PRINT"GLUCOSE            "J
1040PRINT:PRINT
1050PRINT"--- Na CASEINATE INFUSION ---":PRINT:I1=Z9*10
1065PRINT"Na CASEINATE       "I1*.1
1075PRINT"Na CARBONATE       "I1*.0053
1085PRINT"VITAMIN SOLUTION   "I1*.0257
1090PRINT:PRINT"TOTAL Na CASEINATE (gms) IN THE INFUSIONS IS
"((I1*.1)+(A*.4))
1100PRINT:PRINT
1110PRINT"--- VFA INFUSION ---":PRINT
1120PRINT"ACETATE            "C1*(T/W)
1130PRINT"PROPIONATE         "C1*(U/W)
1140PRINT"BUTYRATE           "C1*(V/W)
1150PRINT"Ca CARBONATE       "C1*.027
1160PRINT:PRINT
1170PRINT"--- BUFFER INFUSION ---":PRINT:J1=Z*E1
1180PRINT"Na BICARBONATE     "J1*.073
1190PRINT"K BICARBONATE      "J1*.038
1200PRINT"Na CHLORIDE        "J1*.007
1210PRINT:PRINT
1220PRINT"--- TRACE MINERALS ---":PRINT
1230PRINT"FeSO4.7H2O         "Z*.02028
1240PRINT"ZnSO4.7H2O         "Z*.00119
1250PRINT"KI                 "Z*.00108
1260PRINT"MnSO4.4H2O         "Z*.00057
1270PRINT"CuSO4.5H2O         "Z*.00055
1280PRINT"CoSO4.7H2O         "Z*.00021
1290PRINT"NaF                "Z*.00077
1300PRINT:PRINT
1310PRINT"--- MAJOR MINERALS ---":PRINT:K1=G1*Z
```

```
1320PRINT"Ca(H2PO4)2          "K1*.015
1332PRINT"MgCl2.6H2O          "K1*.0075
1340PRINT:PRINT
1350PRINT"--- VITAMIN INFUSION ---":PRINT:L1=I1*.0257
1360PRINT"THIAMINE HCl        "L1*.00009
1370PRINT"RIBOFLAVIN          "L1*.00035
1380PRINT"NICOTINIC ACID      "L1*.00035
1390PRINT"CHOLINE Cl          "L1*.01325
1400PRINT"PYRIDOXINE HCl      "L1*.00004
1410PRINT"AMINO BENZOIC ACID  "L1*.00001
1420PRINT"Ca PENTOTHENATE     "L1*.00027
1430PRINT"FOLIC ACID          "L1*.000001
1440PRINT"CYANOCOBOLAMINE     "L1*.000001
1450PRINT"MYOINOSITOL         "L1*.01325
1460PRINT"BIOTIN              "L1*.0000053
1470PRINT"NAPTHAQUINONE       "L1*.0000442
1480PRINT"TOCOPHEROL Ac       "L1*.0003534
1490PRINT"ETHANOL             "L1*.265086
1500PRINT"LINOLEIC ACID       "L1*.088362
1510FORK9=1TO4:PRINT:NEXT:VDU3
1520VDU2:PRINT"--- VARIABLE CHECK LIST ---":PRINT
1530PRINT"ANIMAL IDENTIFICATION                                          "A1
1540PRINT"LIVEWEIGHT(Kg)                                                 "A2
1550PRINT"METABOLIC LW(Kg^.75)                                           "Z
1560PRINT"LIPID INFUSED(gm/d)                                            "A
1570PRINT"PROTEIN NUTRITION(*M)                                          "F1
1580PRINT"TISSUE N REQUIREMENT @ M (mgN/Kg^.75/d)                        "G
1590PRINT"N CONTENT OF Na CASEINATE(mgN/gm.DM)                           "H
1600PRINT"GROSS ENERGY VALUE OF NaCASEINATE(kJ/gm.DM)                    "H1
1610PRINT"GLUCOSE INFUSED                                                "J
1620PRINT"ENERGY NUTRITION(*M)                                           "N
1630PRINT"METABOLIZABLE ENERGY REQUIREMENT @ M(kJME/KgLW^75/d)           "O
1640PRINT"ACETATE (MOLAR RATIO)                                          "Q
1650PRINT"PROPIONATE                                                     "R
1660PRINT"BUTYRATE                                                       "S
1670PRINT"GROSS ENERGY PROVIDED BY VFA'S(kJ)                             "P
1680PRINT"BUFFER REQUIREMENT(gm/KgLW^.75/d)                              "E1
1690PRINT"MAJOR MINERAL REQUIREMENT(gm/KgLW^.75/d/MULTIPLE OF M          "G1
1700PRINT"TRACE MINERAL REQUIREMENT(gm/KgLW^.75/d)                       "Z
1710PRINT"EFFICIENCY OF UTILIZATION OF CASEIN                            "H3
1720FORK9=1TO5:PRINT:NEXT
1730PRINT"QUICK REFERENCE":PRINT
1740PRINT"QUANTITIES OF STOCK SOLUTIONS TO BE INFUSED(gm/d)":PRINT
1750PRINT"LIPID           "(A)+(A*.4)+(A*.15)+J+((A*18.45)-J)
1760PRINT"Na CASEINATE    "O5*10
1770PRINT"VFA             "P/(P/C2)
1780PRINT"BUFFER          "Z*E1
1790PRINT"TRACE MINERALS  "Z3*Z
1800PRINT"MAJOR MINERALS  "G1*Z
1810VDU3:END
```

APPENDIX 53: Specimen Infusion Formulation designed to supply 1.0 x the Protein Necessary to Meet the Maintenance Requirements and 1.2 x the ME Requirements for Maintenance of a 60kg Sheep. (Refer to Appendix 52 for Program Listing)

THIS PROGRAM IS DESIGNED TO BE USED WITH THE 'MATERIALS & METHODS' SECTION OF INTRAGASTRIC INFUSION AND ITS USE IN THE INVESTIGATION OF RUMINANT NITROGEN AND GLUCOSE METABOLISM' (GIRDLER, 1986)

###INTRAGASTRIC INFUSION FORMULATION###

INFUSIONS FOR BEAST 466

1.---LIPID INFUSION---

LIPID EMULSION DATA

DAILY ALLOWANCE (gms)

LIPID	25
Na CASEINATE	10
EMULSIFIER	3.75
GLUCOSE	10
WATER	451.25

CONSTITUENTS IN THESE PROPORTIONS ARE USED TO PRODUCE A STOCK LIPID SOLUTION

CONTRIBUTION TO THE ME OF THE INFUSIONS (kJ/d)

LIPID	926.25
Na CASEINATE	190
EMULSIFIER	138.9375
GLUCOSE	170

TOTAL CONTRIBUTION TO ME FRACTION OF THE INFUSIONS (kJ/d) IS 1425.1875

CONTRIBUTION TO PROTEIN FRACTION

Na CASEINATE (mg N/d) 1410

2.---PROTEIN INFUSION---

TISSUE N REQUIREMENT (mg N/d) IS 7976.55129

TISSUE N TO BE SUPPLIED BY Na CASEINATE INFUSION (mg/d) IS 6848.55129
THE REQUIREMENT FOR Na CASEINATE (gm/d) IS THEREFORE 60.7141072
OR 607.141072 gm OF THE 10% Na CASEINATE SOLUTION

THE CONTRIBUTION OF THE Na CASEINATE SOLUTION TO ME REQUIREMENTS IS (kJ) 1092.85393

TOTAL NON-VFA ENERGY CONTRIBUTION TO ME (kJ) IS 2518.04143

3.---VFA INFUSION---

TOTAL ME REQUIRED AT THIS LEVEL OF PRODUCTION IS (kJ/d) 9959.90999

ME SUPPLIED BY NON-VFA SOURCES (kJ/d) 2518.04143

ME SUPPLIED BY VFA INFUSION (kJ/d) 7441.86856

THE QUANTITY OF VFA REQUIRED IS (gm) 483.452871

i.e	309.905687 gms	ACETATE
	117.605235	PROPIONATE
	55.9419496	BUTYRATE

ALSO REQUIRED IS 13.0532275 gms CALCIUM CARBONATE

235.925001 gms WATER

TOTAL QUANTITY OF VFA STOCK SOLUTION REQUIRED IS 732.4311

4.---BUFFER INFUSION---

BUFFER ALLOWANCE (gm/d) is 1293.4948

5.---MAJOR MINERAL INFUSION---

MAJOR MINERAL ALLOWANCE (gm/d) is 948.562856

6.---TRACE MINERAL INFUSION---

TRACE MINERAL ALLOWANCE (gm/d) IS 21.5582467

THE QUANTITIES (g/d) OF INDIVIDUAL NUTRIENTS PROVIDED BY THIS FORMULATION

--- LIPID INFUSION ---

LIPID	25
Na CASEINATE	10
EMULSIFIER	3.75
GLUCOSE	10

--- Na CASEINATE INFUSION ---

Na CASEINATE	60.7141072
Na CARBONATE	3.21784768
VITAMIN SOLUTION	15.6035255

TOTAL Na CASEINATE (gms) IN THE INFUSIONS IS 70.7141072

--- VFA INFUSION ---

ACETATE	309.905687
PROPIONATE	117.605235
BUTYRATE	55.9419496
Ca CARBONATE	13.0532275

--- BUFFER INFUSION ---

Na BICARBONATE	94.4251207
K BICARBONATE	49.1528025
Na CHLORIDE	9.05446362

--- TRACE MINERALS ---

FeSO4.7H2O	0.437201243
ZnSO4.7H2O	2.56543136E-2
KI	2.32829065E-2
MnSO4.4H2O	1.22882006E-2
CuSO4.5H2O	1.18570357E-2
CoSO4.7H2O	4.52723181E-3
NaF	1.659985E-2

--- MAJOR MINERALS ---

Ca(H2PO4)2	12.934948
MgCl2.6H2O	6.46747402

--- VITAMIN INFUSION ---

THIAMINE HCl	1.4043173E-3
RIBOFLAVIN	5.46123394E-3
NICOTINIC ACID	5.46123394E-3
CHOLINE Cl	0.206746713
PYRIDOXINE HCl	6.24141021E-4
AMINO BENZOIC ACID	1.56035255E-4
Ca PENTOTHENATE	4.2129519E-3
FOLIC ACID	1.56035255E-5
CYANOCOBOLAMINE	1.56035255E-5
MYOINOSITOL	0.206746713
BIOTIN	8.26986854E-5
NAPTHAQUINONE	6.89675829E-4
TOCOPHEROL Ac	5.51428593E-3
ETHANOL	4.13627617
LINOLEIC ACID	1.37875872

--- VARIABLE CHECK LIST ---

ANIMAL IDENTIFICATION	466
LIVEWEIGHT(Kg)	60
METABOLIC LW($Kg^{0.75}$)	21.5582467
LIPID INFUSED(gm/d)	25
PROTEIN NUTRITION(*M)	1
TISSUE N REQUIREMENT at M ($mgN/Kg^{0.75}/d$)	370
N CONTENT OF Na CASEINATE(mgN/gm.DM)	141
GROSS ENERGY VALUE OF NaCASEINATE(kJ/gm.DM)	18
GLUCOSE INFUSED	10
ENERGY NUTRITION(*M)	1.2
METABOLIZABLE ENERGY REQUIREMENT at M($kJME/KgLW^{0.75}/d$)	420
ACETATE (MOLAR RATIO)	65
PROPIONATE	25
BUTYRATE	10
GROSS ENERGY PROVIDED BY VFA'S(kJ)	7441.86856
BUFFER REQUIREMENT($gm/KgLW^{0.75}/d$)	60
MAJOR MINERAL REQUIREMENT($gm/KgLW^{0.75}$/d/MULTIPLE OF M)	40
TRACE MINERAL REQUIREMENT($gm/KgLW^{0.75}/d$)	21.5582467
EFFICIENCY OF UTILIZATION OF CASEIN	0.8

QUICK REFERENCE

QUANTITIES OF STOCK SOLUTIONS TO BE INFUSED(gm/d)

LIPID	500
Na CASEINATE	607.141072
VFA	732.4311
BUFFER	1293.4948

TRACE MINERALS 21.5582467

MAJOR MINERALS 862.329869

	Concentration and range of concentration		Unit	Ref.
Urea	4.20	(2.65-6.64)	mmol/l	1
Total protein	68.00	(60.0-75.0)	g/l	1
Albumin	33.00	(28.0-34.0)	g/l	1
	41.00	-	g/l	2
Albumin:globulin	0.94	(0.90-1.26)	-	1
Globulin	35.00	(32.0-43.0)	g/l	1
	28.00	-	g/l	2
Glucose	2.50	(2.0-3.0)	mmol/l	1
	-	(2.7-3.5)	mmol/l	2
	4.00	-	mmol/l	2
Lactate	1.38	(1.3-1.6)	mmol/l	2
Glycerol	-	(0.11-0.22)	mmol/l	3
NEFA	-	(0.1-0.6)	mmol/l	3
Acetate	-	(0.4-1.5)	mmol/l	2
	-	(0.6-1.5)	mmol/l	3
Acetoacetate	-	(0.8-1.4)	mmol/l	3
3-OH butyrate	-	(<0.8)	mmol/l	4
Insulin	0.8	-	ng/ml	2
Glucagon	0.28	-	ng/ml	2
Growth hormone	3.60	-	ng/ml	2
Cortisol	-	4.0-6.3	ng/ml	2
γGT	21.00	19.0-23.0	iu	4
GOT	40.00	20.0-60.0	iu	4
SDH	17.0	5.80-28.0	iu	4
Ca	2.50	2.0-3.0	mmol/l	4
Mg	1.02	0.70-1.23	mmol/l	4
P	4.50	4.00-5.50	mmol/l	4
Cu	15.30	9.42-18.84	µmol/l	4
PCV	30.00	22.0-40.0	%	4

Appendix 54: Normal physiological concentrations or activities of metabolites, hormones and enzymes in the blood plasma of sheep. 1. Doxey (1977). 2. Hecker (1983). 3. Lindsay & Leat (1975). 4. WOSAC VIC.