THE RUMINAL METABOLISM OF LACTIC ACID

by

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LIST OF ABBREVIATIONS

LDH	-	Lactate dehydrogenase
GPT	-	Glutamate pyruvate transaminase
NAD	-	Nicotinamide adenine dinucleotide
RF	-	Ruminal fluid
CRF	-	Clarified ruminal fluid
SRF	-	Strained ruminal fluid
RFC	-	Readily fermentable carbohydrate
VFA	-	Volatile fatty acids
NH_3-N	-	Ammonia nitrogen
CP	-	Crude protein
N	-	Nitrogen
SD	-	Standard deviation
CV	-	Coefficient of variation
E	-	Extinction or absorbance
PR	-	Production rate
UR	-	Utilization rate
R	-	Infusion rate
Q	-	Concentration
SA	-	Specific activity

CONTENTS

PART III

LACTATE TURNOVER IN THE RUMEN

PREFACE

In the last 25 years diets containing large proportions of readily fermentable carbohydrate and termed "high concentrate" diets have become economically important in the production of meat and milk from ruminants. The breakdown within the rumen of a large amount of readily fermentable material such as starch and sugars can lead to dramatic changes in rumen conditions with the formation of products, particularly in the early stages of fermentation, other than the volatile fatty acids which are normally encountered. Under these conditions lactic acid is an important intermediate in the fermentation pathway and can accumulate rapidly.

Numerous papers have dealt with severe or acute lactic acidosis induced by grain overload or abrupt changes to high concentrate diets. However, the studies reported in this thesis were performed under "normal" feeding conditions where lactic acid accumulated only transiently after feeding and differs markedly from the "grain overload" or "lactic acidosis" syndrome where lactic acid accumulation persists for long periods of time and is often fatal. Thus literature concerning lactic acidosis will only be quoted when relevant.

The overall fermentation is the result of a series of complex interactions among the bacteria and protozoa present in the rumen. Although many studies have described the numbers and tupes of lactic acid producing and lactic acid utilizing bacteria and their activities in the rumen inferred from pure culture *in vitro* studies, very little is known of the quantitative aspects of lactic acid metabolism *in vivo*. The host animal also has an important influence on the ruminal fermentation since the physiological condition of the animal will control the amount, rate and selection of food at least. This study therefore attempts a complete kinetic description of the whole and the rates of the component processes involved in the ruminal metabolism of lactic acid.

PART I

GENERAL INTRODUCTION

AND

PRELIMINARY STUDIES

CHAPTER 1. GENERAL INTRODUCTION

Notwithstanding the controversy over the competition with man for his food and also for the land on which to grow his food, the ruminant animal is likely to continue fulfilling an important role in the future production of food. Wherever cereals are grown, some are used as livestock food. Man's surplus concentrates would be most effectively used, in descending order, by: milking ruminants, broilers, egg-laying hens, meat-producing pigs, and meat-producing ruminants (Reid, 1970). The figures for meat-producing ruminants were based on calculations involving beef cattle, the least efficient of the domestic animals considered, although this reflects the high feed cost of rearing and maintaining the breeding herd needed to generate the meat producers. The most significant gains in efficiency of output by ruminant animals has resulted from the intensification of the energy input to dilute the energy cost of maintenance. Intensive systems are justified by economic reasons since they increase the turnover rate of animals reducing the large primary costs of food and labour (Blaxter, 1973). It can be expected that the agricultural industry in general will increasingly adopt intensive methods of animal production.

In international terms livestock, in 1970, ate more than 60% of the cereals consumed in the developed countries but less than 10% of those consumed in the rest of the world. By 1990, these proportions are estimated to increase to 67 and 15%, respectively. In absolute terms, the consumption of cereals by livestock is estimated to increase in 20 years by 194 million metric tons in the developed countries (52%) and by 112 million metric tons (221%) in the rest of the world (Greenhalgh, 1977).

The Republic of South Africa produces a surplus of maize grain. Human consumption of maize has decreased from 64,4% to 51,7% of total consumption over the period 1950/51-1954/55 to 1970/71-1974/75, while animal consumption has increased from 32,3% to 45,5%. In 1970/71-1974/75 total production was 8 671 000 metric tons and total consumption was 5 263 000 metric tons. It is estimated that animal consumption of maize may increase to 5 600 000 metric tons in the year 2 000. This should still leave a large enough surplus to allow for considerable increase in the industrial consumption and export. By 1980 surplus production may well be so large that other products will be produced on some of the land currently used for the production of maize (Le Roux, 1977). Thus the future of cereals as livestock foods seems to be assured for the production of meat and milk from ruminants in the Republic of South Africa at least.

/The carbohydrate .....

The carbohydrate components in the diet are fermented anaerobically by micro-organisms in the rumen to yield volatile fatty acids (VFA) mainly acetate, propionate and butyrate, methane, heat and microbial cells. On an energy basis the VFA account for about 65-70% of the heat of combustion of the substrate fermented, methane for 8-12%, growth of microbial cells for up to 8% and about 10% is accounted for as heat (Hungate, 1966). As far as is known all carbohydrates are fermented by a common pathway, the Embden-Meyerhof glycolysis system, to pyruvate and it is in the further metabolism of pyruvate that the different end-products arise (Baldwin, 1965). Initially polysaccharides and soluble sugars are hydrolysed and the resulting hexoses and pentoses are then fermented with the production of VFA and methane. On high roughage diets the molar proportions of VFA are 70% acetic, 18% propionic and 10% butyric acids whereas on high concentrate diets the proportions can vary considerably. When readily fermentable carbohydrates are given, lactic acid is formed in addition to the VFA particularly in the early stages of fermentation.

The first indication of lactic acid formation in the rumen was obtained by Woodman & Evans (1938) who noted a transient accumulation of lactic acid during the fermentation of glucose *in vitro* by micro-organisms from the rumen of sheep. This was subsequently confirmed by other workers who demonstrated that glucose, fructose and sucrose were rapidly fermented in the sheep rumen with a temporary increase in lactic acid concentration which later decreased as VFA increased (Phillipson & McAnally, 1942; Elsden, 1945, 1945-46). Later investigations showed that lactic acid often accumulated transiently in the rumen under practical feeding conditions. Thus, when lambs were fed once daily on flaked maize followed 8h later by hay, rumen pH was low and associated with lactic acid accumulation which reached a maximum *ca* 7h after the maize feed. Lactic acid has been found to accumulate in the rumen when sheep are changed from an indoor to a pasture feeding regime (Annison, Lewis & Lindsay, 1959a) and also on lush spring pasture (Annison *et al*, 1959b). Ingestion of silage by cattle gave rise to concentrations of lactic acid in the rumen which were greater than could be explained by the lactic acid content (*ca* 3-8% of the dry matter) of the silage consumed (Waldo & Schultz, 1956), probably because the animals were not adapted since Kunkle, Fetter & Preston (1976b) have shown that cattle will adapt more rapidly to high concentrate rations when previously fed rations containing lactic acid, such as silage, and/or some concentrate. When a diet containing chaffed wheat and lucerne hays and a high proportion of wheat starch or molasses was fed to sheep, high concentrations of ruminal lactate were found (Briggs, Hogan & Reid, 1957; Chou & Walker, 1964). The highest concentrations of lactic acid found in the rumen after feeding were usually $\leq$20mM.

The amount or proportion of readily fermentable carbohydrate (RFC) in the diet plays an important role in determining the concentration of rumina lactic acid. In general, with adapted animals, the higher the proportion of RFC in the diet the greater the lactic acid concentration in the rumen (Balch & Rowland, 1957; Reid, Hogan & Briggs, 1957; Ghorban, Knox & Ward, 1966; Sutton & Johnson, 1969). However, interactions between the ruminal flora and RFC in the diet can make comparisons between different experiments and also between animals on the same diets difficult especially when additional variables such as time after feeding, length of time on diet and previous dietary history are omitted from the experimental details. When unadapted animals are switched abruptly from high roughage to low roughage or high concentrate diets large amounts of lactate can accumulate temporarily, ruminal pH decreases and there is a reduction in food intake (Dunlop & Hammond, 1965; MacKenzie, 1967; Dirksen, 1970). Once the animals become adapted, food intake and ruminal pH increase while lactic acid concentration decreases (MacKenzie, 1967; Slyter, 1976).

Eadie, Hyldgaard-Jensen, Mann, Reid & Whitelaw (1970) showed that marked changes in the microbiological and biochemical porperties of rumen fluid could be brought about by altering the quantity rather than quality of an all-concentrate diet, although the effect on lactic acid concentration was small. However, most studies on the effect of the amount and rate of food consumption have been confined to those involving "grain overload" (see Slyter (1976) for review) rather than the normal or practical feeding situation. It is common management practice in the intensive feeding of cattle and sheep when changing from forage to high concentrate diets either to limit the amount of concentrates that the animals are allowed to consume if the diet is changed abruptly, or to increase the amount of concentrates in the diet in a stepwise manner (Elam, 1976) in order to control lactic acid accumulation.

Another factor that may affect ruminal fermentation is the physical form of the diet, an aspect which has received considerable attention. Processes such as steaming and rolling which increase the availability of starch to microbial attack promote increased ruminal lactic acid concentration. There are several excellent reviews on the effects of food processing on ruminal fermentation, and on the digestion and utilization of grains by ruminants (Armstrong & Beever, 1969; Armstrong, 1972; Burt, 1973; Hale, 1973; Waldo, 1973; Ørskov, 1976). This aspect will not be discussed further as processing was not one of the variables in the studies reported in this thesis, which entailed the use of finely ground maize grain in order to ensure that most (*ca* 80%) of the starch would be digested in the rumen (Armstrong, 1972) and thus have the greatest influence on the ruminal flora and fermentation.

Since the ruminal micro-organisms are the primary determinants influencing the production and utilization of lactic acid it is perhaps surprising that little is known of the critical factors which control their numbers, types and activity *in vivo*. Although the changes occurring in the rumen of cattle and sheep after excessive intake of starch or sugar containing diets have been studied in detail (Krogh, 1961, 1963; Allison, Bucklin & Dougherty, 1964; Dunlop & Hammond, 1965; Mann, 1970; Allison, Robinson, Dougherty & Bucklin, 1975) this is not representative of the normal or practical feeding situation. Likewise, considerable attention has been focussed on the numbers and types of micro-organisms and the pattern of fermentation end-products produced by them in ruminants fully adapted to moderate or high grain-starch diets (Giesecke, Lawlor & Walser-Kärst, 1966; Slyter, Oltjen, Kern & Weaver, 1968; Eadie *et al*, 1970; Latham, Sharpe & Sutton, 1971; Latham, Storry & Sharpe, 1972; Latham, Sutton & Sharpe, 1974; Ogimoto & Giesecke, 1974) but such studies, which are largely descriptive, are not able to elucidate the controlling factors.

It is likely that the most important alterations and modifications occur during the adaptation itself but only two studies, both of which were published very recently, have attempted to examine microbiological changes during this initial period. Grubb & Dehority (1975) followed at short intervals over a period of 21 days the changes in ruminal micro-organisms of sheep switched abruptly from an all-roughage to a moderately high corn-starch diet while Huber, Cooley, Goetsch & Das (1976) followed changes in lactate-utilizing bacteria in the ruminal fluid of a single steer which was changed from an all-roughage to a high grain ration over a period of 4 weeks. The investigations reported here, which examined the changes which occurred during the all important stepwise adaptation to high concentrate diets were undertaken in an attempt to fill this gap in our understanding of rumen metabolism.

Evidence exists (Preston, 1972) that when ruminants are fed increasing amounts of RFC the recommended protein allowances for the animal are greater than those formulated by the NRC (1968, 1970, 1971) and ARC (1965). It is generally accepted that an increase in the amount of RFC in the diet results in an increased uptake of N by the ruminal micro-organisms. Thus the diet must supply sufficient N in a form which is available to the bacteria in the rumen if N is not to become limiting for growth. If N becomes limiting in the rumen, the fast growing functional groups of bacteria fermenting starch and / or its saccharide components can outgrow the slower fibre digesters and those organisms which are dependent on others for the production of their growth substrates e.g. the lactate-utilizers and methane-producers. This imbalance in the ruminal flora

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results in the accumulation of D- and L- lactic acid at the expense of the VFA which constitute the normal end-products of carbohydrate fermentation produced by a balanced ruminal flora. Even a marginal shortage of N may have an important influence on the adaptation to a high concentrate diet. The protozoa are also implicated in maintaining the concentration of NH_3 in the rumen above growth limiting levels since they ingest large numbers of bacteria (Coleman, 1974) which they digest with the liberation of NH_3. Thus defaunated animals (without protozoa) have lower concentrations of NH_3 in the rumen than faunated animals fed the same diet (Smith, 1969). Although the protozoa are important in maintaining a balanced ruminal flora required to ferment the carbohydrate portion of the diet, from which the host derives energy, nevertheless, this N recycling within the rumen reduces availability of N to the host animal and hence the efficiency of N utilization.

The VFA are the chief sources of metabolizable energy for the host animal and they are rapidly absorbed through the ruminal wall into the bloodstream. The absorption of lactic acid from the rumen is a relatively slow process and it is only when pH falls below 5 that a significant amount of free lactic acid is absorbed (Williams & MacKenzie, 1965). In most circumstances the absorption of lactic acid from the rumen would be negligible since the concentration of lactic acid is usually low and the pH above 5. The absorption of VFA from the rumen was found to be 5-10 times more rapid than lactic acid from solutions at pH 5,2 (Williams & MacKenzie, 1965). It is likely that some lactic acid passes into the small intestine from where it is absorbed rapidly (Shinozaki & Sugawara, 1958; Dunlop & Hammond, 1965). A variable proportion of the lactic acid absorbed under these conditions consists of the D(-)-isomer which is only slowly metabolized, a considerable proportion being excreted in the urine (Dunlop & Hammond, 1965) and thus lost as a source of energy to the animal. In contrast, L(+)-lactate is readily metabolized by the ruminant as a glucogenic substrate (Annison, Lindsay & White, 1963). Information regarding the relative concentrations of the two isomers under certain conditions is thus not without interest.

Rapid fermentation of much RFC leads to a reduction in ruminal pH from the normal (pH 6,6-6,8; Hungate, 1966) as a result of an increased production of acid materials which cannot be balanced by absorption through the rumen wall and neutralization by bicarbonate entering the rumen in saliva (Walker, 1968). This can happen when high concentrate rations are fed, especially when fed in the ground and pelleted forms, since the amount of saliva secreted depends on the coarseness of the diet which influences

/the amount .....

the amount of time spent chewing and ruminating (Kay, 1963). Thus it is necessary to provide a minimal amount of coarse roughage, or buffer salts, or both in the diet to obtain adequate buffering.

The relationship between pH of the rumen contents and accumulation of lactic acid is very complex. Enzymes dissimilating pyruvic acid may be adversely affected by the lower pH and it is known that the rates of production of acetic, propionic and higher acids decline when the pH drops below 6,0 (Bruno & Moore, 1962). Furthermore, below pH 5,5 bicarbonate would be almost eliminated from rumen fluid with the result that the conversion of pyruvic acid to propionic acid through succinic acid would be blocked because this pathway requires CO_2 fixation. The reducing equivalents normally used in the conversion of pyruvate to propionate are then available for the reduction of pyruvate to lactate. It has also been shown that as pH decreases from 6,5 to 5,0 amylase activity increases (Giesecke & Geiges, 1974) but glucose fermentation rates decrease (Myburgh & Quinn, 1943). Therefore at low pH ruminal glucose accumulates and this preceded acidosis problems in sheep fed wheat (Ryan, 1964a).

Hishinuma, Kanegasaki & Takahashi (1968) found that pure cultures of *Selenomonas ruminantium* degraded less lactate to propionic and acetic acids in the presence of glucose in the media than in its absence. *S. ruminantium* ferments lactate by the succinate pathway, but high carbohydrate concentrations would suppress lactate utilization by this organism. Under these conditions lactate might be fermented by *Megasphaera elsdenii*, also found in the rumen which ferments lactate by the acrylate pathway which may not be so susceptible to catabolite repression (Hobson, 1972) as suggested by the relative growth rates on lactate and glucose (Hobson, Mann & Oxford, 1958).

Low pH and high glucose concentration would inhibit the lactate-utilize but would favour the growth of acid-tolerant bacteria which produce lactic acid from glucose (Slyter, 1976). Thus it is clear that some micro-organism such as *Streptococcus bovis*, *Lactobacillus spp*, and *Butyrivibrio fibrisolven* can produce much lactic acid with accumulates when conditions allow, while others, including *S. ruminantium*, *M. elsdenii* and *Propionibacter spp.* are able to utilize lactate for growth.

Although the concentration of lactic acid in the rumen may be low, lact could be an important intermediate in the formation of VFA. The turnover rate of lactic acid in the rumen has been studied *in vitro* by incubating (2-^{14}C)-lactate with rumen fluid obtained from animals fed roughage or concentrate rations. Jayasuriya & Hungate (1959) found that 8-17% of the total substrate was fermented via lactate in grain-fed animals. This value decreased to 0,8% or less in hay-fed animals. However Nakamura & Takahashi (1971) showed that the metabolism of lactate changes not only with diet

also with respect to time after feeding. Rumen content taken from sheep ½ and 1½ h after feeding a 50% concentrate diet and incubated with (2-^{14}C)-lactate *in vitro* showed that 40-47% of acetate was formed via lactate while 32% of propionate in the ½h sample and only 8% in the 1½h sample, were formed via lactate. When the rumen content was obtained ½h after feeding a hay diet 18% of acetate and a negligible amount of propionate were found to have been formed through lactate. Thus it is likely that lactate is an important intermediate in the rumen fermentation especially shortly after feeding but this has not been examined *in vivo*.

Pathways for the microbial conversion of lactic acid to VFA are well established (Wood, 1961). Acetic acid is produced by an initial oxidation of lactate to pyruvate and subsequent decarboxylation to acetate. Butyric acid formation involves the condensation of two equivalents of acetate to give acetoacetate followed by reduction to butyrate. There are two possible mechanisms known for the conversion of lactic acid to propionic acid. The first and most widespread, involves the carboxylation of pyruvate to give oxaloacetate which is converted to succinate which is in turn decarboxylated to propionate. This pathway is operative in most of the known types of ruminal lactate-utilizing bacteria i.e. *Veillonella alkalescens*, some species of *Propionibacterium, Selenomonas ruminantium* and *Anaerovibrio lipolytica* (Johns 1951a, b; Paynter & Elsden, 1970; Prins, Lankhorst, Van der Meer & Van Nevel, 1975). The other pathway involves coenzyme-A esters of lactate, acrylate and propionate and is commonly named the direct reductive or acrylate pathway. This pathway has been detected in *Clostridium propionicum* and the rumen bacterium *Megasphaera elsdenii* (Ladd & Walker, 1959). It is possible to distinguish between the two pathways using lactate labelled in position 2 or 3 as substrate since the first pathway involves the decarboxylation of succinate which, being a symmetrical molecule may lose either carboxyl group resulting in randomisization of label between carbons 2 and 3 of the propionate formed. Propionate formed by the acrylate pathway carries the label in the same position as the lactate used as substrate. Investigations along these lines have shown that 54-88% of lactate is converted to propionate via the acrylat pathway (Baldwin, Wood & Emery, 1962) and that the contribution of the acrylate pathway increased as the concentrates in the diet increased (Baldwin *et al*, 1963; Wallnöfer, Baldwin & Stagno, 1966). Microbiological evidence showing an increase in *M. elsdenii* (the major organism utilizing this pathway) when high grain diets were fed (Gutierrez, Davis, Lindahl & Warwick, 1959; Eadie, Hobson & Mann, 1967; Latham *et al*, 1971) agrees with the biochemical data. However simultaneous determination of the relative importance of the different pathways in the production and fermen-

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tion of lactate and the microbial flora present have not been made.

The formation of propionic acid from ^{14}C-labelled glucose, cellulose and hemicellulose was almost entirely through the randomizing pathway in rumen fluid obtained from animals fed roughage diets but the direct reductive pathway through lactate increased when the rumen fluid was taken from concentrate-fed animals and could account for 30% of the total propionate production from glucose (Baldwin *et al*, 1963; Satter, Suttie & Baumgardt, 1967; Wallnöfer *et al*, 1966). The importance of the direct reductive pathway for cellulose and hemicellulose fermentation was also increased in rumen fluid of animals fed concentrates. These results support the *in vitro* turnover experiments (Jayasuriya & Hungate, 1959; Nakamura & Takahashi, 1971) indicating that lactate increased in importance when animals were fed a high proportion of concentrates.

Metabolic studies with isolated species of rumen micro-organisms have provided useful data on the pathways involved in rumen fermentations and on the identification of the type of organism associated with the different metabolic functions. However, the extrapolation of these results to the *in vivo* situation is difficult since a complex interaction exists between competition for substrates and other beneficial interrelationships in the rumen. Further, more serious problems are associated with culturing all the important species of rumen micro-organisms and estimating their numbers in the rumen. In one study approximately 75% of the organisms in the rumen of animals fed concentrates were cultured, but only 10% of those present in the rumen of animals fed hay could be grown *in vitro* (Maki & Foster, 1957 Interpretation of results is complicated by the fact that the relative increase in numbers is not necessarily related to the same relative increase in activity.

As will be evident from the foregoing discussion the ruminal metabolism of lactic acid has been studied for the past 35 years but confusion and misconception persist. A large portion of the published work has been performed with the use of undefined lactic acid mixtures or without the determination of D- and L- lactic acid concentrations which are extremely variable. The present investigation was undertaken in an attempt to elucidate some of the outstanding deficiencies in our knowledge of lactic acid metabolism in the rumen, using the sheep as test animal. In particular attention was devoted to changes associated with the stepwise adaptation of sheep from a high roughage to a high concentrate diet in which maize grain served as the energy source. In order to avoid some of the weaknesses

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of earlier studies simultaneous experiments were made of the ecology and biochemistry of the rumen. An effort was made to include all the ruminal micro-organisms involved in the metabolism of lactic acid including the ciliate protozoa which engulf bacteria and starch grains thereby controlling the rate of fermentation and hence the production of lactic acid.

Since the work reported in this thesis is dependent on the determination of the two isomers of lactic acid, a separate chapter (Chapter 2) is included which contains details of the methods tested and used for the analysis of D- and L- lactic acid in rumen fluid and culture supernatants.

In any study concerning lactic acid metabolism in the rumen, the lactate-utilizing bacteria merit special attention. Since no generally accepted medium was available for enumeration and isolation of these fastidious organisms some work was done on the development of a suitable medium for this purpose (Chapter 3). Although most of the other microbiological techniques and media were based on the methods and techniques of Kistner (1960) and Van Gylswyk (1970) which were designed for use with high roughage diets, the author made improvements particularly in relation to anaerobiosis, processing of digesta before counting and enumeration of amylolytic bacteria which were suited for use with high concentrate diets. Although much work has been done on lactic acid concentrations in the rumen after excessive intake of starch or sugar containing diets there is, as pointed out earlier, little information available for the normal feeding situation. An exploratory experiment (Chapter 4) was therefore conducted to define the range of concentrations of D- and L- lactic acid, free glucose and ruminal pH values which could be expected under the feeding conditions applicable in the subsequent experiments reported in the thesis. These chapters constitute the first part of the thesis.

The second part of the thesis concerns the stepwise adaptation of sheep from high roughage to high concentrate diets. The microbial ecology during stepwise adaptation at restricted feed intake was studied to determine the critical factors involved in adaptation with particular reference to the effect of pH and N. The results of this ecological study (Chapter 5) are the subject of a publication (Mackie, Gilchrist, Robberts, Hannah & Schwartz, in press). A further stepwise adaptation experiment was carried out at *ad libitum* feed intake and was continued for 120 days on the final diet to be more in line with intensive feeding practice. This is described in Chapter 6 and for the sake of clarity the experimental findings from the two experiments are presented in Chapter 7.

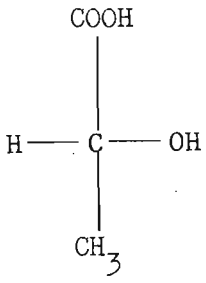
/The third.....

The third and last portion of the thesis contains the experiments on lactate turnover in the rumen, and related studies performed simultaneously on the same sheep. The rates of production and utilization of lactic acid were determined *in vivo* using fully adapted sheep fed diets containing different amounts of RFC (Chapter 8). The contribution of lactate to the formation of acetic, propionic and butyric acids was calculated. A constant infusion, double labelling technique was used which allowed the simultaneous *in vivo* determination of glucose turnover (Chapter 9). Microbiological counts were made during the turnover experiments in order to relate numbers and types of lactate-producing and lactate-utilizing organisms to lactic acid turnover. Degradation studies were also performed on labelled lactate to determine the relative importance of the randomizing and direct reductive pathways of lactic acid utilization (Chapter 10). The qualitative findings in respect of D- and L- lactic acid production and utilization are presented in Chapter 11. The ability of lactate-utilizing bacteria to link sulphate reduction with lactate utilization in the same way as the dissimilatory sulphate-reducers was also tested (Chapter 11). As in the case of Part II, discussion of the results of these different aspects is reserved for a separate chapter (Chapter 12).

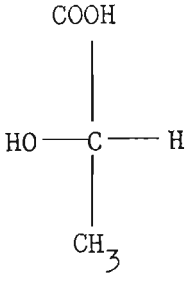
CHAPTER 2. ENZYMIC ANALYSIS OF D(-) AND L(+) LACTIC ACID IN RUMEN FLUID AND CULTURE SUPERNATANT

INTRODUCTION

Lactic acid (2-hydroxy propionic acid) was first discovered in 1780 by the Swedish chemist Scheele. Lactic acid is the simplest hydroxy acid having an asymmetric carbon atom, and it therefore occurs as dextrorotatory L(+)-lactic acid, as laevorotatory D(-)-lactic acid or as a mixture



D-(-)-Lactic Acid



L-(+)-Lactic Acid

of the two optical isomers in various proportions. Since this study is dependent on the determination of the concentration of D- and L-lactic acid in rumen fluid and culture supernatant a short chapter will be devoted to their analysis.

The widely used chemical methods for determination of lactic acid (Barker & Summerson, 1941; Elsden & Gibson, 1954) are unable to discriminate between the two optical isomers of lactic acid and the use of stereospecific lactate dehydrogenase (LDH) is the only method for determining the concentration of each isomer. The advantages of enzymic methods are: excellent specificity and stereospecificity; sensitivity, allowing the use of micromethods; and simplicity, making them suitable for routine work. The basis of all the enzymic methods is the oxidation of lactic acid to pyruvic acid, catalyzed by LDH in the presence of nicotinamide adenine dinucleotide (NAD).

$$CH_3 - CHOH - COOH + NAD^+ \rightleftharpoons CH_3 - CO - COOH + NADH + H^+$$

The lactate concentration is calculated from the amount of NADH formed in the reaction.

The purpose of this chapter is to provide a reference containing details of the methods for the determination of D- and L-lactic acid

which are used repeatedly throughout the thesis. The methods are those of Gawehn & Bergmeyer (1970, 1974) and Noll (1974) and were tested and used as described here. Most biochemicals were obtained from Boehringer Mannheim (West Germany) who also supply kits for L-lactic acid determination.

METHODS

Preparation of standard solutions

L-Lactic acid. The lactate calibration solution containing 1,0 M sodium L-lactate (Boehringer) was used as stock solution and diluted 1 000-fold to give a fresh solution of working standard containing 1,0 mM-L-lactic acid.

D-Lactic acid. A solution was made containing 96 mg lithium D-lactate (Boehringer - 99% pure, < 0,2% L(+)-lactate) dissolved in 100 ml deionized water. This stock solution was diluted 10-fold to give a working standard containing 1,0 mM-D-lactic acid.

Determination of lactic acid. Method 1

Two methods were used for the determination of D- and L-lactic acid. In Method 1, the equilibrium, which lies towards the side of lactate and NAD^+, is displaced towards the right hand side in the presence of excess enzyme and NAD^+; while an alkaline medium removes the protons formed and traps the pyruvate as the hydrazone.

$$\text{L-lactate} + NAD^+ \xrightleftharpoons{\text{L-LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{D-lactate} + NAD^+ \xrightleftharpoons{\text{D-LDH}} \text{Pyruvate} + NADH + H^+$$

Preparation of solutions

Buffer. 22,8 g of glycine (Merck, Darmstadt, West Germany) was dissolved in 550 ml of deionized water and 20 ml of hydrazine hydroxide (Merck, 24%). The pH was checked and adjusted to pH 9,0 if necessary.

NAD solution. 900 mg NAD (Boehringer - 89% pure by enzymic assay) was dissolved in 30 ml of deionized water.

L-LDH suspension. A solution containing 5 mg protein/ml of L-LDH from rabbit muscle (Boehringer - 550 U/mg at 25°C, pyruvate as substrate) was used.

D-LDH suspension. A solution containing 5 mg protein/ml of D-LDH from *Lactobacillus leichmanii* (Boehringer - 300 U/mg at 25°C, pyruvate as substrate) was used.

/All

All solutions were stored in a refrigerator, but allowed to reach room temperature (20-25°C) before pipetting.

Preparation and dilution of samples

Samples of strained ruminal fluid were diluted with an equal volume of 0,5 N-HCl or 1,0 N-$HClO_4$. The samples were centrifuged at 4 000 g for 15 min and the supernatant neutralized with 5,0 N-KOH and then recentrifuged. The clear, slightly coloured supernatant was stored in a deep freeze at -14°C until required for analysis, when it was allowed to thaw and reach room temperature.

Samples of fermentation media were treated in a similar manner except that centrifugation was at 10 000 g for 20 min at 0°C. Before analysis they were diluted 100-fold with deionized water.

Assay system

The conditions of assay were: wavelength 340 or 366 nm; cuvettes with 1 cm light path; and temperature 37°C. Normally readings were made at 366 nm to minimize interference from other compounds in rumen fluid.

Solutions pipetted into cuvettes or tubes	Blank (ml)	Sample (ml)	Final concentration in assay mixture
Buffer	3,20	3,00	440 mM of glycine 140 mM of hydrazine
NAD solution	0,20	0,20	2,4 mM of NAD
Sample or standard	-	0,20	up to 50 µM of lactate

The solutions were mixed using a rotary shaker and extinction E_1 of blank and sample measured. The LDH suspension was added (0,02 ml of L-LDH (16 U/ml) or D-LDH (9 U/ml)), the solutions mixed, incubated at 37°C for the required time and extinction E_2 measured.

Calculation

The concentration of D- and L-lactate was calculated either from a standard curve or from the following equation after calculating differences in absorbance for both blank ($\Delta E_B = E_2 - E_1$) and sample ($\Delta E_s = E_2 - E_1$) and subtracting the absorbance of blank from sample ($\Delta E = \Delta E_s - \Delta E_B$). The equation used was

/C = ...

$$C = \frac{V}{Exdxv} \times \Delta E \text{ (mM)}$$

where V = final colume of reaction mixture (ml)

v = sample volume (ml)

d = light path (cm)

E = extinction coefficient of NADH at

340 nm = 6,3

366 nm = 3,4 (1 x $mmol^{-1}$ x cm^{-1})

For readings at 366 nm

Concentration (mM) = ΔE x 5,03 x F

and for readings at 340 nm

Concentration (mM) = ΔE x 2,71 x F

where F = dilution factor

Determination of lactic acid. Method 2

In Method 2, the pyruvate formed in the presence of LDH and excess NAD^+ is trapped in a subsequent reaction catalysed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate which also serves as buffer

$$\text{Pyruvate} + \text{L-Glutamate} \underset{\longleftarrow}{\xrightarrow{\text{GPT}}} \text{L-Alanine} + \alpha\text{-Ketoglutarate}$$

Preparation of solutions

Buffer. 4,75 g of glycylglycine (Merck) and 0,88 g of L-glutamic acid (Merck) were dissolved in 50 ml of deionized water. The pH was adjusted to 10,0 with *ca* 4,6 ml of 10 N-NaOH and the solution made up to 60 ml.

NAD solution. 420 mg of NAD (Boehringer - 89% pure by enzymic assay) was dissolved in 12 ml of deionized water.

GPT suspension. 2 ml of suspension (10 mg/ml) from pig heart (Boehringer) was centrifuged at 4 000 rpm for 10 min and 1,0 ml of clear supernatant sucked off and discarded. The pellet was resuspended in the remaining medium to give a suspension containing 20 mg/ml.

L-LDH and D-LDH. These were the same as those used in Method 1.

All solutions were handled as described for Method 1.

Preparation and dilution of samples

This was the same as for Method 1.

Assay system

The conditions of assay were the same as for Method 1 except for temperature which was 20-25°C for L-lactate and 37°C for D-lactate.

Solutions pipetted into cuvettes or tubes	Blank (ml)	Sample (ml)	Final concentration in assay mixture
Buffer	1,00	1,00	220 mM of glycylglycine 36 mM of glutamate
NAD solution	0,20	0,20	3,4 mM of NAD
GPT solution	0,02	0,02	12 U/ml of GPT
Deionized water	1,00	0,90	-
Sample or standard	-	0,10	up to 100 uM of lactate

The solutions were mixed and extinction E_1 of blank and sample measured after 5 min. The reaction is started by addition of 0,02 ml of L-LDH (40 U/ml) or 0,05 ml of D-LDH (27 U/ml) to both blank and sample. The solutions were mixed, incubated for the required time and extinction E_2 measured.

Calculation

The absorbance for blank and sample was calculated in the same way as for Method 1. Concentration was calculated either from a standard curve or by using the equation given in Method 1.

For readings at 366 nm

Concentration (mM) = $\Delta E \times 6,59 \times F$ for L-lactate

= $\Delta E \times 6,68 \times F$ for D-lactate

and for readings at 340 nm

Concentration (mM) = $\Delta E \times 3,56 \times F$ for L-lactate

= $\Delta E \times 3,60 \times F$ for D-lactate

RESULTS AND DISCUSSION

Progress curves

These were performed for D- and L-lactate using both methods. The results are given in Fig. 2.1. It was found that for Method 1 using hydrazine as trapping agent, incubation times of 30 min for L-lactate and 60 min for D-lactate were required at 37°C. After these incubation times over 90% of the ΔE had occurred and absorbance was changing very slowly so that differences in the time taken to read absorbance caused a negligible error. The incubation time recommended by Boehringer Mannheim for this method is 30 min for the determination of both isomers. After this time reaction was complete. For Method 2 the incubation time was 15 min at 20-25°C for L-lactate and 30 min at 37°C for D-lactate. The recommended incubation time is 15 min at 20-25°C for L-lactate and at 37°C for D-lactate.

/Reproducibility .....

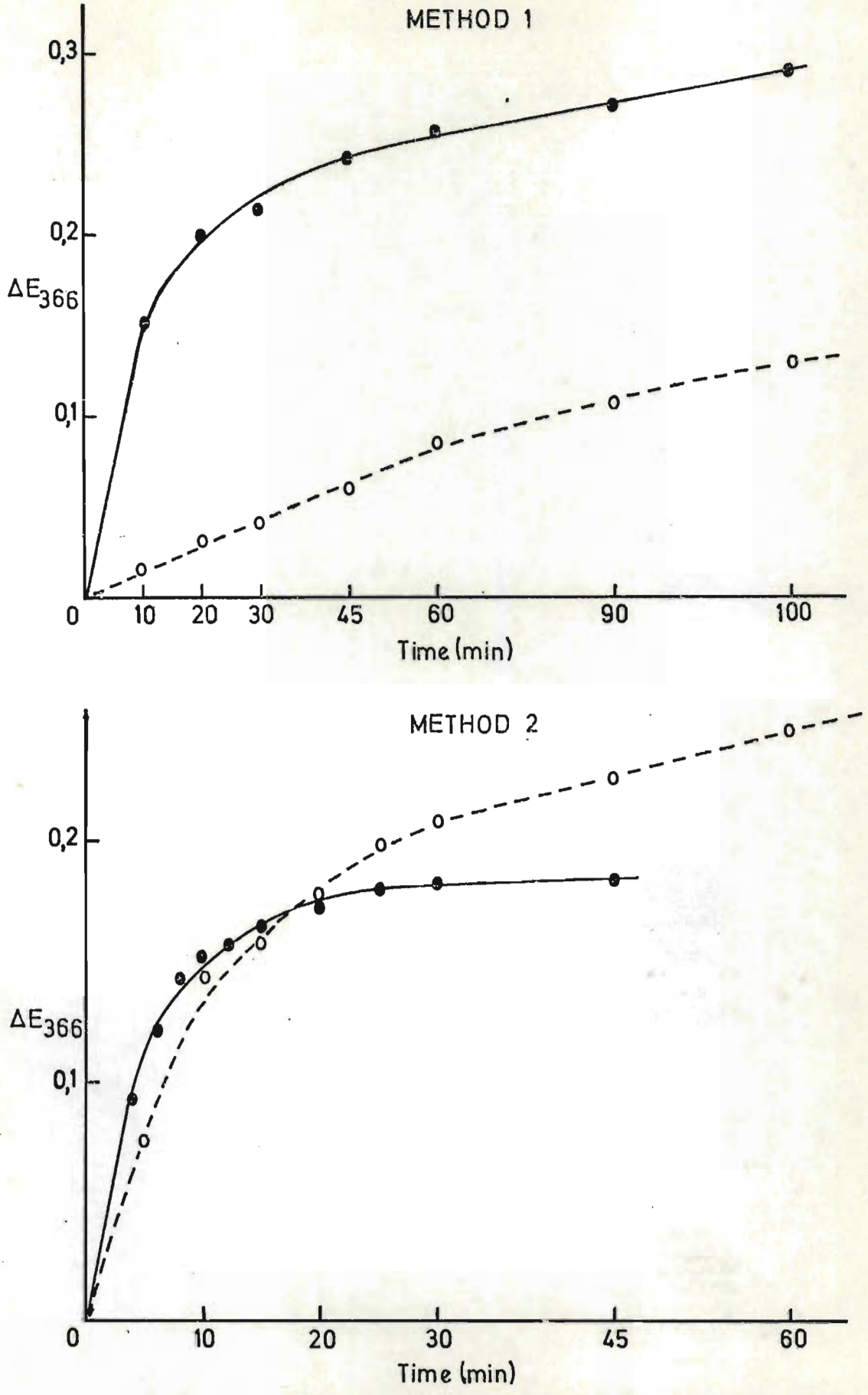


Fig.2.1. The effect of incubation time on the ΔE values for 2,5 mM D-lactate (--o--) and 1,0 mM L-lactate (—o—) using hydrazine (Method 1) or GPT (Method 2) to trap pyruvate. Each point represents the mean of 5 determinations

Reproducibility

Standard curves were drawn up for D- and L-lactate using both methods and a typical set of results is presented in Fig. 2.2 and Fig. 2.3. For Method 1 using D-lactate the standard curve for concentrations between 0-10 mM was almost linear whereas for L-lactate the curve was markedly curvilinear and also gave ΔE values which were at least double those found for D-lactate despite the shorter incubation time. For Method 2 the ΔE values were similar and the curves showed the same slightly curvilinear trend for D-lactate and L-lactate. Incubation times were shorter and ΔE values higher than for Method 1. The recommended concentration of lactate for assay is *ca* 4 - 5 mM at maximum.

Method 2 had lower SD and CV values (CV 1,0 - 3,8%) and was more precise than Method 1 (CV 1,5 - 9,9%) although the SD values were small for both methods (range 0,001 - 0,015 for D-lactate and 0,003 - 0,030 for L-lactate) (see Appendix Table 2). However, Method 1 was more conveneint to use since fewer solutions were involved. The use of an additional enzyme preparation (GPT) in Method 2 increased the cost.

Both methods were reliable and accurate. The use of the equation (page 15) was not accurate over the whole range of concentrations and tends to underestimate values <2,5 mM and overestimate values >5,0 mM especially if the standard curve is markedly curvilinear. These errors are much smaller for curves which are almost linear. In the survey work reported (see Chapter 4) and the fermentation tests (see Chapter 5) when most of the concentration values were <1,0 mM, the equation

$$\text{Conc}_{un} = \frac{\Delta E_{un}}{\Delta E_{std}} \times \text{Conc}_{std}$$

was used with little loss in accuracy. A standard containing 1,0 mM D- or L-lactate was run with each set of analyses for this purpose. Concentration values > 5,0 mM were read off the standard curves. This was found to be reliable if the samples were diluted and reanalysed.

There are two additional points relevant to the discussion of the standard curves. Firstly, the activity of the enzyme preparations is expressed in units measured with pyruvate as substrate, namely 550 U/mg for L-LDH and 300 U/mg for D-LDH. However, the activity in the reverse direction as used for the assays in these experiments, is much lower and in the case of D-LDH is only 50 U/mg with D-lactate as substrate (Boehringer Mannheim, personal communication). For L-LDH from beef muscle (phospate buffer pH 7,5; 25°C) the K_M values were 2,5 x 10^{-2} M with L-lactate as substrate and 1,0 x 10^{-3} M with pyruvate as substrate (Pesce, McKay, Stolzenbach, Cahn & Kaplan, 1964). The second point concerns Method 1 in

/which

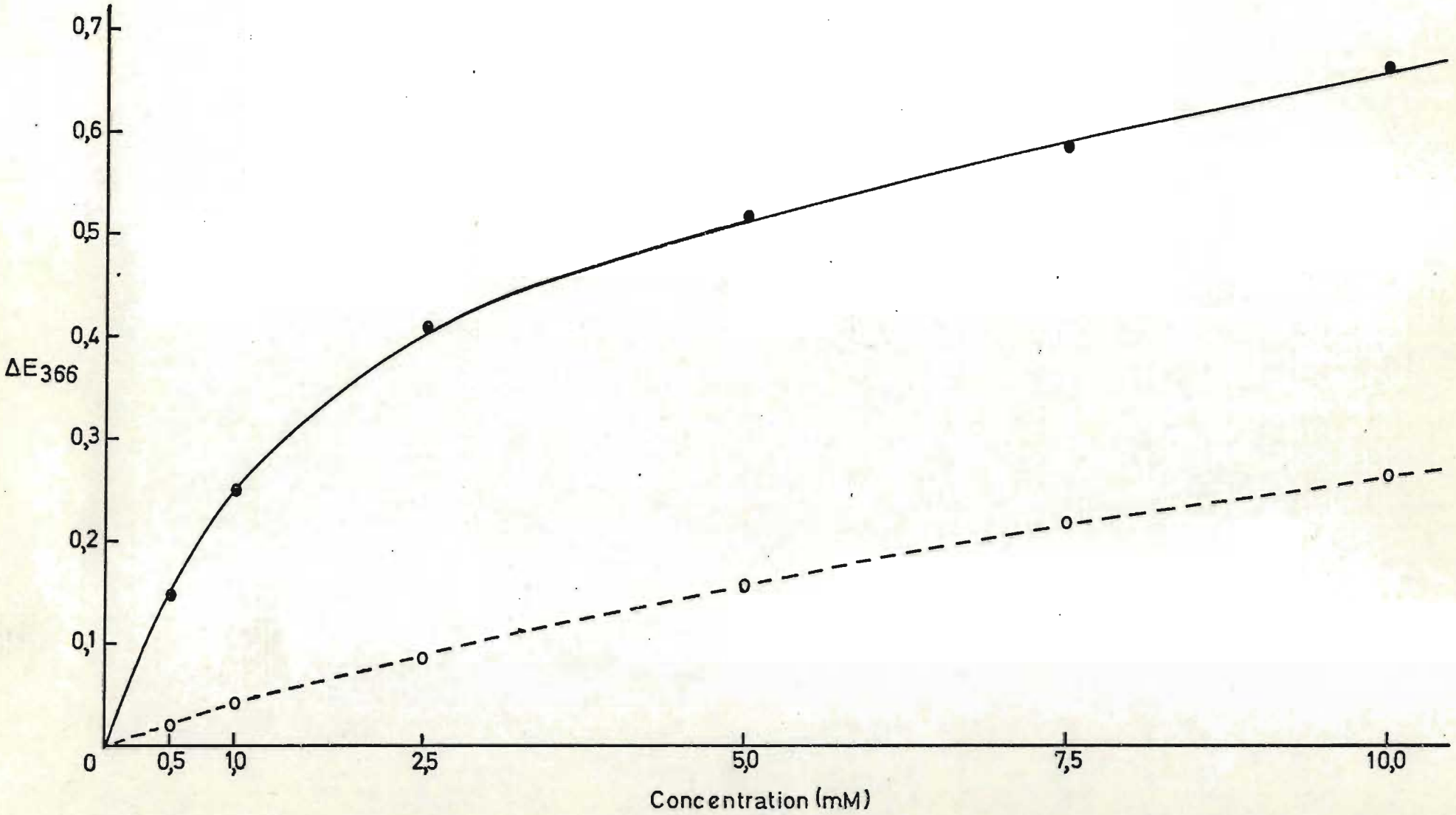


Fig.2.2. Standard curves for D-lactate (--o--) and L-lactate (——●——) using Method 1 with hydrazine to trap pyruvate

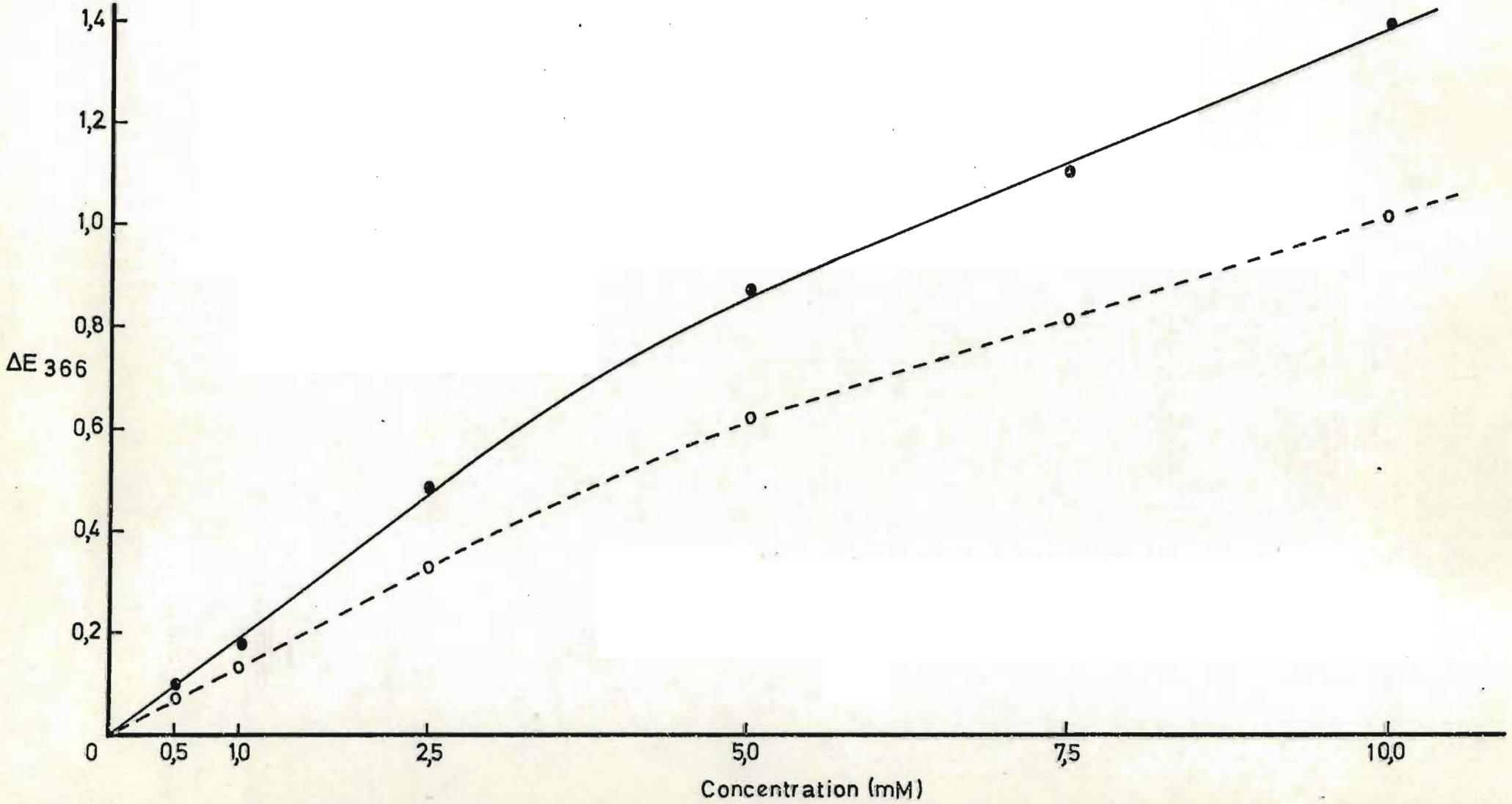


Fig.2.3. Standard curves for D-lactate (--o--) and L-lactate (——o——) using Method 2 with GPT to trap pyruvate

which hydrazine was used to trap pyruvate. There is an increase in extinction in the absence of lactate. This is caused by the formation of an addition compound of hydrazine with NAD which absorbs at the same wavelength as NADH (Boehringer Mannheim, personal communication). The coupled enzymic method for the determination of lactic acid (Method 2) was developed to overcome these problems.

Stability of solutions

All solutions must be stored in a refrigerator at 4°C in stoppered glass vessels. Under these conditions the recommended maximum storage times are as follows: buffer solutions, 3 months; NAD solutions, 4 weeks; L-LDH and GPT, 1 year; and D-LDH, 6 months (Boehringer). It has been found that all solutions remain stable even after storage for double the stated times but care was taken to test the solutions before use when this was done.

Stability of samples

After deproteinization D- and L-lactate are stable both under acid or neutral conditions at 4°C provided no microbial growth occurs (Noll, 1974; Gawehn & Bergmeyer, 1974).

Specificity

The specificity of the methods is dependent on the purity and specificity of the LDH enzyme used in the assay. The enzyme L-LDH reacts only with L-lactate and D-LDH with D-lactate although both can react with other L- or D-2-hydroxy acids respectively but at much slower rates. The enzyme preparations are also free of any NADH oxidase activity (Boehringer).

In conclusion, the concentration of D- and L-lactic acid can only be determined directly using an enzymic method employing the sterospecific D- and L-LDH enzymes. Details of the two methods which are used repeatedly throughout the thesis are presented in this chapter. In one method hydrazine was used to trap pyruvate formed in the reaction to displace the equilibrium, and in the other a coupled enzyme reaction with LDH and GPT was used. The reaction normally proceeds in the direction of pyruvate to lactate and not as used in the assay.

CHAPTER 3. DEVELOPMENT OF A METHOD FOR ENUMERATION AND ISOLATION OF LACTATE-UTILIZING BACTERIA

INTRODUCTION

In any study concerning lactate in the rumen special attention must be paid to the enumeration and isolation of bacteria utilizing lactic acid. However, viable bacteria belonging to the functional group metabolizing lactate are difficult to enumerate. Several workers have attempted direct counts of lactate-utilizing bacteria using either "habitat-simulating" media containing rumen fluid as a general growth stimulant and lactate as the only added energy source (Gutierrez, 1953; Kistner, Gouws & Gilchrist, 1962), or semi-defined media without rumen fluid but containing yeast extract, peptones, volatile fatty acids (VFA), trace elements and haemin with lactate as energy substrate (Giesecke, 1968; Latham *et al*, 1971). It has generally been found that "habitat-simulating" media support the growth of larger numbers of bacteria but are not as specific as the semi-defined media (Hungate, 1966).

Thus no single medium has been universally accepted for direct counts of this group of bacteria. Some work was therefore done on the development of a highly specific medium without rumen fluid which would support the growth of all species of lactate-utilizing bacteria known to occur in the rumen. The media tested ranged from a low lactate/low protein medium (LL) to a high lactate/high protein medium (LH).

METHODS

Animals and management

Three mature South African Mutton Merino wethers with large 80 mm ID permanent ruminal cannulae (Taljaard, 1972) were used to obtain samples of ruminal ingesta. The sheep and diets were as follows:

A20 - 1 500 g Medium quality teff hay (*ca* 5% CP), 200 g maize meal, 20 g biuret fed once daily;

A63 - 2 000 g Hammermilled lucerne hay fed once daily;

A69 - 750 g Hammermilled maize stalks, 25 g maize meal, 5 g urea fed twice daily at 08h00 and 16h00.

Each sheep received 15 g of a 50/50 mixture of NaCl (animal feed grade) and a commercial mineral mix (Kerolik No 3, composition given in Table 4.1).

/The sheep .....

The sheep had been on their respective diets for at least 3 months prior to the first sampling. Water was available at all times except on sampling days when it was removed before feeding at 08h00 and offered again after sampling 2,5 h later.

Sampling and processing of samples

The contents of the rumen and reticulum were mixed manually through the cannula while CO_2 was bubbled vigorously into the ruminal ingesta. A pre-gassed, wide-mouthed bottle was inserted into the rumen, filled, withdrawn, stoppered and immediately taken to the laboratory. Using a scoop approximately 10 g of sample was accurately weighed out. This sample was diluted 10-fold with anaerobic diluent (Appendix Table 3.1) and processed in an Ultra-Turrax Homogenizer (Janke & Kunkell KG, Staufen i.Br., 20 000 U/min) for 1 min with CO_2 being bubbled through the fluid by means of a bent needle.

Media

All media were prepared and inoculated using anaerobic techniques based on those of Kistner (1962) and Van Gylswyk (1970). All cultures were incubated at 39°C.

Rumen fluid. The clarified rumen fluid was obtained by sampling sheep 6 h after feeding lucerne hay, straining through 2 layers of cheesecloth and centrifuging at 1 500 g for 30 min to yield a cloudy supernatant. In this way several batches were collected and pooled as a single, uniform batch which was used throughout the experiments described in this thesis.

Total culturable count. Two media were used to determine the total culturable count, one with rumen fluid and the other without. Their composition is given in Appendix Table 3.1. The colonies appearing in the agar films in the roll bottles were counted after 7 days of incubation.

Lactate-utilizing bacteria. Several media differing in amounts of lactate and trypticase/yeast extract were prepared (see Table 3.1 and Appendix Table 3.1). None contained rumen fluid. Colonies were counted after 3, 5 and 7 days of incubation.

Anaerobic diluent. The composition is given in Appendix Table 3.1.

Maintenance slopes. Slopes of LH and LL media were made as described in Appendix Table 3.1.

Isolation of lactate-utilizing bacteria

Bacterial colonies from LH and LL media were isolated randomly from roll bottles of the highest dilutions containing well spaced colonies using a bent platinum needle and stabbed into appropriate maintenance

/slopes .....

slopes (Appendix Table 3.1). The slopes were incubated and examined daily. When growth was sufficiently heavy 0,5 ml of anaerobic diluent was injected onto the slope, the growth washed off and then transferred onto 2 fresh maintenance slopes. The duplicate slopes were incubated until growth was heavy and then stored in dry ice (CO_2 atmosphere) until required for fermentation tests. All transfers were made aseptically and anaerobically.

Morphological examination

Smears made from the colonies taken at random from roll bottles of the highest dilutions showing growth and at each subsequent transfer were stained using Gram's method.

Fermentation tests

The isolates obtained from LH and LL media were transferred into liquid medium containing 1,0% lactate (LM, see Appendix Table 3.1) from which agar was omitted. The isolates were incubated for 7 days and disappearance of D- and L-lactate compared to control bottles inoculated with the same volume of sterile anerobic diluent. D- and L-lactate were analyzed using the methods given in Chapter 2.

RESULTS

The counts of lactate-utilizing bacteria obtained after 7 days of incubation on the different media containing varying amounts of lactate and trypticase/yeast extract are given in Table 3.1. Counts were lowest on the medium containing the highest concentration of lactate and trypticase/yeast extract and highest on the medium containing the lowest concentration of these constituents. The SD is high because the values from the three different diets were used in the calculation.

Table 3.2 records the total culturable count obtained from media with and without rumen fluid and the counts of lactate-utilizing bacteria on LH and LL media as a percentage of the total counts. Counts on LL medium were 5-9 times higher than on the LH medium. The counts on LL medium, which contained no rumen fluid, were 9,3 - 35,4% of the total culturable count in medium without rumen fluid but only 4,2 - 11,5% in medium with rumen fluid. The counts on LH medium, which contained no rumen fluid, were only 1,9 - 5,3% of the total culturable count in medium without rumen fluid and less in medium with rumen fluid. The lactate-utilizing bacteria in the rumen of the sheep fed the maize stalk diet obviously formed a smaller part of the flora than on the other two diets.

/Table 3.1 .....

Table 3.1 The effect of varying the concentration of lactate and trypticase/yeast extract on counts of lactate-utilizing bacteria from sheep fed three different diets. The count on each diet was repeated twice

Medium	Variable components in media (%)	Count (x 10^{-8}/g ingesta)			
		Teff hay & maize meal	Maize stalks & maize meal	Lucerne hay	Mean ± SD (n = 6)
LH	2,0 Lactate 2,0 Trypticase 0,2 Y. extract	1,0	0,4	0,6	0,7 ± 0,4
LA	2,0 Lactate 0,2 Trypticase 0,05 Y. extract	1,1	0,3	0,6	0,7 ± 0,4
LB	0,35 Lactate 2,0 Trypticase 0,2 Y. extract	2,2	1,7	2,8	2,2 ± 0,6
LM	1,0 Lactate 1,0 Trypticase 0,1 Y. extract	2,5	1,1	3,8	2,5 ± 1,6
LL	0,35 Lactate 0,2 Trypticase 0,05 Y. extract	10,0	3,3	4,3	5,9 ± 3,9

The survival rate of lactate-utilizing bacteria after the initial transfer from roll bottle to medium slope was higher on LH medium than LL medium. The percentage of isolates failing to show growth was 2,25 times higher on the LL medium (Table 3.3). However, the biggest difference between the two media was the almost complete absence of *Veillonella*- and *Megasphaera*-like organisms in LL medium. Further losses occurred with each successive transfer with a better survival rate on the LH medium. After 4 transfers the surviving isolates were tested for ability to utilize more than 10% of DL-lactate in the medium. On the LH medium all survivors were found to utilize lactate whereas on the LL medium only 80% of survivors utilized lactate.

Two considerations led to the selection of the LH medium for the enumeration of the lactate-utilizing bacteria. As shown in Table 3.3 the LL medium was not capable of supporting the growth of lactate-utilizing cocci and seemed to be a selective medium for the growth of *Propionibacter* since even the percentage of *Selenomonas* was much less on this medium than on the LH medium. This would give an inaccurate and unrepresentative indication of the types of lactate-utilizing bacteria present in the rumen if used

Table 3.2. Counts of lactate-utilizing bacteria as a percentage of the total culturable count with and without rumen fluid (RF)

Diet	Total culturable count ($x10^{-9}$/g ingesta) with RF	without RF	Counts of lactate-utilizing bacteria					
			LL medium			LH medium		
			Counts ($x10^{-8}$/g ingesta)	Percent of total culturable bacterial count with RF	without RF	Counts ($x10^{-8}$/g ingesta)	Percent of total culturable bacterial count with RF	without RF
Teff hay, maize meal & biuret	3,4	1,5	3,9	11,5	26,0	0,8	2,4	5,3
Maize stalks, maize meal & urea	4,1	1,3	2,5	4,2	9,3	0,5	0,8	1,9
Lucerne hay	6,0	2,7	4,6	11,2	35,4	0,5	1,2	3,8

Table 3.3. The effect of medium on the morphological types of lactate-utilizing bacteria and survival rate after isolation onto slopes

Medium	No. of isolates	Morphological types resembling: *Veillonella*	*Megasphaera*	*Selenomonas*	*Propionibacter*	No growth
LH	101	12	13	19	37	20
		11,9%	12,9%	18,8%	36,6%	19,8%
LL	124	0	2	5	61	56
		0%	1,6%	4,0%	49,2%	45,2%

during an ecological study. The second factor was the higher survival rate of isolates on the LH medium. It is possible that if the picked isolates were placed on the same medium after isolation, survival rates would not have differed. In order to minimize losses when working on lactate-utilizing isolates from the Adaptation Experiment (Chapter 5), the maintenance slopes were modified to include 40% clarified rumen fluid. This reduced the number lost on each successive transfer from 20% to about 10%. The survival rate also improved with operator's experience and was better during the Adaptation Experiment than that reported in this section. The counts after 3, 5 and 7 days of incubation showed that the longer incubation was necessary since most colonies only showed up in the clear medium after 5-7 days. This also indicates that the medium is not perfect. Specificity was not impaired by the 7 day incubation.

The application of the LH medium, which was similar in composition to that of Giesecke (1968), for the direct counts of lactate-utilizing bacteria is shown in Table 3.4. This is a summary of a selected portion of the results to be presented in the following section (Chapter 5). The results show the progressive increase in numbers and percentage of lactate-utilizing bacteria during the adaptation to a high concentrate diet.

Table 3.4 Counts of lactate-utilizing bacteria as a percentage of total culturable bacteria during the stepwise adaptation of sheep to a high concentrate diet

Diet	Days on diet	Percent starch & soluble sugar in diet	Bacterial count		Lactate-utilizers as percentage of total culturable bacteria
			Lactate-utilizers ($\times 10^{-7}$/g	Total culturable ingesta)	
A	17	5,6	0.5	262,5	0,2
AE	7	14,9	0,9	205,0	0,4
E	7	29,8	2,4	322,5	0,7
EB	7	44,3	6,5	682,5	1,0
B	7	48,7	112,3	3 075,0	3,7
	21		42,0	210,0	20,0
	54		189,5	850,0	22,3

The total medium contained rumen fluid and thus the percentage of lactate-utilizers is an underestimate. Isolations from the lactate medium gave a reliable indication of the predominant species of lactate-utilizing bacteria. This amount of information over the short period of time involved

/would .....

would not be possible if indirect counts from islates of the total culturable bacteria were made. The counts were similar to the highest reported in the literature for this type of diet (Giesecke, 1968; Latham *et al*, 1971).

DISCUSSION

The results of the counts of lactate-utilizing bacteria on media containing differing amounts of lactate and trypticase/yeast extract are interesting. The counts on medium LH and medium LA were the same although they differed in the amount of trypticase/yeast extract. When the amount of trypticase/yeast extract was kept at 2,0 and 0,2% respectively but the lactate decreased to 0,35% (Medium LB) counts increased 3-fold relative to the LH medium. When all three components were at a low concentration (Medium LL) the counts were *ca* 8-fold higher than when all three components were at a high concentration. It would appear that the amount of lactate in the medium plays an important role in regulating the growth of bacteria which do not utilize lactic acid. This conclusion is supported by: the fact that lactic acid bacteria (streptococci and lactobacilli) inhibit the growth of other bacteria in the intestine of monogastric animals (Schaedler, 1973; Speck, 1976; Roach, Savage & Tannock, 1977); the interrelationship between lactic acid-producing and -utilizing bacteria in the rumen of milk fed lambs and calves (Eadie & Mann, 1970); and the lactic fermentation used in making silage (McDonald & Whittenbury, 1973). Although several lactate-utilizing species, including *Selenomonas ruminantium*, *Megasphaera elsdenii* and *Anaerovibrio lipolytica* (Hobson & Mann, 1961; Bryant & Robinson, 1962; Prins *et al*, 1975), are stimulated by or have an absolute requirement for peptides, amino acids and vitamins present in trypticase and yeast extract, this would also be beneficial to other bacteria present which do not utilize lactate.

Lactate-utilizing bacteria form $^{1}/10$ to $^{1}/3$ of the total culturable bacteria if the comparison is made between media without rumen fluid. However if the total culturable bacteria are counted on a medium containing rumen fluid the fraction drops to $^{1}/50$ - $^{1}/20$. The maize stalk diet supported a small population of lactate-utilizing bacteria while the teff hay diet, which contained 200 g maize meal, and the lucerne hay diet contained a higher proportion of lactate-utilizers. Kistner *et al* (1962) found high numbers of lactate-utilizing bacteria in the rumen of sheep fed lucerne hay alone. These results demonstrate that care must be taken when calculating the percentage of the total culturable count formed by direct counts of a functional group of bacteria. Rumen fluid is extremely difficult to replace even in semi-defined media containing trypticase and yeast extract as sources of growth factors.

CONCLUSIONS

1. Counts of lactate-utilizing bacteria were highest on the medium containing 0,35% lactate and 0,2% trypticase/0,05% yeast extract and lowest on media containing 2,0% lactate and either 2,0% trypticase/0,2% yeast extract or 0,2% trypticase/0,05% yeast extract.
2. Counts on both the LL and LH media which contained no rumen fluid formed a larger percentage of the total culturable count in the medium without rumen fluid than the total culturable count in the medium which contained rumen fluid.
3. There was an almost complete absence of cocci (*Veillonella*- and *Megasphaera*-like organisms) on the LL medium whereas 25% of the isolates on the LH medium were cocci. In addition, survival of isolates was twice as high on LH as on LL media. The specificity in respect of lactate utilization by the surviving isolates was 100% on LH and 80% on LL media.
4. On the basis of these results the LH medium was selected as the most suitable medium for obtaining direct counts of a complete spectrum of lactate-utilizing bacteria. Using this medium it was shown that the numbers and percentage of lactate-utilizing bacteria increased progressively with stepwise additions of maize grain during the adaptation of sheep to a high concentrate diet.

CHAPTER 4. EXPLORATORY EXPERIMENT TO ESTABLISH THE CONCENTRATION OF LACTIC ACID IN THE RUMEN OF SHEET UNDER DIFFERENT FEEDING CONDITIONS

INTRODUCTION

Prior to this experiment problems were experienced in getting the sheep to consume their ration without selecting for either roughage or concentrate. However, it was found that if they were offered a complete, mixed, hammermilled diet containing fixed proportions of maize stalk to maize grain, and using 10% molasses as binder, very little selection was possible. The molasses also made the diet less dusty, more palatable and served as a source of readily fermentable carbohydrate (RFC). Having thus overcome the selection problem it was possible to proceed with the exploratory experiment reported in this chapter. This experiment was planned to establish the concentrations of D- and L-lactic acid, free glucose and pH values in the rumen of sheep fed either a high roughage (A) or two high concentrate diets (B and C) at high and low levels of intake under "normal" feeding conditons as defined in the preface. Valuable information was obtained on the effects of the amount of RFC in the diet, and of the amount and rate at which food is consumed, on ruminal lactic acid concentration. The two high concentrate diets differed in crude protein (CP) content since if nitrogen (N) is limiting in the rumen the slower growing bacteria and those that depend on others to produce their growth substrate, e.g. lactate-utilizing bacteria, will be most affected. The lactate-utilizers are important in preventing the accumulation of lactic acid in the rumen. The CP content of Diet C was greater than the recommended allowance (NRC, 1968; ARC, 1965) to ensure an adequate supply of ruminal NH_3-N.

METHODS

Animals and management

Six mature sheep, each provided with a ruminal cannula, were used in the experiment. They were individually housed in covered pens where water was freely available. They were offered half a ration twice daily at 08h00 and 16h00 except on sampling days when they were fed at 07h00 and 15h00. The full ration consisted of 1 500 g or 1 000 g of food at high or low intake respectively. Any food remaining was weighed back before the morning feed. The composition of the 3 experimental diets is given in Table 4.1.

/Table 4.1 .....

Table 4.1. Composition of diets used in the exploratory experiment

Component	kg/100 kg of Diet A	B	C
Maize stalks	83,0	17,7	16,8
Maize grain	-	60,8	57,2
Molasses	10,0	10,0	10,0
Urea	1,0	-	4,5
Fishmeal	-	5,0	5,5
NaCl	1,0	1,0	1,0
Minerals[Φ]	2,0	2,0	2,0
$CaCO_3$	3,0	3,0	3,0
Crude protein (CP)	7,8	11,3	17,8

[Φ]Commercial mixture Kerolik No. 3 (Cooper & Nephews, South Africa) containing 12% P; 24% Ca; 5% S; 0,002% Co; 0,05% Cu; 0,002% I; 0,1% Mn; 0,3% Fe; 0,3% Zn.

The allotment of sheep to these diets is in Table 4.2 which also indicates their previous dietary history. Initially all sheep were fed their respective diets at a high level of intake for a period of 21 days, except A85 which was introduced to Diet C only 3 days before the first sampling. After a further 3 weeks at the high level of intake, the ration was reduced to low intake. Two weeks later the sheep were smapled for the second time.

Sampling and analysis of ruminal fluid

The sheep were sampled before (0) and ½, 1, 1½, 2, 3, 4½, 6 and 8 h after the first feed and ½, 1, 1½, 2, 3 and 4½ h after the second feed. Samples of ruminal fluid were taken from the rumen using a suction tube (15 mm ID) inserted through the plug of a large rumen cannula. One portion (*ca* 15 ml) was strained through 2 layers of cheesecloth and 10 ml of strained ruminal fluid pipetted into a wide-mouth McCartney bottle containing 10 ml of 0,5 N-HCl. The other portion (*ca* 25 ml) was placed in a beaker and a layer of liquid paraffin mixture pipetted onto the surface to prevent CO_2 escaping. The mixture contained liquid paraffin and xylol in a ratio 4:1. This beaker was immediately taken to the laboratory and pH measured using a Radiometer PHM 26 (Radiometer, Copenhagen, Denmark).

/The .....

The acid-treated samples were centrifuged at 4 000 rpm for 20 min and the supernatant stored at 4°C until required for analysis. L- and D-lactate were analyzed using specific enzymic methods (Method 1, Chapter 2) and total lactate calculated as the sum of the two isomers. Free glucose was measured using the GOD-Perid kit (Boehringer) containing glucose oxidase.

RESULTS

Food intake

The food consumption of the sheep on the different diets at high and low levels of intake respectively is given in Table 4.2. The two sheep offered Diet A never consumed all their food, and despite the reduction in food offered they actually consumed more in the second half of the experiment. Differences in food intake had little effect on pH, concentration of lactic acid and glucose and data for the two levels of intake were pooled for Diet A.

Table 4.2. Previous diet and mean food intake of sheep over the 5 day period before and including the sampling day

Sheep	Previous diet	Experimental diets		
		Diet	Food intake (g)	
			High level[a]	Low level[b]
A52	Lucerne hay	A	1 090	1 215
A63			745	870
K1	Maize stalks & maize grain	B	1 500	1 000
A26			1 500	1 000
A35	Maize stalks & maize grain	C	1 275	1 000
A85	Lucerne hay	C	1 500	1 000

[a] offered 750 g twice daily

[b] offered 500 g twice daily except Diet A where 650 g offered twice daily

Effect of diet on ruminal pH, concentration of lactic acid and free glucose

Figure 4.1 shows the effect of diet on the mean pH, total lactic acid and free glucose in the ruminal fluid. The pH tended to be lower on Diet B

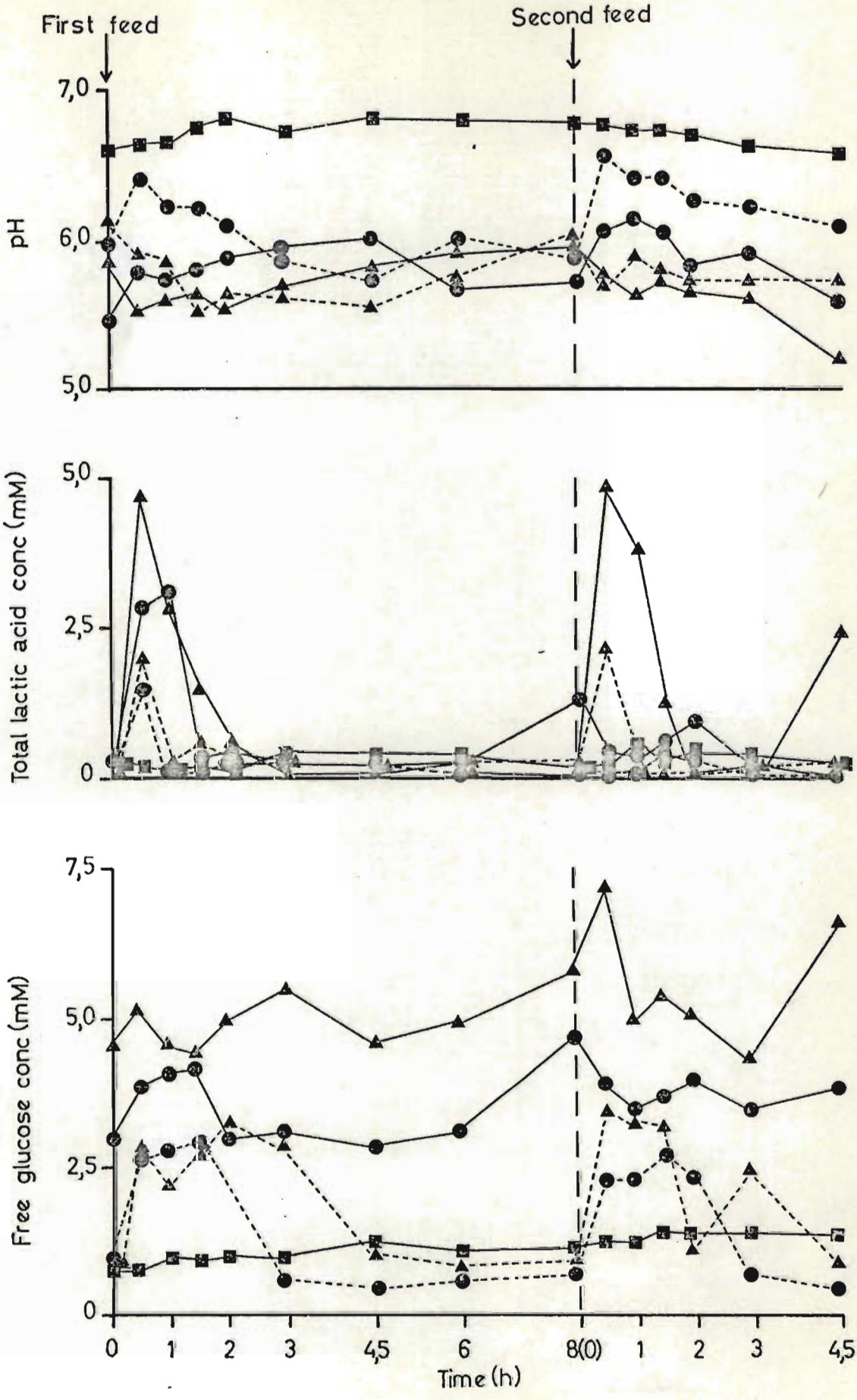


Fig.4.1. The mean pH, total lactic acid (mM) and free glucose (mM) in ruminal fluid of sheep fed Diet A (—■—) or high (——) and low levels (------) of Diet B (▲) or Diet C (●)

than on Diet C and was highest on Diet A. The pH was found to increase for a short time (½ h) after feeding with Diet C and never fell as low as the values obtained on Diet B. Peak lactic acid and free glucose concentration showed a similar trend being highest with Diet B (4,9 and 7,3 mM respectively), followed by Diet C (3,1 and 4,8 mM respectively) and lowest on Diet A (0,5 and 1,5 mM respectively). The concentrations were also highest on the high level of food intake and lower with the low level. At both levels of intake on the high concentrate diets, lactate concentration decreased to almost zero *ca* 2 h after feeding whereas the free glucose concentration at the high intake tended to remain elevated without falling as it did on the low intake.

Effect of rate of food consumption on ruminal pH, concentration of lactic acid and free glucose

The effect of the rate at which food is consumed on ruminal pH, total lactate and free glucose is shown in Fig. 4.2. This comparison is possible only with the two sheep on Diet B because at both levels of intake they both consumed all the food offered, the only difference being the rate at which it was consumed. Sheep K1 ate all food within 20-30 min whereas sheep A26 required from 2-4 h to consume the same amount of food. The fast eater had higher concentrations of lactic acid than the slow eater which was most marked at the high level of intake. The pH values were generally lower for the fast eater than for the slow eater. In contrast the values for free glucose were higher for the slow eater than for the fast eater at each level of intake.

Effect of diet on the proportion of lactate isomers at peak accumulation

The proportion of total lactate contributed by L-lactate was usually >50%. L-lactate was the predominant isomer at all times in the feeding cycle for Diet A, Diet C (both levels of intake) and Diet B (low level of intake). However, on Diet B at peak accumulation of total lactic acid (Table 4.3) on the high level of intake the proportion of D-isomer increased to form 82% of the total lactate.

Table 4.3. The proportion (%) of L-lactate in total lactate at peak accumulation of lactic acid

Diet	L-lactate as % of total lactate	
	High level of intake	Low level of intake
B	18	68
C	75	53

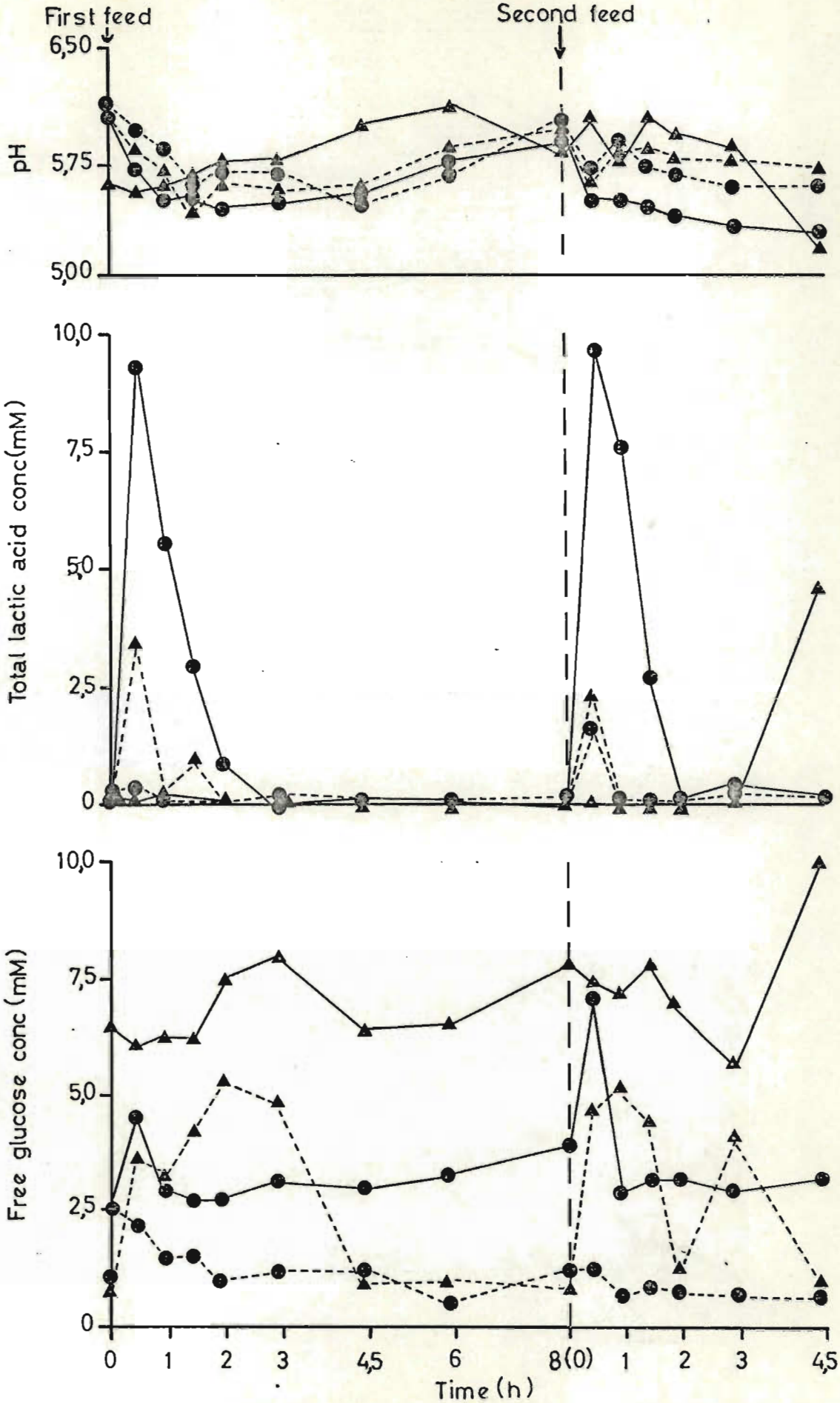


Fig.4.2. Values for pH, total lactic acid (mM) and free glucose (mM) in the ruminal fluid of sheep K1 (●), a fast eater, and sheep A26 (▲) a slow eater, at high (——) and low (- - - -) levels of food intake

CONCLUSIONS

In conclusion the main findings of this exploratory experiment are summarized below.

1. Food intake on the high roughage diet never exceeded 1 250 g/day whereas on the high concentrate diets (especially Diet B) the sheep easily consumed the 1 500 g offered.
2. Peak lactic acid and free glucose concentrations were highest on Diet B (4,9 and 7,3 mM respectively), followed by Diet C (3,1 and 4,8 mM respectively) and lowest on Diet A (0,5 and 1,5 mM respectively). The concentration of lactic acid had returned to almost zero about 2 h after feeding.
3. On Diet B it was possible to examine the effect of rate of food consumption, since both sheep on this diet consumed all food at both levels of intake only at different rates. The fast eater had higher concentrations of lactic acid and lower pH values than the slow eater. The difference was more marked at the high level of intake.
4. The proportion of total lactate contributed by L-lactate was usually 50% on all diets at all times in the feeding cycle except at the high level of feeding on Diet B when D-lactate formed 82% of total lactate at peak accumulation.
5. Further discussion of the results is presented in Chapter 7.

PART II

RUMEN BIOCHEMISTRY AND MICROBIOLOGY

DURING STEPWISE ADAPTATION

TO HIGH CONCENTRATE DIETS

CHAPTER 5. AN ECOLOGICAL STUDY OF THE STEPWISE ADAPTATION OF SHEEP TO A HIGH CONCENTRATE DIET

INTRODUCTION

The ruminal microbiology and related biochemistry and physiology of ruminants fed high roughage and high concentrate diets have been studied extensively. However, very little is known about the changes which occur in the numbers and types of ruminal micro-organisms during adaptation from high roughage to high concentrate diets. Thus little is known of the critical changes that take place during the all-important stepwise adaptation from low to high concentrate diets practised routinely in the dairy industry and in intensive fattening of sheep and cattle. This experiment was designed to further evaluate microbiological methods and to study some of the critical factors involved in the microbiological adaptation to high concentrate diets. For this reason changes that occurred in respect of the amylolytic and lactate-utilizing bacteria and the ciliate protozoa were studied. Counts of total culturable bacteria were also included in order to distinguish between general trends and changes in any one of the groups of functional bacteria investigated. The pH and concentrations of total volatile fatty acids (VFA), individual VFA, D-lactate, L-lactate and NH_3-N were determined on the representative samples of ruminal ingesta taken for the microbiological counts.

METHODS

Animals and management

Eight mature South African Mutton Merino wethers with large 80 mm ID permanent ruminal cannulae (Taljaard, 1972) and weighing 56-69 kg at the start of the experiment were used. Four were used for the microbiology study (A18, A57, A58 and A72) and four (A19, A21, A28 and A31) to make more detailed studies on biochemical changes occurring during the experiment. It was considered advisable to use two groups of sheep since previous experience in this laboratory has shown that it is not possible to remove more than 1 kg of ingesta per week, even with sheep containing 6-10 kg of ruminal ingesta, without upsetting microbial activity.

The sheep were changed from a lucerne hay diet to the basal diet containing 10% molasses as binder (see Chapter 4) and 83% maize stalks for 17 days. Thereafter increasing amounts of maize grain were substituted for the stalks at weekly intervals until the final diet contained 18%

/stalks .....

stalks, 61% grain and 10% molasses. Details of the diets are given in Table 5.1. The stalks and grain were hammermilled to pass a 6 mm mesh screen. All diets were fed at a rate of 600 g twice daily at 08h00 and 16h00 except on days the diet was changed, when 600 g of the old diet was given at 08h00, 600 g of the new diet at 10h00 immediately after a sample of ruminal ingesta was taken for microbiological examination, and a further 600 g at 16h00. With the last change of diet, 600 g of Diet EB was given at 08h00 and 300 g of Diet B at 10h00 and 16h00. Water was available at all times except on sampling days when it was removed before feeding and replaced after sampling.

Sampling

Samples were taken 2 h after the first feed of the day. Microbiological examinations were made 24 h and 7 days after a change in diet during

Table 5.1. Composition of diets used in the stepwise adaptation experiment

Diet	A	AE	E	EB	B
Days on diet	17	7	7	7	54
Composition (kg/100 kg)					
Maize stalks	83,0	67,5	44,2	28,3	17,7
Maize grain	-	13,7	34,3	50,2	60,8
Molasses	10,0	10,0	10,0	10,0	10,0
Urea	1,0	0,6	-	-	-
Fishmeal	-	2,2	5,5	5,5	5,5
$CaCO_3$	3,0	3,0	3,0	3,0	3,0
NaCl	1,0	1,0	1,0	1,0	1,0
Mineral mixture[Φ]	2,0	2,0	2,0	2,0	2,0
Analysis (g/100 g Dry Matter)					
Mono- + di-saccharides	2,4	2,6	2,0	2,4	5,0
Starch + fructosans	3,2	11,9	27,8	41,9	43,7
Hemicellulose + cellulose	51,0	45,2	30,0	22,9	18,7
Crude protein (CP)	8,3	9,7	12,2	12,5	12,3
Ca	2,3	2,3	2,3	2,0	2,2
P	0,42	0,49	0,57	0,53	0,61

[Φ]Commercial mixture given in Table 4.1 (Kerolik No. 3, Cooper & Nephews, South Africa)

the adaptation period and also after 1, 7, 21, 40 and 54 days on the final diet. The contents of the reticulo-rumen were thoroughly mixed by hand

/in situ, ...

in situ, and a representative sample was scooped out with a 200 ml beaker which was filled to the brim, the pH measured, and sealed with Parafilm (American Can Coy., Wisconsin). The four sheep were sampled, and the samples were ready for processing in the laboratory within 10-15 min of removal of ingesta from the first sheep.

Processing

Anaerobic precautions were taken throughout. Approximately 20 g of sample was weighed rapidly but accurately in a stoppered bottle and transferred quantitatively with *ca* 80 ml of anaerobic diluent to a plastic bag. This was loaded into a Colworth Stomacher 400 (Seward & Co, 6 Stamford St, London) and processed for 2 min. A further quantity of anaerobic diluent, sufficient to give a final 10-fold dilution, was added and the contents mixed and poured through 2 layers of cheesecloth into a 25 ml wide-mouthed McCartney bottle which was filled to capacity and sealed. The bottles were stored in crushed ice until the dilution series was made ¼-2h later. Preliminary experiments showed that no change occurred in viable counts of the groups examined during this period (see Table 5.2).

Table 5.2. Effect of holding samples of ingesta in ice for different lengths of time after processing with the Colworth Stomacher

Diet	Counts (per g of ingesta)	Length of time (min) 0	30	60	90	120
Lucerne hay	Total culturable x 10^{-9}	3,4	3,0	3,4	3,5	3,6
	Cellulolytics x 10^{-7}	3,8	1,9	4,1	4,8	3,4
	Ciliate protozoa x 10^{-5}	1,7	1,2	1,1	1,0	0,9
High concentrate	Total culturable x 10^{-9}	7,1	8,2	9,0	7,6	12,2
	Cellulolytics x 10^{-7}	7,2	7,2	6,8	3,7	8,1
	Ciliate protozoa x 10^{-5}	9,2	11,0	12,4	10,4	12,0

Protozoal counts

Counts of the ciliate protozoa were made on processed samples of ingesta (Colworth Stomacher - see above) obtained from the 10^{-2} dilution of the dilution series used for the bacterial counts. A portion (5 ml) from the 10^{-2} dilution was added to a bottle containing 5 ml of 40% formalin (as preservative) and 5 ml anaerobic diluent using a syringe. The contents were mixed gently and thoroughly and 10 ml added to a bottle containing an equal volume of glycerine to give a suspension suitable for counting in a counting chamber described by Boyne, Eadie & Raitt (1957). Subsequent investigations showed processing reduced the numbers of both large and small protozoa compared to the minimal treatment which involved a 10-fold dilution and gentle manual swirling to mix (see Table 5.3).

Table 5.3. Mean count ($x10^{-5}$/g of ingesta) and percentage of different ciliate protozoa$^{\Phi}$ in the ruminal ingesta of sheep fed a high concentrate diet (Diet B) and processed by the Stomacher and Minimal treatments

Numbers and types of ciliates	Counts on different treatments	
	Minimal	Stomacher
Total protozoal count	16,1±0,3	7,0±2,1
Differential counts as a percentage of the total count		
1) Holotrichs (a) large	0,1	0
(b) medium	0,6	0
2) Entodiniomorphs		
(a) large	3,9	0,9
(b) medium	11,3	12,9
(c) small	84,1	86,2

$^{\Phi}$Differential counts were made on the basis of size (m):
Holotrichs (a) 100-140x60-90 (b) 60-80x40-50
Entodiniomorphs (a) 120-150x70-90 (b) 70-100x50-80 (c) 50-80x30-50

Bacterial counts

The techniques employed for counts of viable bacteria were essentially those of Kistner (1960), except that an Astell roll bottle apparatus (Astell Laboratory Service, 172 Brownhill Rd., Catford, London) modified for use with anaerobic bacteria (Toerien & Siebert, 1967) was employed in place of the Julius apparatus. Amounts of 2,5 ml of inoculated agar growth medium were injected in triplicate into the larger Astell roll bottles.

Total culturable bacteria were counted using the medium containing ruminal fluid given in Appendix Table 3.1.

Lactate-utilizing bacteria were counted on the LH medium given in Appendix Table 3.1.

Amylolytic bacteria were counted by the method of Kistner (1960). The medium was identical to the total medium containing ruminal fluid except that 1 g soluble starch (amylodextrin) (Merck, Darmstadt) replaced all the other carbohydrates. A short incubation period (20 h) was employed to obtain specific counts (Kistner, 1960). Considerably higher counts are obtained with longer incubation periods (Table 5.4) but only counts obtained with the 20 h incubation period are reported in the results as these represent the fastest growing starch digesters which are the most important with respect

Table 5.4. Effect of incubation time on mean bottle count of total culturable, amylolytic, glucolytic and lactate-utilizing bacteria in representative samples of ruminal ingesta obtained 2 h after feeding a high concentrate diet (Diet B) to sheep. (NC = not counted)

Medium	Incubation time (days) 1	2	5	7
Total culturable $x10^{-9}$	NC	NC	14,6	16,6
Amylolytic $x\ 10^{-9}$	1,1	2,8	4,5	NC
Glucolytic $x10^{-9}$	2,5	6,0	8,6	10,1
Lactate-utilizing $x10^{-8}$	NC	NC	4,0	4,4

Morphological examinations

Smears stained by Gram's method were made from colonies taken at random from roll bottles contributing to the counts reported for each of the above groups of bacteria. Morphological examination occurred at each subsequent transfer of the isolates obtained from the media for counting amylolytic and lactate-utilizing bacteria.

Biochemical analyses

The sample of whole ruminal ingesta remaining after a sub-sample (*ca* 20 g) had been taken for microbiological examination was immediately strained through 2 layers of cheesecloth and frozen in dry ice until required for analysis, with the exception of the samples obtained after 21, 40 and 54 days on Diet B. In this case the whole ruminal ingesta remaining after taking the sub-sample for microbiological examination was held in the refrigerator for 90 min before straining for chemical analysis. The frozen samples were then thawed and one portion treated with an equal volume of 0,5 N-HCl for analysis of ammonia, lactic acid and glucose. The sample for VFA analysis was treated with 50% (w/v) NaOH (*ca* 4-5 drops) to pH >10. Ammonia was determined by steam distillation by the method of Schwartz, Schoeman & Färber (1964). D- and L-lactic acids were measured enzymically as described in Chapter 2. Volatile fatty acids were steam distilled by the method of Briggs *et al* (1957) and titrated with 0,05 N-NaOH. The sodium salts were evaporated, dissolved in 0,1-0,2 ml formic acid and the free acids separated by gas chromatography using a column of Chromosorb W AW-DMCS, 80-100 mesh (Johns Manville, New York) coated with 10% SP 1200 (Supelco, Philadelphia) and 1% phosphoric acid (Ottenstein & Bartley, 1971).

/The pH .....

The pH was measured on whole ruminal ingesta immediately after sampling using a portable Polymetron PM 1 meter

Isolation of bacteria

Isolations of lactate-utilizing bacteria were performed as described previously in Chapter 3 (page 23). Isolations of amylolytic bacteria were made onto slopes of maintenance medium (Van Gylswyk, 1970) which contained 40% rumen fluid, 0,05% of each of the carbohydrates and 0,05% Casitone (Difco) using the same techniques as for the lactate-utilizers. Survival of the amylolytic isolates after three transfers was excellent (96,1%).

Identification of the amylolytic and lactate-utilizing bacteria

All isolates taken from the media for counting amylolytic and lactate-utilizing bacteria were placed into groups on the basis of cell morphology and size, Gram reaction and the ability to ferment soluble starch with the production of lactate or to utilize lactate respectively. These fermentation tests were performed in media containing 1,0% of their respective substrates and the culture supernatant tested for an increase or decrease in lactic acid respectively as compared to uninoculated control bottles. Since it was not possible to identify all the isolates, only 25 isolates selected from the different groups of amylolytic and lactate-utilizing bacteria were put through a further series of carbohydrate fermentation and other biochemical tests detailed in Appendix Tables 5.1, 5.2 and 5.3.

End-product analysis by gas chromatography: The procedure used for the preparation of standard solutions, and the extraction and methylation of volatile and non-volatile fatty acids was based on that reported in the Anaerobe Laboratory Manual (Holdeman & Moore, 1975). The column packing used was 15% SP 1220/1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Bellefonte, Pennsylvania). The separation of an ether extract of the volatile acid standard (C_1-C_7) and a chloroform extract of the methylated non-volatile acid standard are shown in Figs. 5.1 and 5.2. Operating conditions are given on the figures.

The isolates were identified according to characteristics detailed in Bergey's Manual of Determinative Bacteriology (1974) and the Anaerobe Laboratory Manual (Holdeman & Moore, 1975).

/RESULTS .....

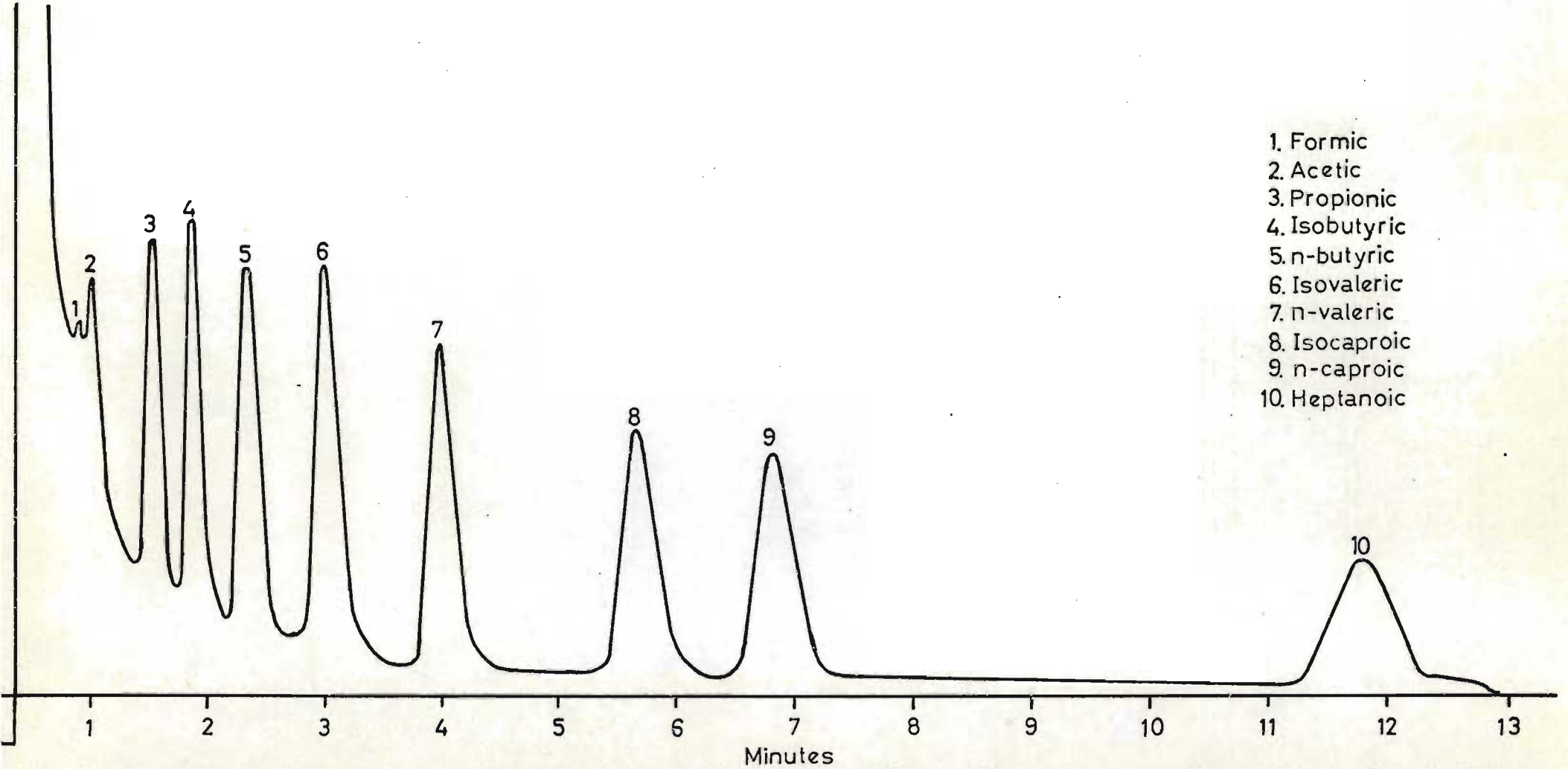


Fig.5.1. Ether extract of volatile acid standard containing 1mM of each acid (C_1-C_7). Operating conditions: Packing, 15% SP-1220/ 1% H_3PO_4 on 100/120 Chromosorb W AW; Column, 1800X 4mm ID, Glass; Temperature, Inlet 150°C, Column 135°C, Detector 150°C; Flow rate, 80 ml/ min, helium; Sample size, 15 µl; Detector, 200 mA, 1x attenuation; Instrument, Perkin Elmer Model 881.

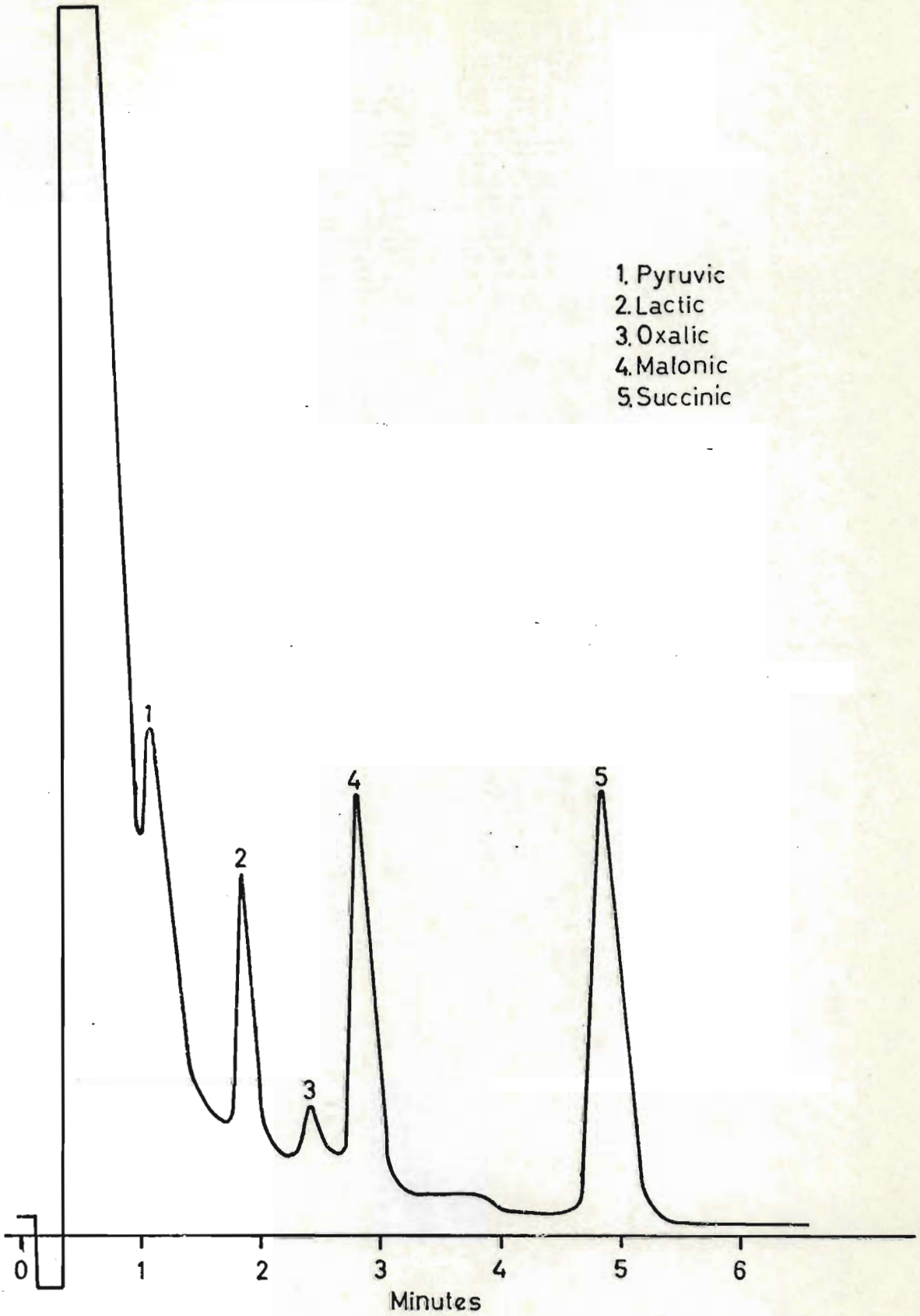


Fig.5.2. Chloroform extract of methylated non volatile acid standard. Operating conditions are identical to those given in Fig.5.1.

RESULTS

Apart from a few occasions involving sheep A57 and A72, all the ration was consumed within 1 h and often within ½ h. This eliminated selection of the fibrous portions of the diet and also variations in intake between experimental animals.

Biochemical changes in the rumen during adaptation

Biochemical measurements were made on the same samples used for the microbiological counts and were taken 2 h after the morning feed. The results are presented in Tables 5.5, 5.6, 5.7 and Fig. 5.3 for which values may be found in Appendix Table 5.4.

Ruminal pH

The mean ruminal pH decreased as the amount of grain and molasses in the diet increased (Table 5.5). The mean pH on Diet A, containing 10% molasses, was 6,71 and reached the lowest value of 5,75-5,80 after 7 days on the final high concentrate diet. The mean pH values were always lower 1 day after each change in diet during the adaptation than they were 7 days later.

Volatile fatty acids

The values for total VFA concentration are also presented in Table 5.5. The mean value was lowest on Diet A and increased as the amount of grain and molasses in the diet increased despite the decrease in ruminal pH. The values for sheep A72 were exceptionally high starting with Diet E containing 44% grain and molasses. The total VFA concentration of sheep A72 from this stage of the experiment and especially on the final high concentrate diet must be viewed with scepticism and has been omitted from the calculation of mean $\pm$ SD from day 7 to day 54 on the final high concentrate diet.

The general trend in the molar percent of acetic, propionic and butyric acids can best be seen in Fig. 5.3 which is based on values in Appendix Table 5.4. Little variation in the mean proportion of acetate (range: 62,5-64,5%) or propionate (range: 18,5-19,8%) occurred from the first sampling on Diet A through to that on the first day on Diet E. The ratio of acetate/propionate was 3,2-3,5. After 7 days on Diet E the proportion of acetate decreased and propionate increased with the ratio between the two falling to 2,6 and reaching its lowest value of 2,1 after 21 and 40 days on the final diet containing 71% grain and molasses. The mean molar percent of butyrate was usually 13,5-18,8% except on one occasion when the mean value was 20,8% after 21 days on the high concentrate diet. This corresponded with the lowest mean value of 48,1% for acetic acid and the lowest ratio (2,1) for acetate/propionate.

Table 5.5. Values for pH and total VFA concentration in the rumen fluid of sheep adapted stepwise to a High concentrate diet. The samples were taken 2 hours after the morning feed and were also used for the microbiological counts. The sample for A57 on Diet A was lost on workup. (NS = not sampled)

Diet	Days on diet	pH values A18	A57	A58	A72	Mean±SD	Total VFA concentration (mM) A18	A57	A58	A72	Mean±SD
A	17	6,70	6,80	6,65	6,70	6,71±0,06	103	-	100	74	92±15
(10% grain & molasses)											
AE	1	6,50	6,65	6,45	6,65	6,56±0,10	135	116	128	133	128± 7
(24% grain & molasses)	7	6,75	6,65	6,50	6,55	6,61±0,11	129	114	124	126	123± 6
E	1	6,20	6,30	6,15	6,10	6,19±0,08	146	114	125	216	150±45
(44% grain & molasses)	7	6,50	6,25	6,20	6,35	6,33±0,13	127	108	105	171	128±30
EB	1	6,10	5,95	5,95	6,10	6,02±0,08	144	140	132	202	156±32
(60% grain & molasses)	7	6,10	6,10	6,05	6,30	6,14±0,11	144	121	125	198	147±35
B	1	5,90	6,10	5,90	6,10	6,00±0,11	134	115	122	176	137±27
(71% grain & molasses)	7	5,80	5,95	5,70	5,55	5,78±0,16	150	122	131	430	134±14
	21	5,75	NS	NS	5,85	5,80	188	NS	NS	300	188
	40	5,70	NS	NS	5,80	5,75	158	NS	NS	272	158
	54	5,80	5,85	5,80	5,65	5,78±0,08	180	140	168	292	163±21

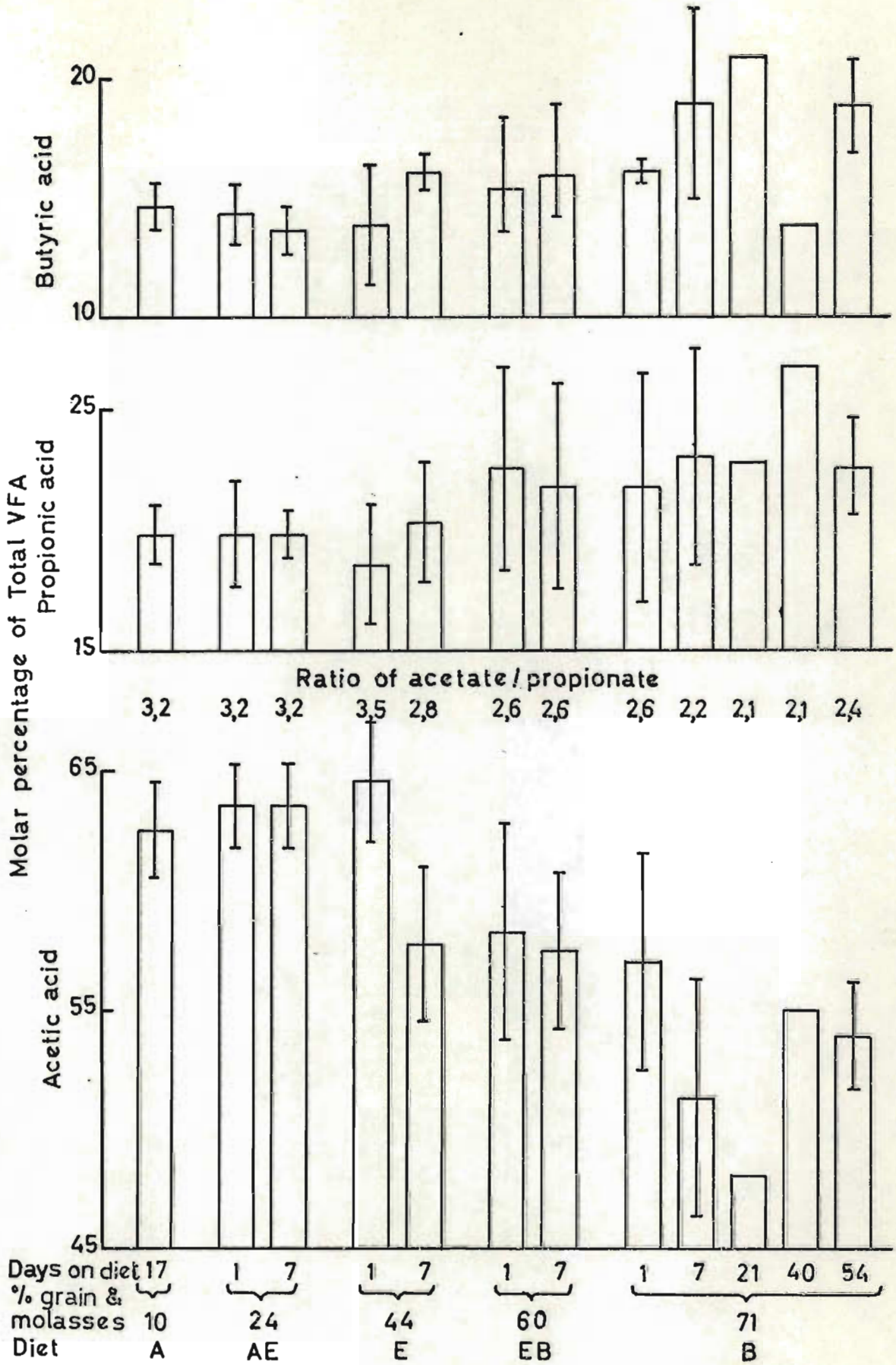


Fig.5.3. Changes during stewise adaptation to high concentrate diet in the mean molar percentage of acetic, propionic and butyric acids in the rumen fluid of sheep. The vertical bars represent the SD

The values for isobutyric, isovaleric and valeric acids (Appendix Table 5.4) showed a tendency to increase as the grain content of the diet increased and reached the highest values on the final high concentrate diet. The highest mean value for caproic acid was 1,0% on day 21 of the final diet.

Lactic acid

Table 5.6 gives the values for concentration of D- and L-lactate acid in the rumen fluid of the different sheep. It is unlikely that any of the values reported are peak values since it had been shown previously (Chapter 4) that the peaks occur ½-1 h after feeding. Nevertheless the values reported do show a trend, increasing until 7 days after feeding the 44% grain and molasses diet then decreasing again with some fluctuation on the 71% grain and molasses diet. The D-lactate value for sheep A72 after 54 days on the final diet is rather high and at variance with the figure for L-lactate and must be viewed with scepticism. The L-isomer tended to predominate up to day 1 on the 71% grain and molasses diet and thereafter the trend was reversed with the D-isomer predominating. The percentage D-lactate varied from 27-60%, excluding the unusually high value for D-lactate on day 54 on the final diet. If this value is included the upper value increases to 70%.

Ammonia

The values for NH_3-N concentration in the rumen fluid are given in Table 5.7. The mean NH_3-N concentrations were all above 10 mM except on the first day of feeding the 44% grain and molasses diet when the mean value was 6,2 mM and only 3,0 mM in sheep A58. However, after 7 days on this diet the values had all increased to a mean of 16,2 mM. This decrease in the ruminal NH_3-N concentration corresponds with the change in N source supplied in the diet (Diets A and AE contained 1,0 and 0,6% urea respectively; Diet AE also contained 2,2% of fishmeal whereas Diet E contained 5,5% fishmeal and no urea) when there may have been a lack of a proteolytic flora capable of breaking down the fishmeal at a sufficient rate to maintain ruminal NH_3-N concentration.

/Table 5.7. .....

Table 5.6. D- and L-lactic acid concentration in the rumen fluid of sheep adapted stepwise to a high concentrate diet. The samples were taken 2 hours after the morning feed and were also used for the microbiological counts. The sample for sheep A57 and Diet A was lost on workup. (NS = not sampled)

Diet	Days on diet	D-lactic acid concentration (mM)					L-lactic acid concentration (mM)				
		A18	A57	A58	A72	Mean±SD	A18	A57	A58	A72	Mean±SD
A	17	0,00	-	0,10	0,48	0,19	0,24	-	0,48	0,81	0,51±0,28
(10% grain & molasses)											
AE	1	0,17	0,30	0,24	0,78	0,37±0,27	0,23	0,60	0,49	0,93	0,56±0,28
(24% grain & molasses)	7	1,18	1,27	0,48	0,86	0,95±0,35	1,50	1,56	0,69	0,83	1,15±0,44
E	1	0,92	0,57	1,45	0,50	0,86±0,43	0,98	0,62	0,45	0,53	0,65±0,23
(44% grain & molasses)	7	1,13	2,11	1,07	1,71	1,51±0,49	1,21	1,98	1,54	1,54	1,57±0,31
EB	1	0,64	1,89	0,28	1,05	0,97±0,69	0,69	1,47	1,38	1,34	1,22±0,35
(60% grain & molasses)	7	0,52	1,86	0,32	0,42	0,78±0,72	0,51	0,55	0,62	0,68	0,59±0,07
B	1	0,37	0,44	0,38	0,29	0,37±0,06	0,41	0,68	0,56	0,41	0,52±0,13
(71% grain & molasses)	7	0,28	0,58	1,52	0,53	0,73±0,54	0,32	0,49	0,55	0,55	0,48±0,47
	21	0,54	NS	NS	1,45	1,00	0,51	NS	NS	1,27	0,89
	40	0,07	NS	NS	0,25	0,16	1,54	NS	NS	0,30	0,92
	54	1,27	1,68	0,28	8,78	3,00	1,45	1,43	2,15	0,00	1,26±0,89

Table 5.7. NH_3-N concentration in the rumen fluid of sheep adapted adapted stepwise to a high concentrate diet. The samples were taken 2 hours after the morning feed and were also used for the microbiological counts. The sample for sheep

Diet	Days on diet	NH_3-N concentration (mM)				
		A18	A57	A58	A72	Mean+SD
A (10% grain & molasses)	17	10,9	-	10,7	12,5	11,4±0,9
AE	1	11,9	13,8	13,4	12,2	12,8±0,9
(24% grain & molasses)	7	16,6	15,9	17,9	13,6	16,0±1,8
E	1	8,8	6,6	3,0	6,2	6,2±2,3
(44% grain & molasses)	7	19,4	15,3	14,6	15,4	16,2±2,1
EB	1	12,3	12,9	8,9	13,3	11,9±2,0
(60% grain & molasses	7	13,7	17,0	13,3	20,1	16,0±3,1
B	1	15,1	18,9	13,1	23,9	17,8±4,7
(71% grain & molasses)	7	11,0	28,1	12,1	7,1	14,6±9,2
	21	15,2	NS	NS	24,1	19,7
	40	15,9	NS	NS	22,6	19,3
	54	22,5	18,5	16,9	27,6	21,4±4,7

Microbiological changes in the rumen during adaptation

The counts of all groups of bacteria tended to be higher 24 h after a change in diet, when altogether 1 800 g of food was given, than they were 7 days later (Table 5.8 and Fig. 5.4). Thus the trend of change in bacterial numbers in each group can be seen most easily by following the count after 7 days on each successive diet.

Total culturable bacteria and ciliate protozoa

The number of total culturable bacteria generally increased only after the change to the diet containing 60% grain and molasses, and reached a peak when the sheep had been on the final diet for 7 days. In contrast, the number of ciliate protozoa changed little in the 24 h following a change in diet but showed an increase after 7 days on each diet. The increase in protozoal numbers appeared to be roughly proportional to the increase in the grain content of the diet. The number of protozoa reached a peak either after 7 days on the diet containing 60% grain and molasses or after 1 day on the final 71% grain and molasses diet. As a result of these changes in population numbers, the ratio of total culturable bacteria to ciliate protozoa fell from $11\text{-}20\times10^3$ at the beginning of the experiment to as low as 2×10^3 when the protozoa reached their peak.

Table 5.8. Numbers of total culturable, amylolytic and lactate-utilizing bacteria ($x10^{-7}$/g of ingesta) and of ciliate propozoa ($x10^{-5}$/g of ingesta) obtained 2 h after feeding from the rumen of sheep A18, A57, A58 and A72 changed stepwise from low to high maize concentrate diets

Diets		A	AE		E		EB		B				
Grain & molasses (%)		10	24		44		60		71				
Days on diet		17	1	7	1	7	1	7	1	7	21	40	54
Total culturable bacteria	A18	280	250	210	340	270	650	1 800	2 700	2 900	350	1 300	1 760
	A57	360	250	150	370	350	280	400	460	1 700	NS	NS	850
	A58	240	370	270	110	250	410	290	560	3 400	NS	NS	260
	A72	170	310	190	350	420	240	240	240	3 300	70	370	530
Amylolytic bacteria	A18	4	8	8	53	20	20	93	268	280	80	400	426
	A57	1	7	2	32	48	48	27	65	130	NS	NS	253
	A58	4	20	10	40	4	160	53	268	27	NS	NS	16
	A72	8	52	20	44	9	9	27	26	932	9	29	27
Lactate-utilizing bacteria	A18	0,6	2,3	0,9	1,1	5,1	4,4	6,1	16	280	80	31	650
	A57	0,3	1,1	1,0	1,2	2,7	6,3	2,6	4,7	37	NS	NS	50
	A58	0,4	1,3	0,7	3,9	0,9	8,4	15	31	22	NS	NS	50
	A72	0,6	1,3	1,1	1,9	0,9	2,7	2,4	7,6	110	4,0	3,6	8
Ciliate protozoa	A18	1,4	1,9	5,0	5,8	13,4	12,2	16,6	17,0	9,1	8,9	9,1	10,8
	A57	2,6	2,2	4,1	4,1	6,2	9,8	14,9	7,9	10,6	NS	NS	3,8
	A58	2,2	3,6	3,8	2,2	5,0	4,1	7,2	5,5	6,7	NS	NS	4,1
	A72	1,4	5,5	4,8	3,1	4,8	6,2	8,4	10,8	1,7	3,8	18,5	7,0
Ratio of: $\left[\frac{\text{Total culturable}}{\text{Ciliate protozoa}}\right] x10^{-3}$	A18	20	13	4	6	2	5	11	16	93	4	14	16
	A57	14	11	4	9	6	3	3	3	26	NS	NS	22
	A58	11	10	7	5	5	10	4	10	51	NS	NS	6
	A72	12	6	4	11	9	4	3	2	194	2	2	8
$\left[\frac{\text{Amylolytics}}{\text{Lactate-utilizers}}\right]$	A18	6,7	3,5	8,9	48,1	3,9	4,5	15,2	16,8	1,0	1,0	12,9	0,7
	A57	3,3	16,4	2,0	26,7	17,8	7,6	10,4	13,8	3,5	NS	NS	5,1
	A58	10,0	15,4	14,3	10,3	4,4	19,0	3,5	8,6	1,2	NS	NS	0,3
	A72	13,3	40,0	18,1	23,2	10,0	3,3	11,3	3,4	8,5	2,3	8,1	3,4

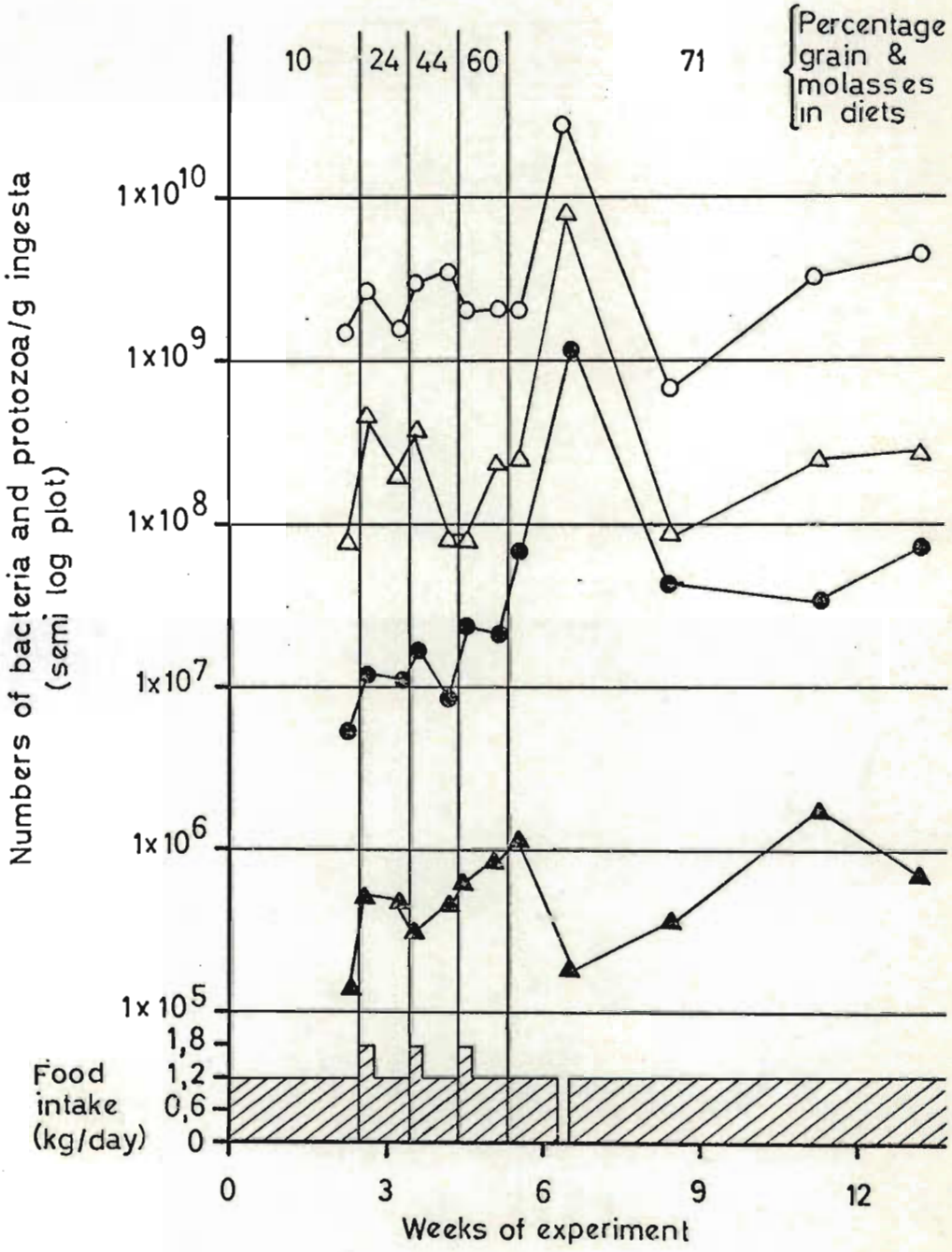


Fig.5.4.Numbers of ciliate protozoa (▲) and of lactate utilizing (●), amylolytic (△) and total culturable (○) bacteria in the rumen and food intake (▨) of Sheep A72 changed stepwise from a low to a high maize concentrate diet

Within a week after the final change in diet the number of protozoa fell in all 4 sheep and the number of bacteria increased to a peak, the ratio of total culturable bacteria to protozoa rising steeply to 26-194x10^3. One sheep, A72, refused all food on the fifth day after the change in diet. At this stage the ruminal pH was 5,60 at 10h00 and the number of protozoa had fallen from 10,8 to 3,6x10^5/g of ingesta, with even lower counts 2 days later.

From this stage onwards only A18 and A72 were examined regularly until the end of the experiment. After they had been on the final diet for 21 days the number of total culturable bacteria had fallen while the protozoa had started to increase and the ratio of total culturable bacteria and ciliate protozoa decreased to 2-4x10^3. After 40 days on the final diet the numbers of both protozoa and bacteria had increased, and after 54 days on the diet the number of bacteria had increased relative to the protozoa and their ratio had increased to 8-22x10^3 indicating that conditions in the rumen again tended towards instability.

Amylolytic and lactate-utilizing bacteria

As expected, the number of amylolytic bacteria increased with each increase in grain content of the diet from 1-8x10^7 on the initial diet to 27-932x10^7 per g of ingesta at peak on the final diet. The number of lactate-utilizing bacteria, initially 0,3-0,6x10^7 per g of ingesta, followed those of the amylolytic bacteria which produced their growth substrate, lactic acid. The numbers of these two groups tended to be higher 1 day after the change in diet when the animals had received 1 800 g of food and thus when an increase in lactic acid production could be expected. Moreover, a surge in the number of amylolytic bacteria was invariably followed by one in the number of lactate-utilizing bacteria. In one instance (sheep A18) after 40 days on the final diet, the number of amylolytic bacteria increased to 400x10^7 per g of ingesta while that of the lactate-utilizers was 31x10^7, but after 54 days the latter had risen to 650x10^7. This tendency for these groups to balance each other can be seen from the values for the ratio of the number of amylolytic to lactate-utilizing bacteria. The effect was most marked during and after the decrease in number of protozoa when values as low as 1,0 were obtained.

Variations in numbers of organisms in stable populations

The variations in the numbers of bacteria and protozoa per g of ingesta associated with a change in diet during the adaptation experiment were greater than those found for a stable population of bacteria and ciliate protozoa from the rumen of sheep fed lucerne hay (Table 5.9).

/Table 5.9. .....

Table 5.9. Variations in numbers/g of ingesta for stable populations of bacteria and ciliate protozoa from the rumen of sheep fed lucerne hay

Micro-organisms	No of sheep	No of observations	Mean±SD	CV
Total culturable bacteria $x10^{-9}$	4	12	3,6±1,6	45%
Lactate-utilizing bacteria $x10^{-7}$	3	9	1,6±0,7	44%
Ciliate protozoa $x10^{-5}$	3	9	1,9±0,4	24%

Increases and decreases in number over the entire course of the experiment were >1-19 fold for total culturable bacteria, >1-253 fold for amylolytic bacteria, >1-1 083 fold for lactate-utilizing bacteria and >1-13 fold for ciliate protozoa. By contrast, on a lucerne hay diet with a stable microbial population the fluctuations in number for repeated samplings over a 6 month period were <1 for total culturable and lactate-utilizing bacteria and 0,5 fold for ciliate protozoa.

On the final diet containing 71% grain and molasses the amylolytic bacteria constituted a mean of 11,5-19,3% of the total bacterial count (Appendix Table 5.5) whereas the lactate-utilizing bacteria constituted a mean of 14,3 and 15,9% on days 21 and 54 respectively, but only 2,7 and 3,8 on days 1 and 7 respectively. The lactate-utilizing bacteria constituted 0,1-5,2% of the total count before the change to the final diet and the amylolytic bacteria 0,3-34,1%. There was a big difference between individual sheep with respect to both amylolytic and lactate-utilizing bacteria.

The low counts of these two groups of bacteria relative to the total culturable count can be explained as follows. The amylolytic bacteria were counted after 20 h of incubation compared to 7 days for the total culturable bacteria. The bottle count of amylolytic bacteria increased 4-fold by increasing incubation time from 1 to 5 days (Table 5.4) and hence the amylolytic flora as a proportion of the total are underestimated by the short incubation. Although counts of lactate-utilizing and total culturable bacteria were both made after 7 days of incubation, the lactate medium contained no rumen fluid in contrast to the total medium which contained 40% rumen fluid. This would also tend to underestimate the contribution of the lactate-utilizing bacteria to the total bacterial flora. This aspect was discussed in Chapter 3 (Table 3.2).

/Identification of .....

Identification of the predominant types of amylolytic and lactate-utilizing bacteria

The results of the carbohydrate fermentation tests (Appendix Tables 5.9 and 5.11) and biochemical tests (Appendix Tables 5.10 and 5.12) of the amylolytic and lactate-utilizing isolates selected from the different morphological groupings are summarized in Tables 5.10 and 5.11 respectively. The characteristics listed were found to be the most useful for differentiating between the isolates. The results show that all of the amylolytic isolates characterized were producers of lactic acid. In the case of the lactobacilli, eubacteria and streptococci, lactic acid is a major end product of the fermentation of glucose. The butyrivibrios are also major producers of lactic acid. The bacteriodes, however, produce small amounts of lactic acid and mainly acetic acid from glucose fermentation. *Selenomona ruminantium*, depending on strain, is able to produce lactate from glucose and propionate. The veillonellas and *Anaerovibrio* are unable to ferment glucose in contrast to the other lactate-utilizing isolates.

On the basis of the identification of the isolates selected from the different morphological groupings of amylolytic and lactate-utilizing bacteria it was possible to determine the changes in average proportions of the predominant types of bacteria in the rumen during successive periods on diets containing increasing amounts of maize grain (see Table 5.12). *Bacteroides* predominated throughout the whole of the adaptation but was superseded by *Lactobacillus* and *Eubacterim* on the final diet containing 71% grain and molasses. *Butyrivibrio* occurred among the predominant amylolytic bacteria throughout the study. *Anaerovibrio* were the predominant lactate-utilizing bacteria while *Propionibacter* were present throughout the experiment. *Megasphaera* occurred sporadically. *Veillonella* and *Selenomonas* did not appear among the predominant lactate-utilizers after the 24 and 44% grain and molasses diets respectively.

Changes in species in relation to the shifts in ruminal pH

When the results of the changes in the average proportions of the predominant types of bacteria in the rumen during the experiment were plotted in the form of a histogram (Fig. 5.5) it was apparent that there was an orderly change in species type over the course of the experiment. This was related to the gradual fall in ruminal pH. On searching the available literature it was found that there was a dearth of information on the pH optimum for growth of the predominant ruminal bacteria. However, from the limited data available (references detailed below) it could be seen that acid-sensitive species predominated on the initial high roughage diet. The proportions of these decreased as the ruminal pH fell with the increase in grain content of the diet, while the proportions of acid tolerant bacteria increased. Thus in the case of the amylolytic bacteria,

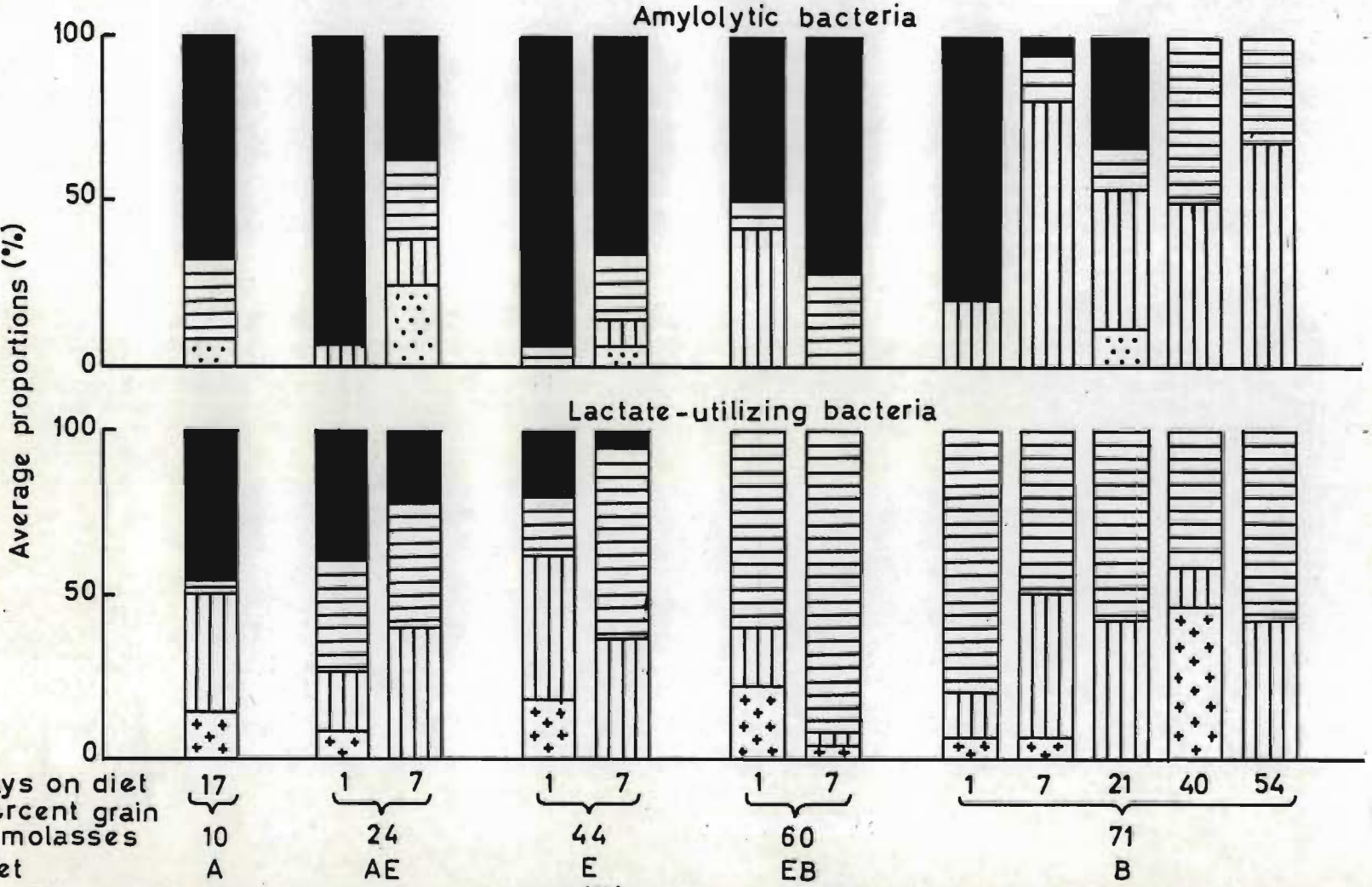


Fig.5.5. Changes in the average proportions (%) of the predominant types of bacteria in the rumen of sheep during successive periods on diets containing increasing amounts of grain & molasses
Amylolytic bacteria: Bacteroides (■), Butyrivibrio (▤), Lactobacillus+Eubacterium (▥), Selenomonas+Streptococcus (⚄)
Lactate-utilizing bacteria: Veillonella+Selenomonas (■), Anaerovibrio (▤), Propionibacter (▥), Megasphaera (⚄)

Table 5.10 Characteristics used to identify the isolates of amylolytic bacteria

Isolate	Morphology and size	Gram reaction	Φ End products on glucose	Φ Other characteristics
A10 *Bacteroides*	Rods with round ends. Some very short. 0,8-1,2 by 1,5-5 *um*	-	A p ib b iv 1 S	-
A32 *Selenomonas*	Rods, curved to crescent shaped. Some chains 0,7-1,0 by 3-6 *um*	-	A P L s	NO_3 redn + Catalase + Mannitol +
A62 *Streptococcus*	Cocci, in pairs and chains. 0,711,0 *um* in diam.	+	f a L	-
A65 *Bacteroides*	Rods with round ends. Some very short 0,7-1,0 by 1,5-5 *um*	-	A p ib b iv	-
A75 *Butyrivibrio*	Rods, curved. Singly, pairs and chains 0,3-0,6 by 1,5-4 *um*	-	f A p B L s	NO_3 redn - Catalase - Mannitol -
A88 *Lactobacillus*	Rods, straight. Singly and in chains 0,6-0,9 by 2-6 *um*	+	f a L s	Galactose + Melibiose + Raffinose +
A101 *Lactobacillus*	Rods, straight. Singly and in chains 0,6-0,9 by 2-6 *um*	+	f a L s	Galactose + Melibiose + Raffinose +
A132 *Butyrivibrio*	Rods, curved. Singly, pairs and chains 0,3-0,6 by 1,5-4 *um*	-	f A p B L s	NO_3 redn - Catalase - Mannitol -
A152 *Eubacterium*	Rods, straight. Singly and in short chains 0,7-0,8 by 2-4 *um*	+	F a p L	Galactose - Melibiose - Raffinose -
A155 *Bacteroides*	Rods with round ends. Some very short. 0,8-1,2 by 1,5-5 *um*	-	A p ib b iv S 1	-
A156 *Lactobacillus*	Rods, straight. Singly and in chains 0,6-0,9 by 2-6 *um*	+	f a L s	Galactose + Melibiose + Raffinose +

Φ Explanation of symbols: + = positive reaction, - = negative reaction. End products: upper case letters represent >1 mM, lower case < 1 mM where A = acetic, P = propionic, IB = isobutyric, B = butyric, IV = isovaletic, V = valeric, IC = isocaproic, C = caproic, L = lactic and S = succinic acids

Table 5.11. Characteristics used to identify the isolates of lactate-utilizing bacteria

Isolate	Morphology and size	Gram reaction	End products on Glucose	End products on Lactate	Other characteristics
L7 *Selenomonas*	Rods, curved to crescent shaped. Some chains 0,7-1,0 by 3-6 *um*	-	A P L s	A P s	Glucose + Lipase - Flagella +
L23 *Veillonella*	Cocci, in pairs, short chains and masses 0,4-0,6 *um* in diam	-	-	A P s	NO_3 redn + Glucose - Catalase +
L30 *Selenomonas*	Rods, curved to crescent shaped. Some chains 0,7-1,0 by 3-6 *um*	-	A P L s	A P s	Glucose + Lipase - Flagella +
L77 *Anaerovibrio*	Rods, curved to "S" shaped. Singly 0,4-0,6 by 2-4 *um*	-	-	A P s	Glucose - Lipase + Glycerol +
L80 *Megasphaera*	Cocci, in pairs and chains of pairs (4-8) 1,5-2,0 *um* in diam	Var	A p ib B iv v ic C s	A P	NO_3 redn - Glucose + Catalase -
L88 *Anaerovibrio*	Rods, curved to "S" shaped. Singly 0,4-0,6 by 2-4 *um*	-	-	A P s	Glucose - Lipase + Glycerol +
L121 *Megasphaera*	Cocci, in pairs and chains of pairs (4-8) 1,5-2,0 *um* in diam	Var	A p ib B iv v ic C s	A P	NO_3 redn - Glucose + Catalase -
L143 *Propionibacter*	Rods, pleiomorphic with coccoid cells 0,3-0,5 by 1-4 *um*	+	A P b l s	A P s	Glucose + Lipase - Flagella -
L146 *Selenomonas*	Rods, curved to crescent shaped. Some chains 0,7-1,0 by 3-6 *um*	-	A P L s	A P s	Glucose + Lipase - Flagella +
L150 *Propionibacter*	Rods, pleiomorphic with coccoid cells 0,3-0,5 by 1-4 *um*	+	A P b l s	A P s	Glucose + Lipase - Flagella -
L151 *Anaerovibrio*	Rods, curved to "S" shaped. Singly 0,4-0,6 by 2-4 *um*	-	-	A P s	Glucose - Lipase + Glycerol +
L166 *Anaerovibrio*	Rods, curved to "S" shaped. Singly 0,4-0,6 by 2-4 *um*	-	-	A P s	Glucose - Lipase + Glycerol +
L172 *Propionibacter*	Rods, pleiomorphic with coccoid cells 0,3-0,5 by 1-4 *um*	+	A P b l s	A P s	Glucose + Lipase - Flagella -
L176 *Propionibacter*	Rods, pleiomorphic with coccoid cells 0,3-0,5 by 1-4 *um*	+	A P b l s	A P s	Glucose + Lipase - Flagella -

Table 5.12. Changes in the average proportions (%) of the predominant types of amylolytic and lactate-utilizing bacteria in the rumen of sheep during successive periods on diets containing increasing amounts of maize grain

Diet	A	AE		E		EB		B				
% Grain & molasses	10	24		44		60		71				
Days on diet	17	1	7	1	7	1	7	1	7	21	40	54
Types of amylolytic bacteria:												
Selenomonas	-	-	25	-	-	-	-	-	-	-	-	-
Bacteroides	67	94	38	94	67	50	71	79	6	33	-	-
Butyrivibrio	25	-	23	6	19	8	29	-	13	13	50	31
Eubacterium	-	6	15	-	8	-	-	8	13	17	13	13
Lactobacillus	-	-	-	-	-	42	-	13	69	25	38	56
Streptococcus	8	-	-	-	6	-	-	-	-	13	-	-
Types of lactate-untilizing bacteria:												
Veillonella	22	11	-	-	-	-	-	-	-	-	-	-
Selenomonas	36	28	23	19	6	-	-	-	-	-	-	-
Megasphaera	13	8	-	18	-	22	4	6	6	-	46	-
Anaerovibrio	4	36	38	18	58	59	92	81	50	58	42	58
Propionibacter	25	17	40	46	36	19	4	13	44	42	13	42

Bacteroides with an initial pH optimum for growth of 7,8-6,8 (Hamlin & Hungate, 1956) clearly predominated until one day after the final change in diet. At this stage the ruminal pH of all animals examined fell below 6,0 for several hours each day, and the *Bacteroides* were superseded by *Lactobacillus* and *Eubacterium* with lower pH optima (6,0-5,5; Rogosa, 1974; Holdeman & Moore, 1974). *Butyrivibrio* were usually present (pH optimum *ca* 6,5; Bryant, 1974) while *Streptococcus bovis* (pH optimum 5,0; Sims, 1964) occurred sporadically throughout the experiment. Among the lactate-utilizers *Veillonella* (pH optimum 8,5-7,0; Rogosa, 1964) and *Selenomonas* pH optimum 6,7; Kingsley & Hoeniger, 1973) which contributed largely to the early counts failed to appear among the predominating bacteria; *Veillonella* within 7 days on the diet containing 24% grain and molasses and *Selenomonas* by the time 60% grain and molasses was given. *Megasphaera* appeared intermittently throughout the experiment (pH optimum 7,4- 5,0; Rogosa, 1971). *Anaerovibrio* (pH optimum *ca* 7,0- 5,8; Prins *et al*, 1975) and *Propionibacter* (pH optimum 7,0-6,5; Moore & Holdeman, 1974) occurred throughout the experiment mostly in large proportions. The proportions of *Anaerovibrio* reached a peak (92%) after 7 days on the diet containing 60% grain and molasses while *Propionibacter* accounted for 40-50% of the lactate-utilizing bacteria on 5 of 12 occasions.

Although pH values at sampling (Table 5.5) were available for the microbiology group of sheep, and these also showed a decrease as the grain content of the diet increased, it was considered desirable to evaluate the length of time the pH remained below a certain critical level (say pH 6,00). As this factor appeared to have the most marked effect on growth rate and hence on the predominating ruminal bacteria a means was sought for expressing this value. For this purpose a value designated "pH_6-hours" was developed which was calculated as that area of the pH-time curve which was below pH 6,00. This is illustrated in Fig. 5.6. The area under the curve was calcu-

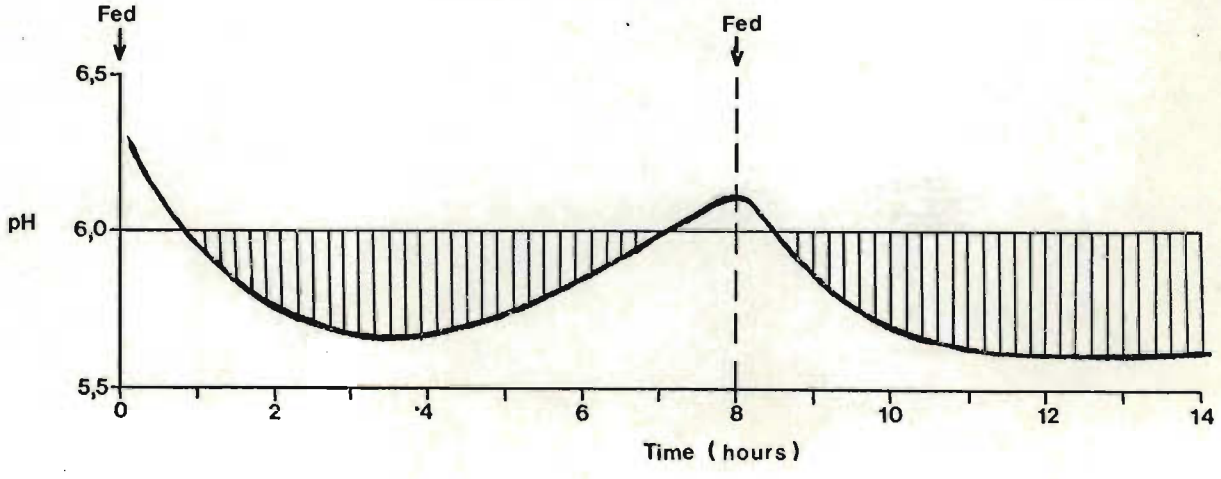


Fig. 5.6. Calculation of "pH_6-hours" by counting the squares in the shaded portion of the

lated by counting the squares although a computer programme for the integration of the data could be used if there are a large number of calculations.

Values for the length of time below pH 6,00 expressed as "pH_6-hours" are given in Table 5.13 for the four sheep in the group used for detailed biochemical analyses starting with Diet E and also for four sheep in the microbiology group starting with Diet B. If sampling had been continued for the full 24 h period these values would obviously have been higher and more accurate since pH values tended to reach a minimum 4-6 h after the second feed and remain low for the following 3-4 h.

Table 5.13. Values for the length of time below pH 6,00 over the 14 h sampling period expressed in units of "pH_6-hours"

Diet	Days on Diet	Biochemical group A19	A21	A28	A31	Microbiology group A18	A72	A57	A58	Mean
E	7	0	0	0	0,31	-	-	-	-	0,08
EB	1	1,99	0,77	1,13	2,93	-	-	-	-	1,71
	7	0	0,02	0,03	0,79	-	-	-	-	0,21
B	1	3,07	0,67	0,78	1,75	0,96	0,62	0,85	1,58	1,29
	7	1,19	0,44	1,84	0,76	-	-	-	-	1,06
	21	2,50	-	2,43	2,18	0,78	1,02	1,62	0,91	1,63
	40	5,28	-	-	5,00	0,35	1,73	4,46	3,74	3,43
	54	3,09	-	-	4,68	1,27	3,48	2,29	3,65	3,08

Correlation coefficients were then calculated for proportions or numbers of amylolytic and lactate-utilizing bacteria and the corresponding pH values at sampling or "pH_6-hours". The results are presented in Table 5.14. Correlation coefficients were calculated for *Bacteroides*, *Anaerovibrio*, *Propionibacter* and for *Lactobacillus* + *Eubacterium* since both species were acid tolerant and behaved similarly and also for lactate-utilizing *Veillonella* + *Selenomonas* since both species were acid sensitive. Despite the shortcomings of the data some significant correlations were obtained. The most consistent correlations were obtained with the acid sensitive *Veillonella* + *Selenomonas* and the acid tolerant *Lactobacillus* + *Eubacterium*. In general the best correlations were obtained between the mean values for "pH_6-hours" and the percentage proportions especially when values over all diets were used. Mean values were better than individual values since the

/individual .....

Table 5.14. Correlation coefficients obtained for pH at sampling or "pH_6-hours" and the numbers ($x10^7$/g ingesta) or proportions (%) of *Bacteroides*, *Lactobacillus* + *Eubacterium*, *Anaerovibrio*, *Propionibacter* and *Veillonella* + *Selenomonas*.
Statistical significance: $^{\Phi\Phi}$= P > 0,99, $^{\Phi}$= P > 0,95, NS = not significant

Correlation between	*Bacteroides*	*Lactobacillus* + *Eubacterium*	*Anaerovibrio*	*Propionibacter*	*Veilonella* + *Selenomonas*
	Individual values for microbiology group on Diet B only				
pH at sampling vs numbers	0,34(NS)	-0,03(NS)	-0,06(NS)	-0,29(NS)	-
pH at sampling vs proportions	0,67(Φ)	-0,34(NS)	0,64(Φ)	-0,43(NS)	-
pH_6-hours vs numbers	0,41(NS)	0,08(NS)	0,35(NS)	0,18(NS)	-
pH_6-hours vs proportions	-0,50(NS)	0,39(NS)	-0,40(NS)	0,64(Φ)	-
	Individual values for microbiology group from Diet A to B				
pH at sampling vs proportions	0,39(Φ)	-0,57(ΦΦ)	-0,39(Φ)	-0,03(NS)	0,64(ΦΦ)
	Mean values on Diet B only				
pH_6-hours vs numbers	0,71(NS)	0,98(Φ)	0,59(NS)	0,99(ΦΦ)	-
pH_6-hours vs proportions	0,89(NS)	0,89(NS)	-0,69(NS)	0,79(NS)	-
	Mean values from Diet A to B				
pH at sampling vs proportions	-0,29(NS)	-0,78(ΦΦ)	-0,49(NS)	-0,08(NS)	0,87(ΦΦ)
pH at sampling vs log proportions	0,65(Φ)	-0,67(Φ)	-0,58(Φ)	-	0,90(ΦΦ)
pH_6-hours vs proportions	-0,76(Φ)	0,63(NS)	-0,57(NS)	0,08(NS)	-
pH_6-hours vs log proportions	-0,84(ΦΦ)	0,69(NS)	-0,59(NS)	0,20(NS)	-

individual variation between the animals lowered the correlation. The correlation coefficients on Diet B alone were good even when using numbers, since the large increase in numbers which occurred with the increase in grain content of the diet was not so marked on the final diet and thus an increase in proportion would also mean an increase in number.

CONCLUSIONS

The main findings of the experiment may be summarized as follows:

1. A stepwise adaptation was carried out with sheep through a series of diets containing 10, 24, 44, 60 to a final diet containing 71% ground maize grain and molasses. Biochemical measurements were made on the samples used for microbiological counts and taken 2 h after feeding.
2. Mean ruminal pH decreased and total VFA increased as the amount of grain & molasses in the diet increased. The lowest value (2,1) for the ratio of acetate/propionate was reached after 21 and 40 days on the final diet. The values for D- and L-lactic acid, although not peak concentrations, increased until the 44% grain & molasses diet was fed, decreased on the 60% grain & molasses diet and then began increasing again after 40 days on the final diet. The mean values for NH_3-N concentration in the rumen fluid were all above 10 mM except on the first day of feeding the 44% grain & molasses diet when the mean value was 6,2 mM, and in the case of sheep A58 only 3,0 mM.
3. The number of total culturable bacteria generally increased only after the change to the diet containing 60% grain & molasses and reached a peak ($2\ 825 \times 10^7$/g of ingesta) when the sheep had been on the final diet for 7 days. In contrast, the number of ciliate protozoa changed little in the 24 h following a change in diet but showed an increase after 7 days on the diet which was roughly proportional to the increase in the grain content of the diet. The number of protozoa reached a peak either after 7 days on the diet containing 60% grain & molasses or after 1 day on the final 71% grain & molasses diet. After the sheep had been on the final diet for 21 days the number of total culturable bacteria had decreased while the protozoa had started to increase.
4. The number of amylolytic bacteria increased with each increase in grain content of the diet from $1\text{-}8 \times 10^7$ on the initial high roughage diet to $27\text{-}932 \times 10^7$/g of ingesta **at** peak on the final diet. The

number of lactate-utilizing bacteria followed those of the amylolytic bacteria which produced their growth substrate, lactic acid. This tendency for these groups to balance each other can be seen from the values for the ratio of amylolytic to lactate-utilizing bacteria.

5. On the basis of the identification of isolates selected from the different morphological groupings of amylolytic and lactate-utilizing bacteria, the changes in the average proportions of the predominant types of bacteria present in the rumen during the experiment were determined. Among the amylolytic bacteria, *Bacteriodes* tended to predominate throughout the adaptation but was superseded by *Lactobacillus* and *Eubacterium* on the final diet containing 71% grain & molasses. *Butyrivibrio* were present among the predominant amylolytic bacteria throughout the experiment. *Anaerovibrio* were found to be the predominant lactate-utilizers although *Propionibacter* were also present throughout the study. *Veillonella* and *Selenomonas* had disappeared from the predominant lactate-utilizing bacteria by the time the 24% and 44% grain and molasses diets had been fed respectively.

6. The changes in species were related to the gradual fall in ruminal pH. From the limited data available in the literature it could be seen that there was an orderly shift from acid sensitive species which predominated on the initial high roughage diet to acid tolerant species which predominated as the grain content of the diet increased and the ruminal pH decreased. A value was calculated which took into account the length of time that pH stayed below a certain critical value (chosen as pH 6,00) since this would have the most effect on growth rate and hence on the proportions of predominating ruminal bacteria. This value was given the unit of "pH_6-hours". Correlation coefficients were calculated for the proportion or number of the different amylolytic and lactate-utilizing bacteria and the corresponding values for pH at sampling or "pH_6-hours". The best correlations were obtained between the mean values for "pH_6-hours" and the percentage proportions for *Lactobacillus* + *Eubacterium* which were acid-tolerant and *Veillonella* + *Selenomonas* which were acid sensitive.

CHAPTER 6. EFFECT OF STEPWISE ADAPTATION AT *AD LIBITUM* INTAKE ON LACTIC ACID CONCENTRATION IN THE RUMEN

INTRODUCTION

This experiment was planned to supplement information obtained during the Stepwise Adaptation Experiment. While the first experiment was carried out at restricted intake (i.e. 2x600 g per day) and was terminated after 54 days on the final diet, the present experiment was designed to be more in line with intensive feeding practice where the food is offered *ad libitum* for 100-150 days. Since the balance of the ruminal flora in the earlier experiment was unstable during the first week after the change to and again after 54 days on the final diet, the present experiment was continued for 120 days on the final diet in order to ascertain whether a balance could be maintained without excessive accumulation of lactic acid.

Whereas in the first experiment the protein content of the diet increased from 8,3 to 12,3% as the adaptation progressed, it was decided in this experiment to use a uniform but higher level of protein in the diets, namely 15%. Although analyses of samples taken 2 h after the morning feed at each stage during the first experiment had given mean NH_3-N values above 6 mM, (the lowest was 3,0 mM in Sheep A58 on the first day after the change to Diet AE) more detailed analyses on the ingesta from sheep receiving Diets E to B revealed NH_3-N levels were at a maximum *ca* 2 h after feeding and that lowest values occurred at subsequent sampling times, minimum values of 3,4 mM being obtained on several occasions (Mackie *et al*, in press). Growth limiting concentrations of 3,4-5,0 mM NH_3-N have been reported for the ruminal bacteria (Satter & Slyter, 1974; Mercer & Annison, 1976; Okorie, Buttery & Lewis, 1977). Thus at certain times during the feeding cycle marginal concentrations of NH_3-N existed in the rumen during the first experiment. Furthermore, Preston (1972) has shown that the CP requirements of sheep and cattle increase as the energy concentration or proportion of concentrate in the diet increases and for rapidly growing ruminants is as high as 13,9% CP. It was envisaged that increasing the level of CP to 15% would completely remove the possibility of NH_3-N limitation.

The earlier experiment showed clearly the influence of low ruminal pH over prolonged periods of time on changes in predominating species of ruminal bacteria, particularly the lactate-utilizers. Thus a mixed buffer

(McManus, Bigham & Edwards, 1972) which would be more effective in resisting rapid changes in ruminal pH, and also operate over a wider pH range than single or dual component mixtures, was used in the present experiment.

No microbiology was included in this study which concentrated rather on the biochemical aspects. Food intake, weight gain, ruminal pH and D- and L-lactic acid were determined by the writer while NH_3-N was determined on the same samples by Schwartz & Hannah (Internal Progress Report, October, 1975).

METHODS

Animals and management

Six sheep were used in the experiment. Four of the sheep (A27, A35, K1 and K29) had been on a lucerne hay diet for at least 6 months before the experiment started. Sheep A85 had been on lucerne hay-teff hay (50:50) for 6 months. These 5 sheep followed the same experimental plan and diets which are given in Table 6.1. Sheep A52 was on Diet B (see Chapter 5) for 2 months before introduction to the experiment and followed a different experimental plan and diets as detailed in Table 6.2. Four of the sheep had previously been used in the Exploratory Experiment (Chapter 4).

Sampling and analysis of ruminal fluid

Sampling was performed in the same way as in the Exploratory Experiment (Chapter 4) with the exception that, when the ruminal fluid became viscous as the adaptation progressed, it was not possible to pipette accurately and samples were weighed into the 0,5 N-HCl. Ruminal pH was measured on SRF using a Polymetron portable meter. D- and L-lactate analyses were performed as reported previously (Method 1, Chapter 2) and total lactate calculated as the sum of D- and L-lactate. NH_3-N was determined by steam distillation using the method of Schwartz, Schoeman & Färber (1964).

RESULTS

Food intake and weight gain

The food intake and weight gain of the six sheep during successive periods on diets containing increasing amounts of maize grain are given in Table 6.3. Food intake and weight gain was higher for Sheep A85 and K1 than the other three sheep (A27, A35 and K29) on the same diets. Food intake and weight gain were lower for A52 than the other five sheep.

Table 6.1. Experimental plan and diets during adaptation of Sheep A27, A35, A85, K1 and K29 to a high grain-high protein diet

Period Date	– 15/10/74– 29/10/74	1 30/10/74– 5/11/74	2 6/11/74– 12/11/74	3 13/11/74– 19/11/74	4 20/11/74– 26/11/74	5 27/11/74– 25/3/75
Amount of food	1 230 g	*Ad lib*	*Ad lib*	*Ad lib*	*Ad lib*	*Ad lib*
Diet	Lucerne	HR	HR/Int	Int	Int/HC	HC
Composition (kg/100 kg)						
Lucerne	1 200 g	–	–	–	–	–
Maize stalks	–	67,0	51,0	35,0	24,5	14,0
Maize grain	–	–	16,5	33,5	44,0	55,0
Molasses	–	10,0	10,0	10,0	10,0	10,0
Fishmeal	–	17,0	16,5	16,0	15,5	15,0
NaCl	15 g	0,5	0,5	0,5	0,5	0,5
Minerals	15 g[a]	1,5[b]	1,5[b]	1,5[b]	1,5[b]	1,5[b]
Buffer[c]	–	4,0	4,0	4,0	4,0	4,0
%CP	14,5	16,7	15,5	15,2	14,2	12,9

[a] Commercial mixture given in Table 4.1 (Kerolik No. 3, Cooper & Nephews, South Africa)

[b] Commercial mixture Kimtrafos 25 (Kynoch Feeds, South Africa), containing 24% Ca; 12% P; 0,15% Fe; 0,15% Zn; 0,05% Cu; 0,1% Mn; 1,0% Mg; 0,002% Co; 0,002% I; 5,0% S; 3,0% dried molasses

[c] Containing 2% $CaCO_3$; 0,5% Na_2HPO_4; 0,5% $NaHCO_3$; 0,5% $KHCO_3$; 0,5% K_2HPO_4

Table 6.2. Experimental plan and diets fed to Sheep A52 during adaptation to a high grain-high protein diet

Period Date	1 19/10/74- 29/10/74	2 30/10/74- 5/11/74	3 6/11/74- 12/11/74	4 13/11/74- 19/11/74	5 20/11/74- 25/3/75
Amount of food	2x750 g	2x750 g	2 x 750 g	2 x 750 g	2 x 750 g
Diet	A[a]	AF	F	FC	C[a]
Composition (kg/100 kg)					
Maize stalks	83,0	62,5	42,0	29,4	16,8
Maize grain	-	16,0	32,0	44,6	57,2
Molasses	10,0	10,0	10,0	10,0	10,0
Urea	1,0	2,75	4,5	4,5	4,5
Fishmeal	-	2,75	5,5	5,5	5,5
NaCl	1,0	1,0	1,0	1,0	1,0
Minerals[b]	2,0	2,0	2,0	2,0	2,0
$CaCO_3$	3,0	3,0	3,0	3,0	3,0
%CP	7,8	15,8	22,1	20,3	17,8

[a]Diets A and C were the same as described in Chapters 4 and 5

[b]Commercial mixture given in Table 4.1 (Kerolik No. 3, Cooper & Nephews)

Sheep K29 had to be removed from the experiment at the beginning of period 5 as his food intake had dropped to <500g on the final diet. Although they reached the last period, sheep A27 and A35 had to be removed from the experiment after 71 and 98 days respectively on the HC diet. The three sheep removed from the experiment were all found to have developed urinary calculi apparently as a result of the dietary buffer which, however, had effectively controlled ruminal pH. That the

Table 6.3. Mean daily food intake and weight gain of sheep during stepwise adaptation at *ad libitum* intake to high concentrate diets

Period	Days on diet	Mean daily food intake [a] (g) for sheep A27	A35	A85	K1	K29	A52[b]
Lucerne hay	14	1 230	1 230	1 230	1 230	1 230	-
1	7	1 110	1 100	1 630	1 210	510	680^{20}
2	7	1 390	1 500	1 860	1 650	1 390	1 030
3	7	1 000	1 325	2 675	2 100	1 675	870
4	7	1 380	1 130	2 310	2 250	1 910	910
5	7	1 500	1 110	2 260	2 070	-	1 090
	22	1 670	1 390	2 450	2 060	-	$1\ 190^{29}$
	42	1 160	1 500	2 340	2 130	-	$1\ 060^{49}$
	71	1 230	1 460	2 100	1 900	-	820^{80}
	98	-	1 410	1 420	1 530	-	$1\ 050^{107}$
	119	-	-	1 540	1 450	-	880^{128}
Weight gain (kg)							
-over whole expt		10,2	17,1	30,2	22,5	-	11,8
-on final diet		7,7	16,4	17,7	14,8	-	9,3

[a] Food intakes represent the mean daily intake over the 7 day period before and including the sampling day

[b] The superscript for A52 refers to the number of days on the diet when this differs from the other 5 sheep

dietary buffer, rather than other components of the diet, was demonstrated by subsequently maintaining sheep A85 and A52 for 214 and 224 days respectively without any trouble on a high concentrate diet containing 15% CP with all additional N as fishmeal but with the mixed dietary buffer replaced by 3% $CaCO_3$.

/Pattern .....

Pattern of ruminal pH and lactic acid

The pattern of ruminal pH and total lactic acid over the sampling period is shown in Fig. 6.1 for the two sheep (A85 and K1) which completed the experiment and had consistently high food intakes. Details of pH and total lactic acid values for all the sheep are given in Appendix Table 6.1. The same trends were shown here as in the Exploratory Experiment (Chapter 4). Peak lactate accumulation during the adaptation occurred on or before the feeding of the Intermediate diet and then for Sheep K1 lactate accumulation was high after 42 and 71 days on the final High concentrate diet. The ruminal pH fell as the adaptation progressed and on the 98th and 120th day the values for A85 were below pH 6,00 for the entire 24 hours with the exception of ½ h on the 98th day.

The individual maxima for D- and L-lactate are not necessarily at peak total lactate accumulation and hence their sum does not always give the maximum total lactate value. Correlations for individual sheep between food intake and maximum total lactate, food intake and minimum pH, and maximum total lactate and minimum pH were weak to moderate and not significant ($P<0,95$). The peak total lactate values are shown in the histogram presented in Fig. 6.2.

Proportions of D- and L-lactic acid

The percentage of D-lactate in total lactate at peak accumulation is given in Table 6.4. For Sheep A52 the L-isomer predominated except for the period on Diet AF and the first day on Diet F. On the 15% CP diets this was reversed and the D-isomer formed more than 50% of the total lactate at peak accumulation on all except two occasions, when the High concentrate diet was first fed. Similar trends in the proportions of the two isomers were found at times other than peak lactic acid accumulation.

Minimal ruminal ammonia concentrations

The values for the minimum ruminal ammonia concentrations reported in Table 6.5 were taken from the report of Schwartz & Hannah (1975) and were not determined by the author. The sheep were sampled for 8 h after the second feed until the 7th day on the final diet when sampling was continued for a full 24 h. The minimum recorded values for the sheep on the 15% CP diets were mostly >6 mM although on several occasions were between 3-5 mM. The values tended to rise as the adaptation progressed. For sheep A52 the minimum value on the initial diet was only

/Table 6.4. .....

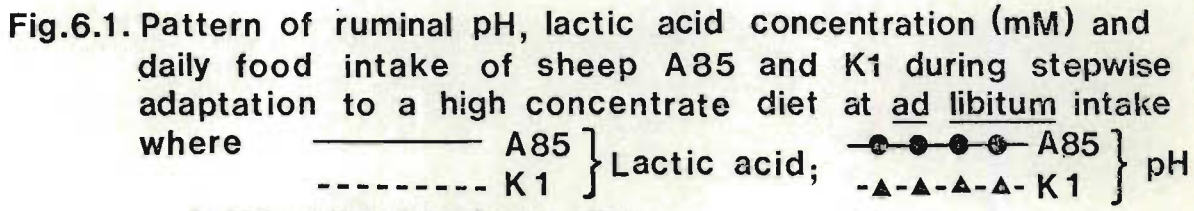


Fig.6.1. Pattern of ruminal pH, lactic acid concentration (mM) and daily food intake of sheep A85 and K1 during stepwise adaptation to a high concentrate diet at ad libitum intake where ———— A85 ---------- K1 } Lactic acid; —●—●—●—●— A85 -▲-▲-▲-▲- K1 } pH

and HR = High roughage diet
Int = Intermediate diet
HC = High concentrate diet

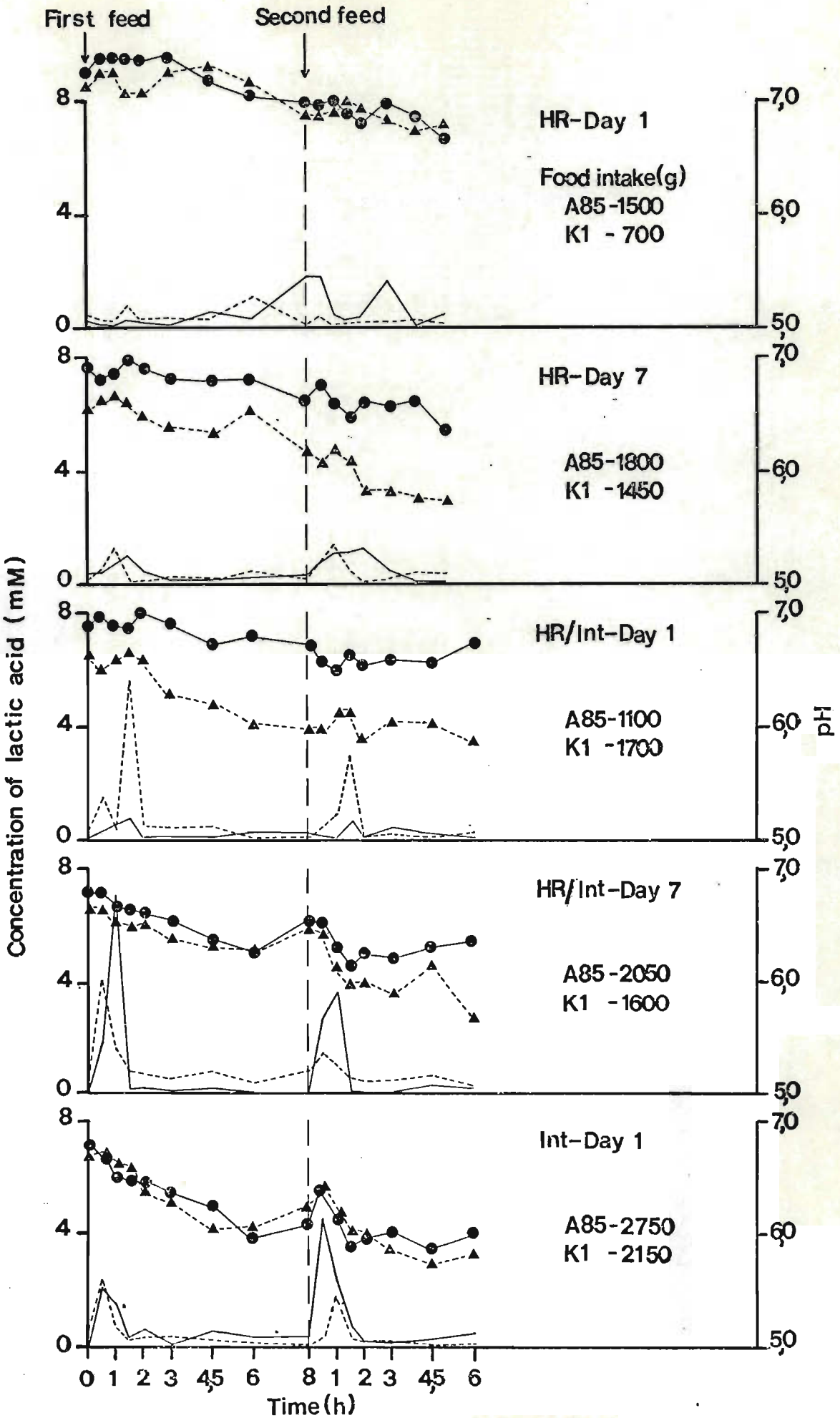


Fig.6.1(a). ——— A85 } Lactic acid —●—●—●— A85 } pH

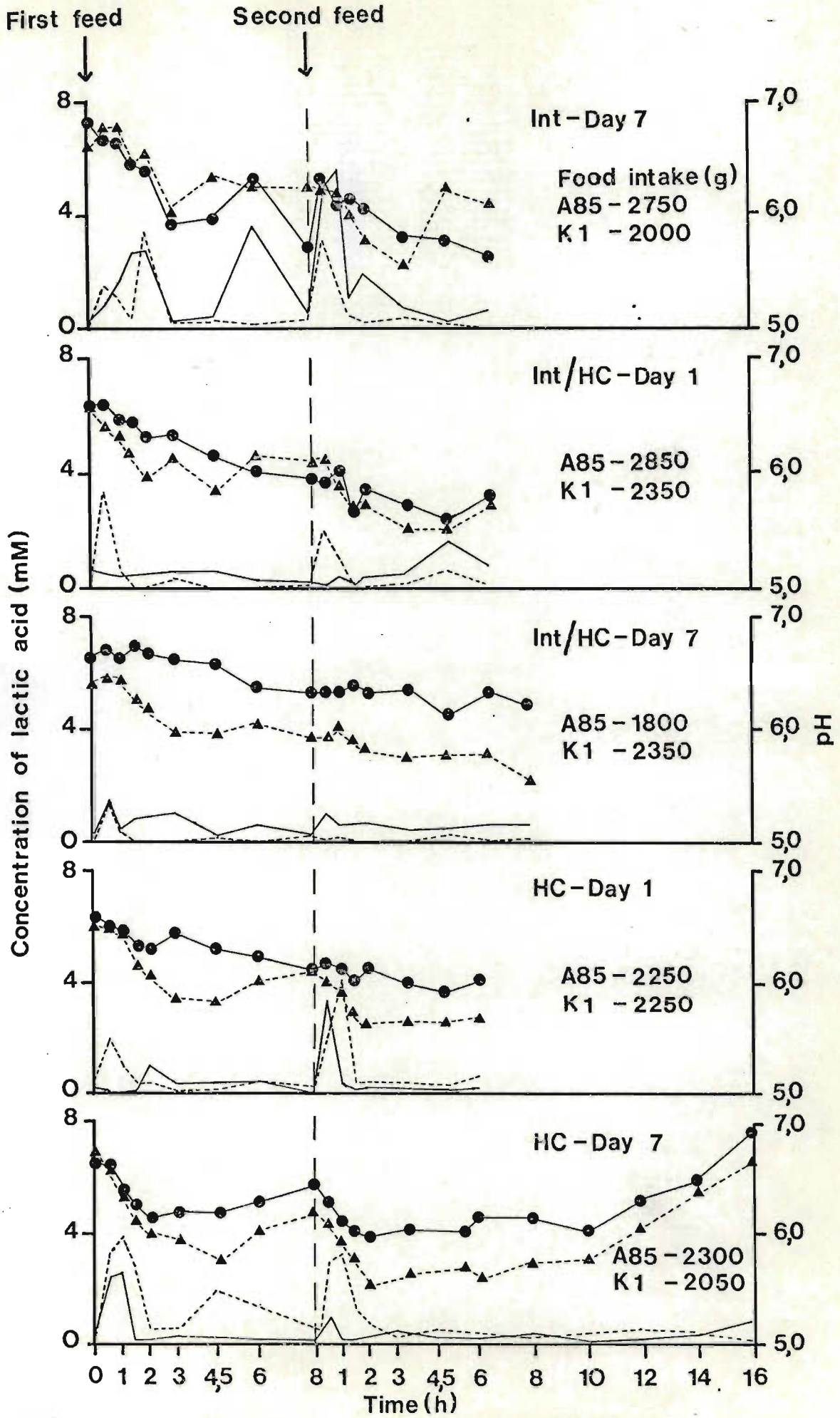


Fig.6.1(b) ——— A85 } Lactic acid
- - - - - - K1 }

—●—●— A85 } pH
—▲—▲— K1 }

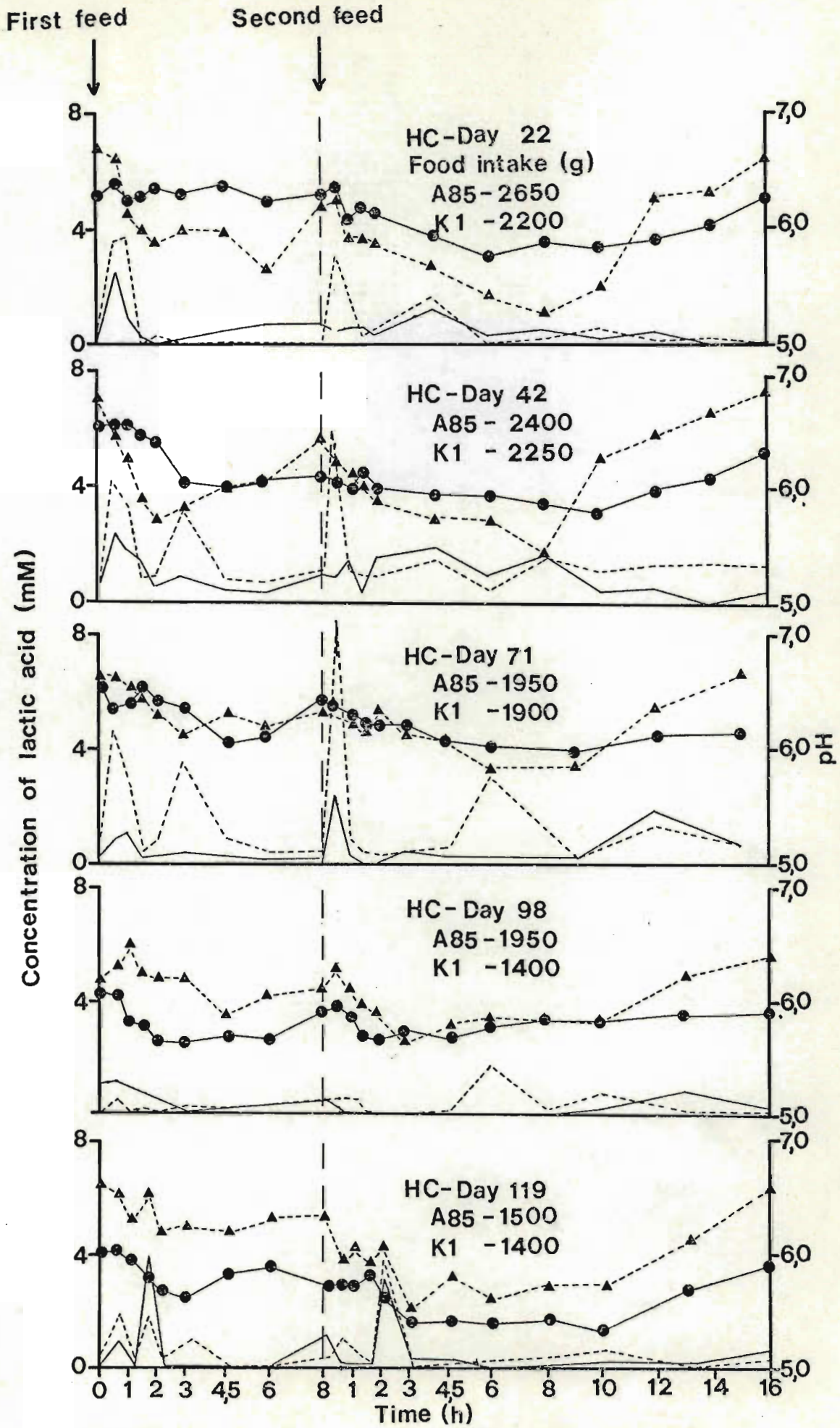


Fig.6.1(c) A85, K1 } Lactic acid , A85, K1 } pH

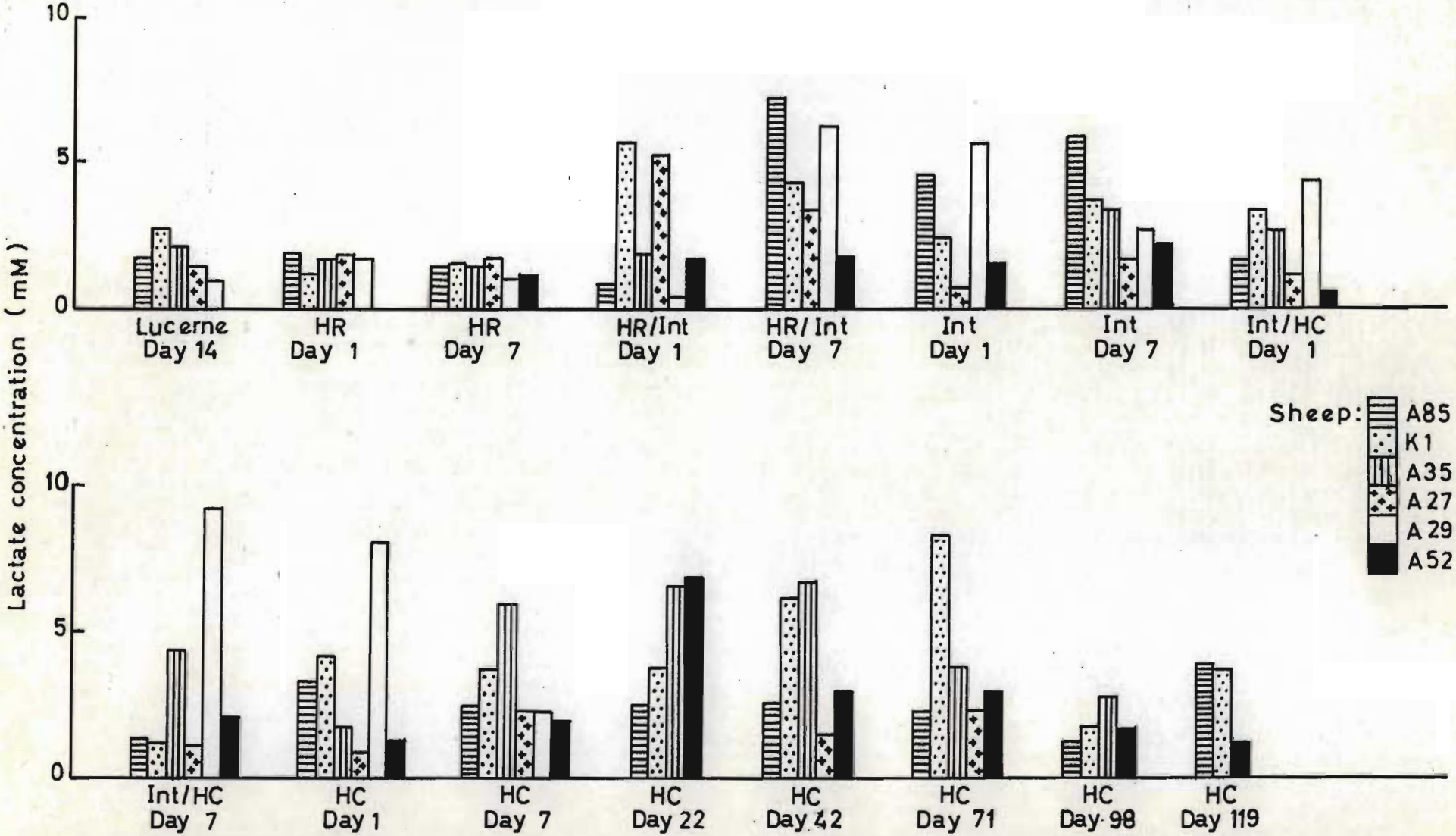


Fig.6.2. Peak accumulation of total lactic acid in the rumen of sheep during adaptation at ad libitum food intake where HR=High roughage, Int=Intermediate and HC=High concentrate diets or mixtures of two diets for the steps in the adaptation

Table 6.4. The percentage D-lactic acid at peak lactate accumulation for the group of sheep fed the 15% CP diets and for sheep A52 fed urea-containing diets

Period	Diets containing 15% CP			Urea containing diets (A52)	
	Days on diet	% D-lactate Mean±SD[a]	CV	Days on diet	% D-lactate
-	14	54±6	12	-	-
1	1	64±24	38	-	-
	7	66±7	10	20	0
2	1	65±19	30	1	79
	7	63±14	22	7	78
3	1	67±13	19	1	77
	7	57±14	25	7	0
4	1	67±5	8	1	0
	7	52±16	31	7	43
5	1	48±22	46	1	44
	7	44±18	41	7	0
	22	63±14	22	29	0
	42	54±17	31	49	17
	71	62±7	11	80	15
	98	61±14	23	107	0
	119	86	15	128	0

[a] n = 5 for periods 1 to 4; 4 for the first 71 days of period 5, 3 for day 98 and only 2 for day 119

3,6 mM NH_3-N but thereafter was always more than 15,6 mM and often >30 mM. The level of urea increased from 1,0% on the initial high roughage diet to 4,5% on the final high concentrate diet while CP increased from 7,8% to 18-22% in the diet of sheep A52.

/Table 6.5. .....

Table 6.5. The minimal ruminal concentrations (mM NH_3-N) during stepwise adaptation at *ad libitum* intake to high concentrate diets

Period	Days on diet	A27	A35	A85	K1	K29	A52$^{\Phi}$
Lucerne Hay	14	10,3	17,5	8,1	19,4	12,3	-
1	1	3,2	6,3	5,3	10,2	12,2	-
	7	6,4	13,1	5,6	9,8	9,3	$3,6^{20}$
2	1	8,1	15,0	5,5	12,4	13,5	17,3
	7	14,6	-	7,1	15,4	16,6	15,6
3	1	10,5	-	9,0	11,1	10,2	21,5
	7	7,9	13,0	10,2	10,0	13,9	50,8
4	1	5,8	15,9	7,8	14,6	6,9	47,8
	7	6,3	14,0	14,6	15,4	7,7	35,4
5	1	4,3	6,0	11,2	12,0	6,5	54,4
	7	12,7	13,1	21,6	11,6	34,6	22,3
	22	-	9,3	6,2	14,6	-	$77,7^{29}$
	42	10,4	17,2	12,1	11,8	-	$67,1^{49}$
	71	15,2	20,3	20,3	16,4	-	$34,4^{80}$
	98	-	12,5	12,5	8,2	-	$19,0^{107}$
	119	-	-	23,1	15,6	-	$27,0^{128}$

$^{\Phi}$The superscript for A52 refers to the number of days on the diet when this differs from those of the other 5 sheep

CONCLUSIONS

1. A stepwise adaptation at *ad libitum* food intake was carried out. Maximum food intakes of 2 000-2 500 g/day were recorded on the diets containing 43, 54 and 65% grain & molasses. Food intakes started to decrease after 22 days on the final diet.
2. Peak lactate accumulation during the adaptation occurred on or before the feeding of the diet containing 43% grain & molasses. On the final diet lactate concentrations began to increase again. The ruminal pH fell as the adaptation progressed and on the last

/two .....

two sampling days on the 65% grain & molasses diet pH values were below 6,00 for the entire 24 hours with the exception of ½ h on the 98th day.

3. On the urea containing diet the L-isomer of lactic acid predominated whereas on the diet containing all supplemental N as fishmeal the D-isomer predominated at peak lactate accumulation. At other times in the feeding cycle similar trends in the proportions of the two isomers were found.

4. The multi-component buffer included in the 15% CP diets at a level of 4% effectively controlled the decrease in ruminal pH and despite the fact that all diets were fed *ad libitum* and the sheep remained on the final diet for 120 days total lactate concentration never exceeded 10 mM, indicating that a balanced ruminal flora was maintained throughout the experiment. Immediately after feeding the pH values showed an increase before the gradual decrease.

5. Minimal ruminal ammonia concentrations (taken from report of Schwartz & Hannah, 1975) were usually >6 mM NH_3-N but on several occasions the values decreased to 3-5 mM for the sheep on the 15% CP diets. For sheep A52 the values increased from 3,6 mM on the initial diet containing 1% urea to >19 mM on the final diet containing 4,5% urea.

CHAPTER 7. DISCUSSION OF PART II

The results presented in these chapters form the foundations on which subsequent studies in the thesis are based. The diurnal pattern of ruminal lactic acid concentration and the values recorded on the diets differing in amount of RFC are important. The typical pattern was shown in Figs. 4.1, 4.2 and 6.1 where lactic acid accumulated transiently, reaching a peak concentration of *ca* 1-10 mM roughly ½-2 h after each of the two daily feeds, depending on the amount of RFC in the diet. Total lactate values never exceeded 10 mM regardless of the diet. This is in contrast to some of the higher values reported by other workers (Briggs *et al*, 1957; Chou & Walker, 1964; Sutton & Johnson, 1969). The lower values in the present experiments can be ascribed to lower levels of RFC and the gradual adaptation to the high concentrate diets despite being at *ad libitum* intake in the second experiment.

The trends in the peak lactic acid concentration during the *ad libitum* feeding experiment are of interest since they show a tendency to increase when diets containing 26,5-43,0% grain and molasses were fed and then to decrease as the adaptation progressed and the percentage of grain and molasses increased. Once on the final diet the concentrations then tended to increase between 22-71 days on the diet. Sheep K29 reacted differently to the other sheep reaching the highest peak lactic acid concentrations after the other sheep, i.e. on the diet containing 54% grain and molasses and on the final diet. After the first day on the final diet this sheep was removed from the experiment.

The proportion of the two isomers of lactic acid in the rumen is an aspect which has not been studied previously in much detail. The results presented here show that:

1. at times in the feeding cycle other than peak lactate accumulation the proportions of the two isomers were similar. This trend was the same for peak accumulation at low levels of feed intake;
2. at peak lactate accumulation the proportion of the D-isomer increased on the diets containing 10-15% CP (Diet B and the diets used in the *ad libitum* feeding experiment) whereas on the high protein diets (Diet C containing *ca* 20% CP) the L-isomer predominated. However, on the high protein diets fed to A52 the intake of food was both lower and slower possibly related to the high level of urea (4,5%).

/Thus .....

Thus at peak lactate fermentation there is an increase in the proportion of D-lactate. this tendency occurred throughout the diets which ranged from 30-70% RFC and was therefore related to the rate of fermentation which could be very rapid with a fast intake of food containing only 30% RFC. However, the most important factor controlling not only the amount of lactate which accumulates but also the proportion of the two isomers is the ruminal microflora.

These trends in lactate concentration pose the question whether lactate accumulation is due to an increase in production or decrease in utilization or, when no lactate accumulates whether it is due to equal rates of production and utilization or a decrease in production rate. Answers to these questions would assist in explaining the different proportions of the D- and L-lactic acid isomers observed during the feeding cycle since the results obtained for the concentrations of the two isomers indicate that their rates of production and utilization are not the same. The concentration of lactic acid in the rumen is influenced not only by microbial production and utilization, but also by passage of rumen contents to the omasum, abomasum and intestine, diluting and buffering effects of saliva and by changes in the amount of water in the rumen due to osmotic transfer across and absorption through the epithelium.

As shown in the Exploratory Experiment (Fig. 4.1) the peak in lactate accumulation and also of D-lactate corresponds with an increase in free glucose concentration in rumen fluid. Other evidence for this relationship comes from "grain overload" studies. Glucose appeared in the ruminal fluid following engorgement of sheep on wheat (Ryan, 1964a). Although L-lactate was observed in ruminal fluid without the presence of measurable quantities of glucose, D-lactate was not found unless glucose was present (Ryan, 1964b). The increase in glucose concentration together with reduced pH would favour the proliferation of lactic acid producing bacteria (Slyter, 1976). In the present experiments glucose concentrations remained elevated (1,0-7,5 mM) but lactate concentration decreased, suggesting that utilization of lactate was increased relative to production.

The rate of fall in pH and length of time pH remains below critical (as yet undefined) pH values will also influence lactic acid concentration in the rumen. The fall in rumen pH under normal feeding conditions as used in these studies is unlikely to be caused by lactic acid production but rather to the rapid formation of volatile fatty acids. One of the most common methods used to control the fall in ruminal pH is the inclusion of dietary buffers. Diets containing 3% $CaCO_3$ were effective in controlling

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the fall in pH but were not as successful in controlling pH as the dietary buffer used in the *ad libitum* feeding experiment. This buffer contained 2% $CaCO_3$ plus 0,5% each of $NaHCO_3$, $KHCO_3$, Na_2HPO_4 and K_2HPO_4 and was based on that of McManus *et al* (1972). Three of five sheep on these diets died or were slaughtered because of urinary calculi. The incidence of urinary calculi during intensive fattening of castrated male animals is influenced not only by the Ca/P ratio but also the P content of the diet. The most likely reason for the presence of the calculi was the high level of P in the diet related to the high acidity with the fishmeal in the diet compounding the problem (Scott, 1972). The results of the five sheep during the adaptation area not affected since the food intakes of K1 and A85 which completed the experiment were similar to the three that were removed. However, once on the final diet the intake of K29 had dropped by the 7th day, A27 after the 71st day and A35 after the 98th day. The previous drops in intake by the sheep can be ascribed to an "off-feed" reaction to the high concentrate diets. As mentioned in Chapter 6 (page 70) A52 and A85 were placed on a high concentrate diet containing 15% CP, with all additional N from fishmeal, and 3% $CaCO_3$ dietary buffer for a further 220 days without any trouble.

Despite the higher N content of the diet the sheep which received the 15% CP diets *ad libitum* had ruminal NH_3 concentrations which were slightly higher than in the experiment where the feed intake was restricted and the CP was *ca* 12%. These results suggest that due to a high rate of passage on *ad libitum* intake relatively little of the insoluble fishmeal was broken down in the rumen. However, since the sheep were sampled for a full 24 hours while on the final diet containing 67% grain & molasses the concentration of NH_3-N was not limiting for the growth of the ruminal bacteria at this critical stage of the *ad libitum* feeding experiment.

As far as can be ascertained from the available literature there are no reports on rate of food consumption and its effects on lactic acid concentration. However, it is well known that animals that are hungry or greedy eaters are more prone to acidosis than slower eaters. Eadie *et al* (1970) found that marked changes in the microbiological and biochemical properties of rumen fluid could be brought about by an alteration in the quantity rather than the quality of an all-concentrate diet. However, the differences in lactic acid concentrations between the *ad libitum* intake and the restricted intake (80% of calculated appetite) were small and lactate concentrations were low (< 1 mM). Several other studies, as

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reviewed by Slyter (1976), including those on lactic acidosis have shown that t amount and rate at which food is eaten have an important influence on lactic acid accumulation in the rumen.

Despite the fact that sheep are generally less tolerant of high levels of RFC than cattle (Elliott, 1967), the stepwise adaptation of the sheep in the microbiological study (Chapter 5) to a diet containing 71% grain & molasses fed at 2-3 times the maintenance level, was successful in that at no time did the recorded pH fall below 5,3, and lactic acid accumulated only transiently in low concentrations after feeding in both groups of sheep. The adaptation was aided by the fact that the diet contained 3% $CaCO_3$ and that intake was restricted, both of which limited the decrease in pH in the rumen as the amount of grain fed was increased. Nevertheless the maintenance of a balanced ruminal fermentation throughout the experiment may be largely attributed to two gradual changes in the microbial population. The first of these was the increase in the number of protozoa in proportion to the increase in starch and sugar in the diet up to the stage the final diet was fed. The entodiniomorphs, in particular, which predominated throughout the experiment, are known to engulf starch grains rapidly (Eadie *et al*, 1970) and to ferment them at a slower rate than the bacteria (Abou Akkada & Howard, 1960; Whitelaw, Hyldgaard-Jensen, Reid & Kay, 1970). In addition the ciliate protozoa ingest bacteria at a rate of 50 bacteria/protozoon/h which means that if there are 10^5 protozoa/ml and 10^{10} bacterial/ml, the protozoa can ingest bacteria at a rate of $5x10^6$ bacteria/h (Prins & Van den Vorstenbosch, 1975) consequently reducing fermentation rate in the rumen. This effect is clearly shown in the present experiment where there was no increase in the count of total culturable bacteria in the rumen in response to the increase in the amount of RFC fed until the grain & molasses of the diet reached 60% or higher.

Thus the rate at which grain can be introduced into the ration without upsetting the balance of the ruminal population is dependent on the rate at which the protozoa can increase their number in response to the added starch. Minimum division times of 5,5-7,3 h for protozoa in the rumen on several different diets have been reported (Warner, 1962; Hungate, Reichl & Prins, 1971; Potter & Dehority, 1973). However, the net rate of increase in number of protozoa in the rumen is less than this due to passage of protozoa out of the rumen. Several workers have found that it takes 5-6 days for protozoal number to plateau after abrupt changes from all roughage to diets containing increased amounts of concentrates (Nakamura & Kanegasaki, 1969; Grubb & Dehority, 1975). In contrast, minimum doubling times of *ca* 1,5h have been found for *Bacteroides amylophilus*, *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (Hobson, 1965; Hobson & Summers, 1967) while *Streptococcus bovis*

has a doubling time of as little as 9 min under suitable conditions (Brüggemann & Giesecke, 1965). Thus if the amount of grain added to the diet exceeds the capacity of the protozoa to remove a large proportion of it, the bacteria will be able to multiply much more rapidly than the protozoa in response to the excess substrate and the fermentation will then proceed at an uncontrolled rate. This is particularly likely to occur, as it did in the present experiment in the later stages of the adaptation when ruminal pH fell below the growth optimum for protozoa for a substantial part of the day. Purser & Moir (1959) found a marked inhibition of protozoal division 2-6 h after feeding which corresponded with the fall in ruminal pH to *ca* 5,4-5,7 at this time although little inhibition occurred above pH 6.

The second critical factor which required a gradual change in diet during adaptation was the increase in number of acid-tolerant, lactate-utilizing bacteria in response to an increase in lactic acid production and the fall in ruminal pH. On the initial high roughage diet the ratio of lactate-utilizers to total culturable and to amylolytic bacteria was low, and they consisted large of *Veillonella* and *Selenomonas* which are reported to be acid-sensitive. As the grain content of the diet increased the ratio of lactate-utilizing to amylolytic bacteria changed and an orderly succession towards more acid tolerant genera occurred which was related to the decrease in ruminal pH. Thus if an animal has a ruminal flora which is predominantly acid-sensitive and is then changed abruptly to a high concentrate diet the lactate-utilizing bacteria present are unable to multiply in response to the increase in lactic acid production causing an accumulation of lactic acid and a rapid drop in pH. As a result, the more acid-resistant species may not have time to increase to an effective level before the pH falls below their tolerance limit for growth, and lactate utilization ceases with an ensuing acidosis. It is of interest that concomitant work on the numbers and proportions of cellulolytic bacteria by Gilchrist and reported in the paper by Mackie *et al* (in press) showed very little decrease despite prolonged feeding of a diet containing a high proportion of concentrates. The persistence of the cellulolytic *Bacteroides* in the rumen on the 54th day of the final diet when the amylolytic species of the genus had disappeared may have been due to the association of the former with slowly digested fibrous portion of the diet which would tend to prevent them being diluted out the rumen despite a reduction in growth rate due to low pH.

From the data obtained by correlating changes in species with shifts in ruminal pH it was shown that the acid-sensitive species tended to have a negative slope and acid-tolerant species a positive slope. The *Veillonella* + *Selenomonas* species were never a large proportion of the flora and decreased

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rapidly with increase in pH while the *Bacteroides* were a large proportion and decreased more slowly. The acid-tolerant *Propionibacter* were amongst the predominating bacteria and increased slowly as "pH_6-hours" increased, i.e. the length of time spent below pH 6,00, while the *Lactobacillus* + *Eubacterium* species were not present to start with but increased more sharply to form a greater proportion of the predominant flora as "pH_6-hours" increased. These findings are summarized in the diagram shown in Fig. 7.1.

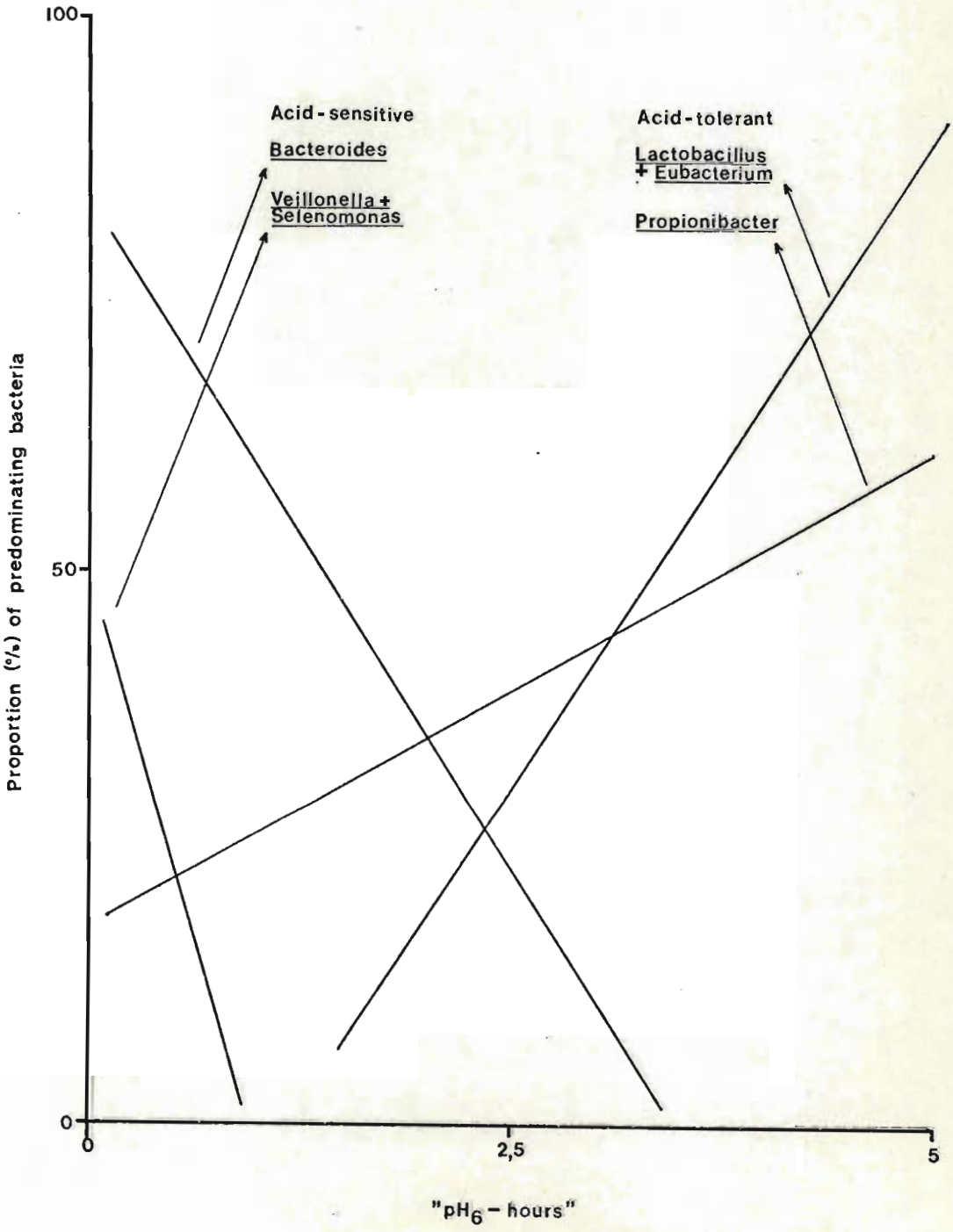

Fig. 7.1 Changes in the proportions (%) of the predominating bacteria in relation to shifts in ruminal pH expressed as "pH_6-hours"

Variations in the quality and number of viable cells in inocula from the different isolates proved to be a problem in the identification of the lactate-utilizing and amylolytic bacteria. Thus although the volume of inoculum added for each carbohydrate fermentation and biochemical test, and the method of obtaining the inoculum were identical, it was not possible to get the same results from the same isolate on repetition. The groups selected on the basis of cell morphology and size, Gram reaction and the ability to ferment soluble stacrh with the production of lactate in the case of the amylolytic bacteria or to utilize lactate in the case of the lactate-utilizers, proved to be reasonably accurate. The *Anaerovibrio* group (L77, L88, L151, L166) was rather heterogeneous and initially included L7 which was subsequently found to be more similar to *Selenomonas*. Poor growth of isolates in the fermentation media used for determination of end products can also be confusing since not only the proportions of end products, such as lactate and propionate in the case of *Selenomonas ruminantium* (see Wolin (1975) for review), but also the quantities produced can change. In explaini results from poor growth the ratios of end products are more useful than the quantities produced, e.g. >1 mM as a major end product and <1 mM as a minor product as indicated in Table 5.10 and recommended by Holdeman & Moore (1975).

In conclusion the most important point to emerge from this section was the effect of a gradual fall in ruminal pH as the adaptation progressed, aided by the inclusion of a dietary buffer and restricted food intake. This allowed the protozoa to increase in number and control the rumen fermentation by engulfing both starch grains and bacteria. The gradual fall in ruminal pH also enabled an orderly succession of lactate-utilizing bacteria from acid-sensitive to acid-tolerant species which prevented the accumulation of lactic acid. Although the proportion of RFC in the diet and the rate and amount of food eaten play an important role in regulating the concentration of lactic acid in the rumen, the microbial flora seem to be the most important since the balance between the organisms fermenting starch and glucose and producing lactic acid and those organisms utilizing the lactic acid produced control the lactic acid accumulation in the rumen. The next section follows this aspect of the work by determining *in vivo* turnover of lactic acid in the rumen of sheep fed diets differing in amount of RFC and relating this to the microbial flora present during the turnover experiments.

PART III

LACTATE TURNOVER

IN THE RUMEN

CHAPTER 8. TURNOVER OF LACTIC ACID IN THE RUMEN OF SHEEP FED DIETS CONTAINING DIFFERENT PROPORTIONS OF READILY FERMENTABLE CARBOHYDRATE AND ITS CONTRIBUTION TO THE PRODUCTION OF THE INDIVIDUAL VOLATILE FATTY ACIDS

INTRODUCTION

In recent years the use of metabolites labelled with radioactive isotope has allowed reasonable estimates ot be made of the production and utilization of various substrates in the intermediary metabolism of the ruminant. However, the use of labelled materials in the study of rumen metabolism has been limited mainly because of problems associated with uniform mixing of infused materials and obtaining representative samples of ingesta. Nevertheless they have been used in the determination of the rates of production of the VFA. *In vitro* studies of the turnover of ruminal lactic acid have been made using rumen fluid from animals fed roughage or concentrate rations, and measuring changes in the specific activity of lactic acid and its fermentation products (Jayasuriya & Hungate, 1959; Nakamura & Takahashi, 1971). However, the *in vivo* rate of disappearance of lactic acid added to the rumen is more rapid than can be expected from the results of the *in vitro* turnover studies (Hueter, Shaw & Doetsch, 1956; Waldo & Schultz, 1960; Kunkle *et al*, 1976a). This is possibly due to the fact that lactic acid is also diluted out of the rumen along with the digesta as well as absorbed through the ruminal wall, albeit much more slowly than the VFA (Williams & MacKenzie, 1965). Thus kinetic studies on ruminal metabolism *in vivo* are essential for a complete explanation of the various processes involved.

This experiment was planned in an attempt to explain the changes in the concentration and proportions of lactic acid isomers possibly caused by differences in the rates of production and utilization of D- and L-lactic acid which occurred in the rumen of sheep during the first Stepwise Adaptation Experiment (Chapter 5). Since it was not possible to follow changes in turnover rate at short intervals during the course of adaptation to new diets, turnover rates were determined on sheep already adapted to a series of diets containing different proportions of RFC. In this way repeated measurements could be made of lactate turnover and related to the microbial flora of the same sheep adapted to the respective diets (see Chapter 10 for the results of the microbiological study). Comparisons were made of the

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continuous infusion and single injection, tracer techniques for measuring the rates of production and utilization of lactic acid. Lactate load experiments were also performed in which large amounts of unlabelled lactate were dosed to another group of adapted sheep fed the same range of diets as those fed to the group used for the tracer experiments since this approach has also been used as a measure of lactate turnover in the rumen (Hueter, Shaw & Doetsch, 1956; Waldo & Schultz, 1960; Kunkle *et al*, 1976a).

METHODS

Animals and management

Four mature South African Mutton Merino wethers with large 80 mm ID permanent ruminal cannulae (Taljaard, 1972) were used. The animals and diets are given in Table 8.1. All sheep had been on their respective diets for 5 months before the start of the experiment. The animals were trained and reacted normally when placed in metabolism cages for the infusion and sampling. The sheep were fed 750 g twice daily at 08h00 and 16h00. Water was available at all times except on days when microbiological counts were made.

Table 8.1. Composition of diets used in the turnover experiment

Sheep	A30	A84	A28	A21
Diet	High concentrate (HC)	Intermediate (Int)	High roughage (HR)	Lucerne hay (Luc)
Composition (kg/100 kg)				
Molasses	10	10	10	-
Maize grain	55	33	-	-
Maize stalks	15	35	67	-
Fishmeal	15	16	17	-
$CaCO_3$	3	3	3	-
Minerals[1]	2	2	2	15 g
Salt	1	1	1	15 g
Analysis (g/100 g Dry Matter)				
Mono- & di-saccharides	3,9	3,4	3,3	4,1
Starch & fructosans	38,8	23,4	2,8	3,3
Cellulose & hemicellulose	12,8	25,6	40,2	28,6
Crude protein (CP)	16,1	16,0	14,6	14,0
Sulphur[2]	0,43	0,38	0,46	-

[1] Commercial mixture given in Table 6.1 (Kimtrafos 25, Kynoch Feeds, South Africa)

[2]

Experimental plan

The experimental plan followed is given in Table 8.2. Microbiological counts were performed on all sheep during the first, third and fith week. Turnover experiments were performed on each sheep. Since A30 was likely to have the least stable ruminal flora the turnover study was conducted 2 days after removal of samples for microbial counts. Turnover studies on sheep A84, A28 and A21 were conducted in that sequence during weeks 2, 4 and 6.

Table 8.2. Experimental plan for turnover and related microbiological experiments (where C = continuous infusion and S = single injection experiment)

Week	Day of week				
	Monday	Tuesday	Wednesday	Thursday	Friday
1	-	Count	-	C(A30)	-
2	-	C(A84)	C(A28)	C(A21)	-
3	-	Count	-	C(A30)	-
4	-	C(A84)	C(A28)	C(A21)	-
5	-	Count	-	C(A30)	C(A84)
6	C(A28)	C(A21)	-	S(A30, A84)	S(A28, A21)
7	-	S(A30, A84)	S(A28, A21)	-	-

Continuous infusion technique

The sheep were placed in a metabolism cage the day before sampling started. The metabolism cages were placed in the animals' normal pens thereby obviating the necessity for a cage-adaptation period longer than 24 h. An infusion catheter was placed in a dorsal, posterior location in each sheep. The infusion point was precisely located and maintained by taping the nylon infusion tube to a length of coiled wire fixed to the large cork stopper of the cannula. In this way sampling through the small hole in the cork stopper was not hindered. The spring in the coiled nylon tube allowed the sheep free movement and enabled them to lie down. The nylon tube was then connected to a length of silicone rubber tubing which passed through a Gilson HP 4 multichannel peristaltic pump (Gilson, France) to a reservoir containing sterile infusate. The infusate was prepared by taking *ca* 60 *u*Ci ^{14}C-labelled lactate and *ca* 125 *u*Ci ^{3}H-glucose and making up in 1 *l* of 2,5 mM carrier lactate and

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glucose. The ^{3}H-glucose was used for the simultaneous determination of glucose turnover reported in Chapter 9. The dual-labelled solution was sterilized by passing through a sterile Millipore filter (90 mm filter, GS with 0,22 *u*m pore size) into a sterile 1 *l* Vacolitre bottle. Radiochemicals were purchased from the Radiochemical Centre (Amersham, England).

The infusion was started at 23h00 to allow the radioactivities to plateau before the morning feed. The sheep was given 750 g of food at 08h00 and the sampling schedule, given below, was followed. The infusion was terminated just before the second feed after the last sample was taken. The infusion catheter was removed and the sheep taken out of the metabolism cage immediately after removing the last sample.

Single injection technique

The sheep were not restrained in metabolism cages and were dosed with 500 ml of filter sterilized solution containing 200-250 *u*Ci DL-(2-^{3}H)-lactate in 2,5 mM carrier lactate solution except in the preliminary experiment when 75 *u*Ci DL-(2-^{14}C)-lactate was used. At 07h55 a sample of ruminal fluid was taken, the first of the two daily feeds placed in the pen and the sheep dosed by means of an enamel funnel placed in the small hole of the large cork stopper. This ensured that all the dose entered the rumen. The sampling schedule given below was then followed.

Sampling of ruminal fluid

For both the continuous infusion and single injection techniques, samples of ruminal fluid were obtained using a suction tube (15 mm ID) inserted through the small hole of the large ruminal cannula. For the preliminary continuous infusion experiment samples were taken 0, ½, 1, 1½, 2, 3, 4 and 8 h after the first of the two daily feeds. The results suggested that the following sampling schedule would give more information and was followed in subsequent experiments i.e. -1¼, -½, 0, ¼, ½, ¾, 1, 1¼, 1½, 1¾, 2, 2½, 3, 4, 6 and 8 h after the first of the two daily feeds.

Each of the samples was treated in the following manner. Approximately 40 ml of rumen fluid was strained through 2 layers of cheesecloth. For the analysis of lactate and glucose concentrations a 5,0 g portion of SRF was weighed into a wide-mouth McCartney bottle containing 0,5 ml of 5N-$HClO_4$ and mixed well. For VFA analysis, 10 g SRF was weighed into a bottle containing 0,25 ml of 10 N-NaOH and mixed well. For the analysis of radioactivity 20 g SRF was weighed into a bottle containing 2,1 ml of 25%-$ZnSO_4.7H_2O$ and mixed well. Then 1,5 ml of 1,0 N-NaOH was added and

/the .....

the contents mixed. In the laboratory 1,4 ml of carrier solution containing 100 mM glucose and lactate was added giving a final volume of 25 ml clarified ruminal fluid (CRF), a concentration of at least 5 mM glucose and lactate, and a dilution factor of 1,20. For the preliminary continuous infusion experiment the final volume was 36 ml giving a dilution factor of 1,80. All samples were centrifuged before analysis.

Analytical techniques

Ruminal pH was measured on ruminal fluid before straining using a portable Metrohm E488 meter. The concentration of D- and L-lactate in rumen fluid was determined using Method 2 (Chapter 2).

The clarified rumen fluid was centrifuged at 10 000 g for 20 min to yield a clear slightly coloured supernatant. For the preliminary experiment 1,0 ml samples were applied to a column of AG 50W-X12, 200-400 mesh for the separation of glucose, lactate and individual VFA. This procedure proved to be slow, however, the low radioactivity in the volume of fluid applied was a disadvantage. The technique was later modified by steam distilling 10 ml of rumen fluid, evaporating the alkaline distillate and redissolving in 1 ml of water which was then applied to the column (Chapter 10). A flow diagram (Fig. 8.1) outlines the steps taken in determining radioactivity in lactate and individual VFA. Initially it was planned to do the extraction step before steam distillation but this was not feasible due to foaming in the continuous extraction apparatus, especially of the viscous fluid from the High concentrate and Intermediate diets. Rather than reduce the volume of ruminal fluid (10 ml) and hence the radioactivity, and in the interests of uniformity, all samples were steam distilled and the lactate in the residual solution extracted. Tests showed that 96,5+1,6% of ^{14}C-lactate remained in the residual solution after steam distillation. Continuous ether extraction of lactate from the residue after distillation using the apparatus of Neish (1952) gave recoveries of 98-100%.

Extraction: The residual solution was evaporated to reduce the volume to *ca* 10 ml and transferred quantitatively to the continuous extraction apparatus described by Neish (1952) and extracted with ether for 3 h. The lactate was trapped in excess NaOH in the receiving flask (usually 5 ml of 0,005 N-NaOH). These samples were then evaporated onto filter paper discs (Whatman No. 40, 7,0 cm diam., W & R Balston Ltd, England).

/Separation of individual VFA: ..

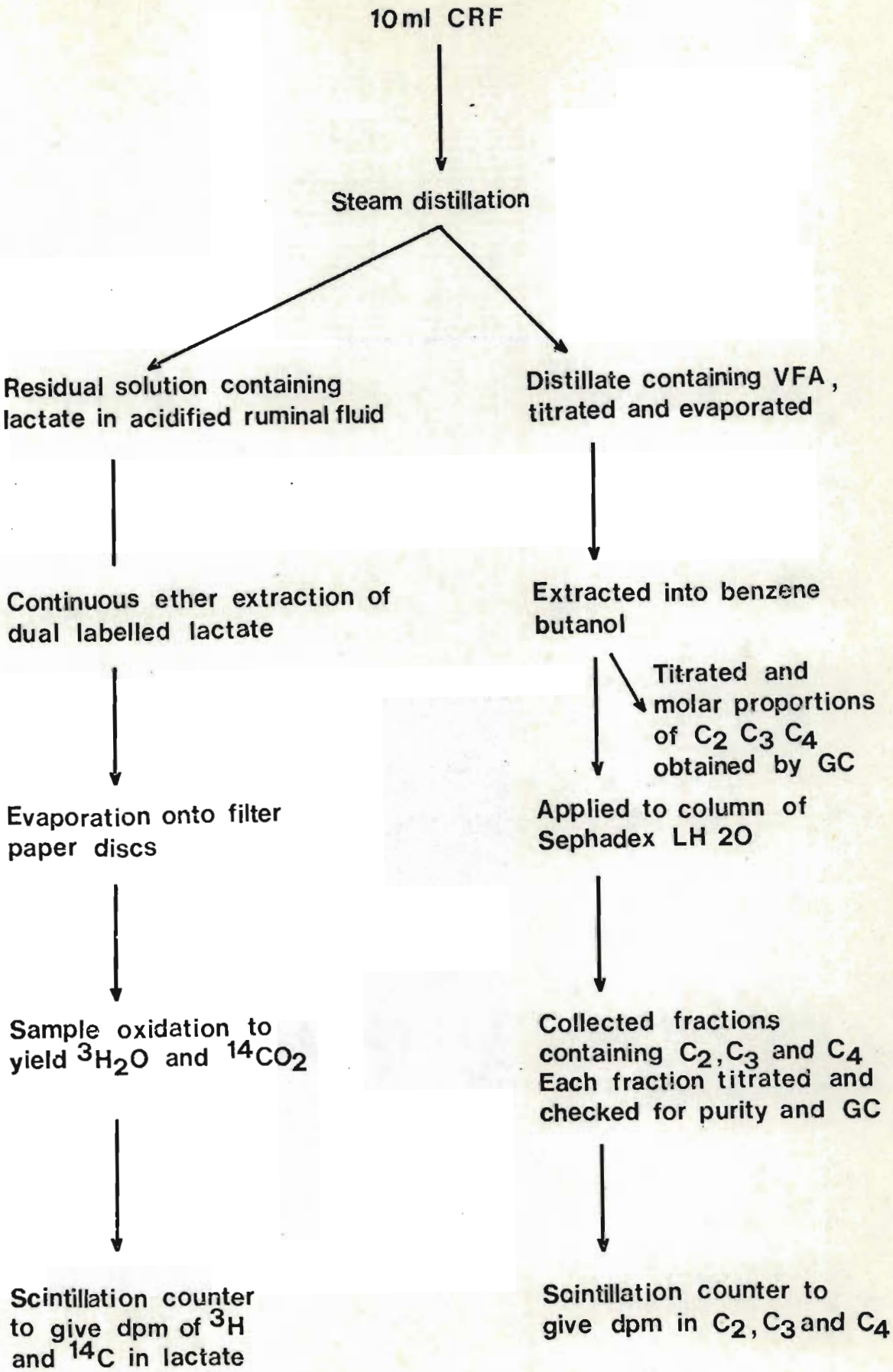

Fig.8.1. Flow diagram of analyses performed on clarified ruminal fluid samples for determination of radioactivity in lactate and in acetate, propionate and butyrate

Separation of individual VFA: The individual VFA were separated from the steam distillate on columns of Sephadex LH 20 using the method of Van der Walt (1977). The extraction was carried out with 5X2 ml portions of dry benzene/butanol (92,5/7,5; v/v) solution and the extracts made up to 10 ml in a volumetric flask to give a higher activity in the 1 ml aliquots used for separation on the columns, estimation of activity in total VFA and for checking the concentration of total VFA and molar proportions of the individual VFA. The separation of the individual VFA and the fractions taken for determination of radioactivity are shown in Fig. 8.2.

Titration: All titrations were carried out with a Metrohm Combititrator apparatus set to an end point of pH 9,00 and using 0,005N-NaOH as titrant. In order to titrate samples containing benzene, 20 ml of water was added to each sample which was then equilibrated under a stream of N_2 for 3 min. After titration to endpoint all samples were taken to pH > 10,5 before evaporation.

Gas chromatography: Separation of fractions on the Sephadex columns were checked using the GC system described in Chapter 5 (page 43). The molar proportions of the individual VFA were estimated using peak height analysis in order to check the values obtained by titration for the acetic, propionic and butyric acids from the column separations.

Radioactivity analysis: The dual labelled lactate samples on filter paper discs were combusted in a Sample Oxidizer (Packard Model 306) in order to determine ^{14}C and ^{3}H activity. The radioactivity in the individual and total VFA was determined by adding 10 ml aliquots of the titrated samples to glass scintillation vials and adding an equal volume of Instagel (Packard). The vials were placed in a Searle Mk III liquid scintillation counter and allowed to stand overnight before counting. Quench curves were entered into the "memory" which enabled the machine to print out activity as dpm.

Production rate of volatile fatty acids estimated *in vitro*

These estimates were made on samples taken 1, 2, 3 and 4 h after the feed i.e. over the peak fermentation period. Approximately 20 g portions of tube samples of whole ruminal fluid were weighed onto a beaker and an equal volume of prewarmed anaerobic diluent (composition given in Appendix Table 3.1) added. The samples were mixed by swirling the beaker and rapidly decanted into test tubes (180x20 mm). The tubes

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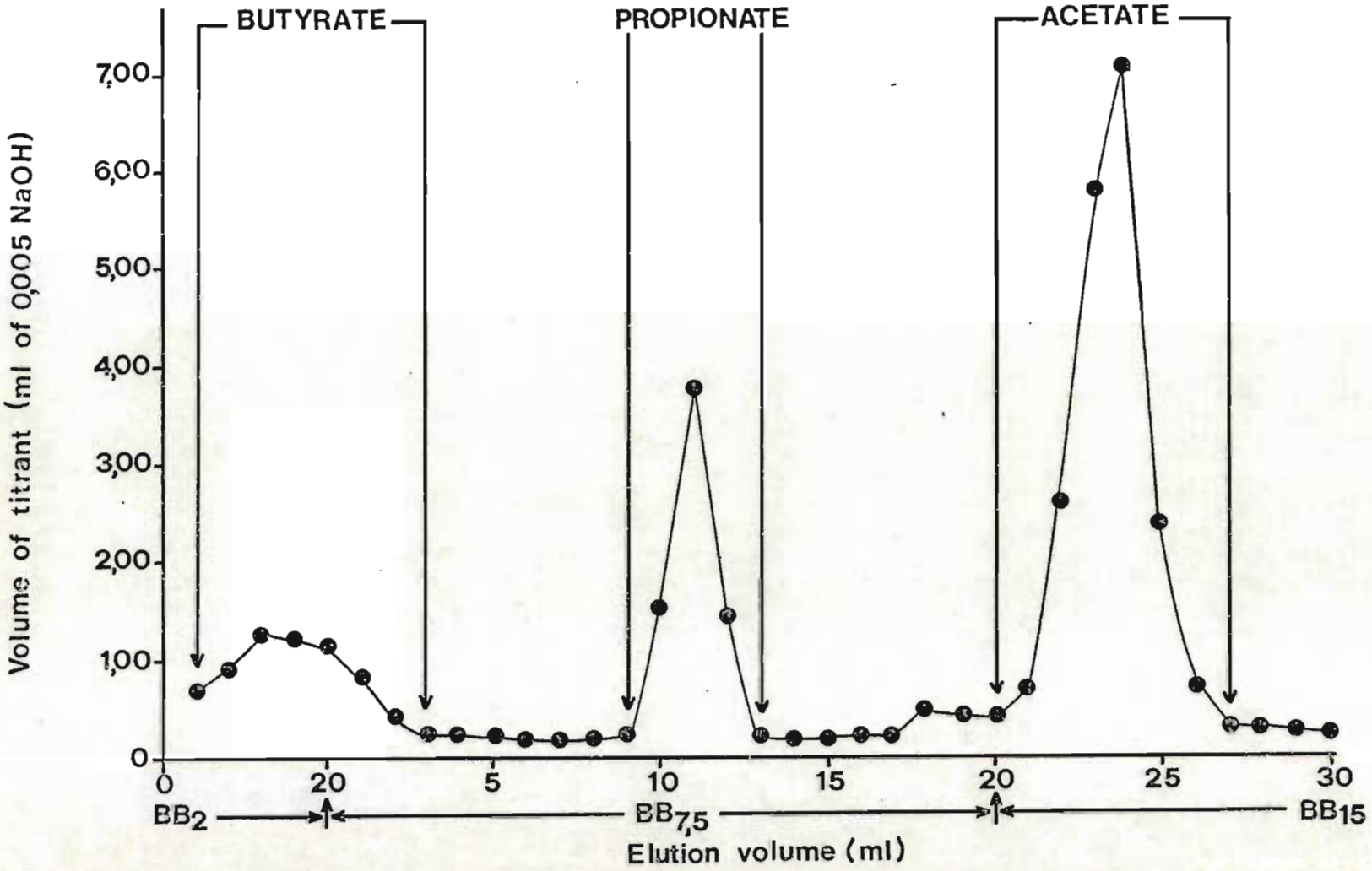

Fig.8.2. Separation of a standard VFA mixture using a Sephadex LH 20 column. The fractions collected for the determination of radioactivity in acetate, propionate and butyrate are indicated by arrows in the figure. (BB_2 = benzene/butanol (98/2; v/v); $BB_{7,5}$ = benzene/butanol (92,5/7,5; v/v); BB_{15} = benzene/butanol (85/15; v/v))

were stoppered using rubber bungs with a simple gas-release valve. Approximately 10 g samples were taken after 0, 15 and 30 min of incubation at 40°C using tubes (7 mm ID) and preserved with 0,25 ml of 10 N-NaOH. Total VFA were determined on the samples as described previously using the method of Briggs *et al* (1957). The data were plotted and zero-time rates estimated by fitting a tangent to the curves.

Separation of D- and L-lactate

Samples containing DL-(2-^{14}C)-lactate were separated for determination of radioactivity in D- and L-lactate in the following manner. Samples (0,5 ml) containing up to 25 *u*mol labelled DL-lactate were incubated with 2,0 ml of glycine buffer, 0,2 ml of NAD solution and 0,02 ml of L-LDH suspension (refer to Chapter 2, Method 1 for details of solution) for 30 min at 37°C. Carrier lactate and pyruvate (0,1 ml) were added followed by 0,1 ml of 60%-$HC1O_4$. The solution was centrifuged at 4 000 g for 15 min, decanted and neutralized with 10 N-NaOH. The neutralized samples were transferred quantitatively together with 2 x 1 ml wash (*ca* 5 ml in all) to glass columns containing 5,0 g AG 1-X8, 100-200 mesh, acetate form (Bio Rad, Richmond, California). The columns were eluted with 20 ml of 1,0 M-HCOOH followed by 20 ml of 1,0 M-NaCl. The separation and fractions collected are shown in Fig. 8.3. The 10 ml of eluant (5 ml sample + wash and 5 ml HCOOH) was discarded. Lactic acid was eluted in the next 15 ml (10-25 ml fraction) and pyruvic acid with 1,0 M-NaCl. The mean recoveries of D-(U-^{14}C)-lactate and (1-^{14}C)-pyruvate standards which were applied to the columns were 94,8 $\pm$ 0,9 and 96,0 $\pm$ 1,2% respectively (n=5). The system was also tested by incubating L-(U-^{14}C)-lactate with L-LDH and then collecting the pyruvate fraction from the columns. In this case the recovery was 92,6 $\pm$ 2,3% (n=5).

Lactate load experiments

Four sheep (A16, A18, A31, A23) were used for these experiments. Prior to the load experiments Sheep A18 which was normally fed the Intermediate diet was used for a preliminary single injection experiment on which day the animal received the HC diet. Sheep A16 was fed the HC diet, Sheep A31 the HR diet and Sheep A23 the Lucerne hay diet. The diets fed and management of the animals were identical to those used in the turnover experiment. The load experiments were performed in

/Fig. 8.3. .....

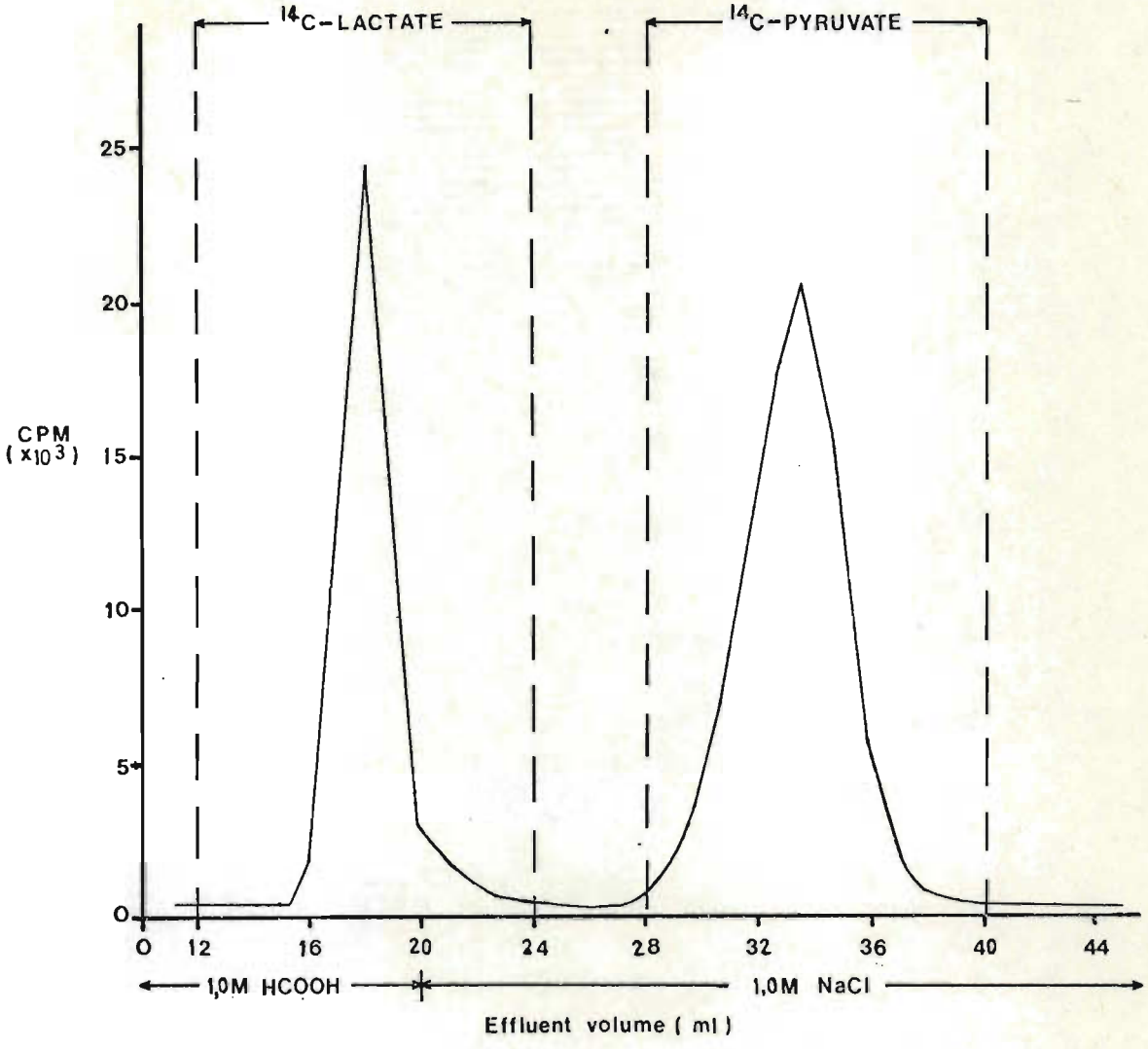

Fig. 8.3 The separation of lactate and pyruvate on AG1-X8 for determination of radioactivity in D-lactate and pyruvate

exactly the same way as the single injection experiments with the exception of the dose which contained no radioactive label and was made up by diluting 50 ml of 70% Na-DL-lactate (BDH) in 500 ml of deionized water. This solution contained *ca* 35 g of Na-DL-lactate and was dosed immediately after taking a sample at zero-time (08h00) and feeding. Samples of ruminal fluid were taken at $^1/3$, $^2/3$, 1, 1½, 2, 2½, 3, 4 and 6 h after the feed in the same way as described previously (page 90) and analyzed for D- and L-lactate concentrations.

/RESULTS .....

RESULTS

Preliminary experiments

Approximately one month before the main experiment (Table 8.2), a preliminary continuous infusion experiment was performed on Sheep A30 fed the High concentrate diet containing 65% grain and molasses since there was likely to be a greater turnover of lactic acid in this than on any of the other diets. The results are presented in Table 8.3.

Table 8.3. Turnover rate of DL-lactate in the rumen of Sheep A30 fed the High concentrate diet as measured by the continuous infusion of DL-(2-^{14}C)-lactate.

Time after feeding (h)	Conc of lactate (mM)	Activity (dpm/ml RF)	Specific activity (dpm/mM)	Calculated rates $^{\Phi}$ (mM/min) Turnover (1)	Production (2)	Utilization (3)
0	0,06	981	16 350 000	0,005		
					0,012	0,009
½	0,15	661	4 407 000	0,019		
1	1,15	295	257 000	0,325		
					0,446	0,431
1½	1,61	229	142 000	0,589		
2	0,11	344	3 127 000	0,027		
					0,028	0,029
3	0,08	230	2 875 000	0,029		
					0,014	0,015
4	0,03	247	8 233 000	0,010		
					0,007	0,007
8	0,02	301	15 050 000	0,006		

$^{\Phi}$Calculated using the following formulae according to Shipley & Clark (1972)

(1) Turnover rate $= \frac{\text{Infusion rate (R)}}{\text{Specific activity}}$

where R = 83 649 dpm/min

(2) Production rate (PR) $= \frac{R-((Q_1+Q_2)/2)\ ((SA_2-SA_1)/(t_2-t_1))}{(SA_1+SA_2)/2}$

Where Q_1 and Q_2 = concentration of lactic acid and SA_1 and SA_2 = specific activity at times t_1 and t_2 respectively

(3) Utilization rate $= PR-(dQ/dt)$

$= PR-(Q_2-Q_1)/(t_2-t_1)$

Further explanation of equations (2) and (3) is provided in the discussion

Turnover rate was low initially reaching a peak of 0,6 mM/min, 1½ hours after feeding and then decreasing sharply. The concentration of lactate were unusually low for this type of diet. It can be seen that equations (1) and (2) gave similar results although equation (2) cannot be used when rates are changing rapidly unless the time intervals are small. Equation (1) assumes steady state conditions which did not exist. Several important points emerged which were incorporated into subsequent experiments. Firstly the radioactivity in 1 ml CRF was low and clearly gave rise to counting errors resulting in overestimation of the SA. This was overcome by extracting 10 ml quantities of CRF to increase counts. Sampling times were also changed so that a baseline could be established before feeding and so that sampling intervals were shorter when peak lactate fermentation occurred 0-2 h after feeding. This would also allow valid use of equations (2) and (3).

A preliminary single injection experiment was then carried out on Sheep A18 which was normally fed the Intermediate diet, but on the day of the experiment was fed the High concentrate diet in order to boost the amount of lactic acid produced. The results are presented in Table 8.4 and Fig. 8.4. Table 8.4 is a summary of the data given in Appendix Table 8.1. Because of mixing and sampling problems which are much more pronounced with single injection experiments the values for activity and hence specific activity fluctuate. This gives rise to negative values for production rate which is not possible. However, these values can be interpreted by assuming the sign to be positive and the utilization rate increases by a corresponding amount. This manipulation changes the values for the rates of production and utilization but the difference in the rates remains the same. This difference in rates is responsible for the observed concentration values. The results show that the concentration of lactate increased to 10,43 mM within the first ¼ h after feeding indicating rapid production in this time interval. Utilization rates were also rapid especially between ¼-¾ h after feeding causing the sharp decrease in lactate concentration.

/Table 8.4. .....

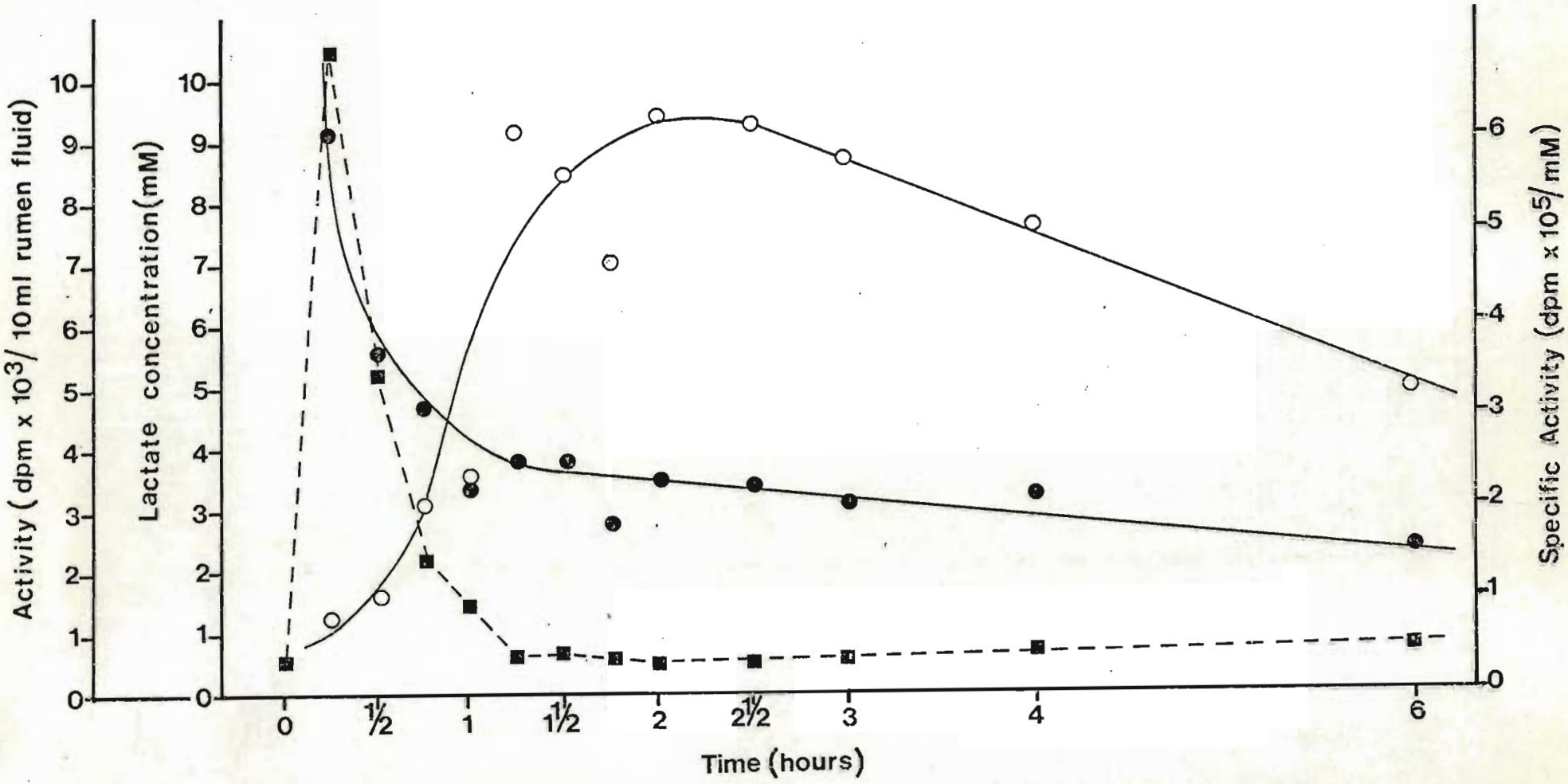

Fig.8.4. Lactate concentration (--■--), activity (—●—) and specific activity (—○—) in the rumen of sheep A18 adapted to an intermediate diet but fed 750 g high concentrate diet immediately before dosing with DL-[2-^{14}C]-lactate as a single injection

Table 8.4. Estimated turnover rate of DL-lactate in the rumen of Sheep A18 adapted to an Intermediate diet but fed a High concentrate diet before dosing with DL-(2-^{14}C)-lactate as a single injection

Time after feeding (h)	Conc of lactate (mM)	$^{\Phi}$Claculated rates (mM/min) Production (2)	Utilization (3)
0	0,56		
¼	10,43	0,102	0,449
½	5,23	0,170	0,375
¾	2,15	0,009	0,054
1	1,48	0,062	0,119
1¼	0,62	0,003	0,000
1½	0,67	0,008	0,012
1¾	0,61	0,012	0,015
2	0,56	0,001	0,001
2½	0,54	0,001	0,001
3	0,54	0,003	0,000
4	0,64	0,002	0,002
6	0,71		

$^{\Phi}$Calculated using the formulae given in the footnote to Table 8.3 except that for a single injection R = 0 and equation (2) becomes

$$\text{Production rate} = \frac{-((Q_1+Q_2)/2)((SA_2-SA)/(t_2-t_1))}{(SA_1+SA_2)/2}$$

Production and utilization rates of D- and L-lactic acid

The rates of production and utilization of the two isomers of lactic acid are presented in Table 8.5 for sheep A30 and in Table 8,6 for sheep A18. The production and utilization rates were higher for L-lactate than for D-lactete. The concentration of L-lactate was also greater than D-lactate. The production and utilization rates for the

/Table 8.5. .....

Table 8.5. The production and utilization rates of D- and L-lactic acid in the rumen of Sheep A30 fed a High concentrate diet as measured by the continuous infusion of DL-(2-^{14}C)-lactate

Time after feeding (h)	Conc of lactate (mM)		Production rate (2) (mM/min)		Utilization rate (3) (mM/min)	
	D-	L-	D-	L-	D-	L-
-1 ¼	0,19	0,20				
			0,006	0,013	0,006	0,012
-½	0,22	0,23				
			0,007	0,009	0,006	0,011
0	0,26	0,19				
			0,351	0,484	0,011	0,009
¼	5,37	7,32				
			0,112	0,100	0,234	0,284
½	3,54	4,54				
			0,082	0,176	0,148	0,221
¾	2,54	3,87				
			0,001	0,101	0,064	0,187
1	1,60	2,58				
			0,083	0,118	0,171	0,261
1 ¼	0,28	0,43				
			0,007	0,001	0,008	0,012
1 ½	0,26	0,26				
			0,003	0,009	0,006	0,010
1 ¾	0,22	0,24				
			0,016	0,023	0,015	0,017
2	0,24	0,33				
			0,002	0,013	0,002	0,014
2 ½	0,23	0,29				
			0,011	0,015	0,010	0,015
3	0,26	0,29				
			0,010	0,014	0,010	0,014
4	0,24	0,27				
			0,009	0,013	0,009	0,014
6	0,22	0,21				
			0,005	0,011	0,004	0,011
8	0,26	0,22				

sheep on the other three diets were similar for both isomers of lactic acid. The concentration of the two isomers was also very similar.

/Table 8.6. .....

Table 8.6. The production and utilization rates of D- and L-lactic acid in the rumen of Sheep A18 adapted to an Intermediate diet but fed on High concentrate diet before dosing with DL-(2-^{14}C)-lactate

Time after feeding (h)	Conc of lactate (mM)		Production rate (2) (mM/min)		Utilization rate (3) (mM/min)	
	D-	L-	D-	L-	D-	L-
0	0,26	0,30	-	-	-	-
¼	4,21	6,22				
			0,054	0,003	0,213	0,190
½	1,82	3,41				
			0,058	0,141	0,131	0,274
¾	0,73	1,42				
			0,002	0,023	0,015	0,055
1	0,54	0,94				
			0,026	0,077	0,043	0,117
1¼	0,28	0,34				
			0,005	0,003	0,003	0,001
1½	0,30	0,37				
			0,021	0,003	0,021	0,006
1¾	0,29	0,32				
			0,021	0,004	0,023	0,005
2	0,26	0,30				
			0,001	0,001	0,000	0,002
2½	0,28	0,26				
			0,004	0,002	0,005	0,002
3	0,27	0,27				
			0,008	0,007	0,006	0,007
4	0,38	0,26				
			0,002	0,004	0,002	0,004
6	0,43	0,28				

The correlations between concentration and the rates of production and utilization for the two isomers are shown in Fig. 8.5 for Sheep A30 fed the High concentrate diet. For the purposes of clarity only the linear regression lines obtained from the data are plotted. The correlation coefficients obtained for both Sheep A30 and A18 are given in Table 8.7. All correlations were highly significant at the 99% level except for the correlation between L-lactic acid concentration and the production rate of L-lactic acid. In general, higher correlation coefficients were obtained from the data of Sheep A30 than from Sheep A18.

/Table 8.7. .....

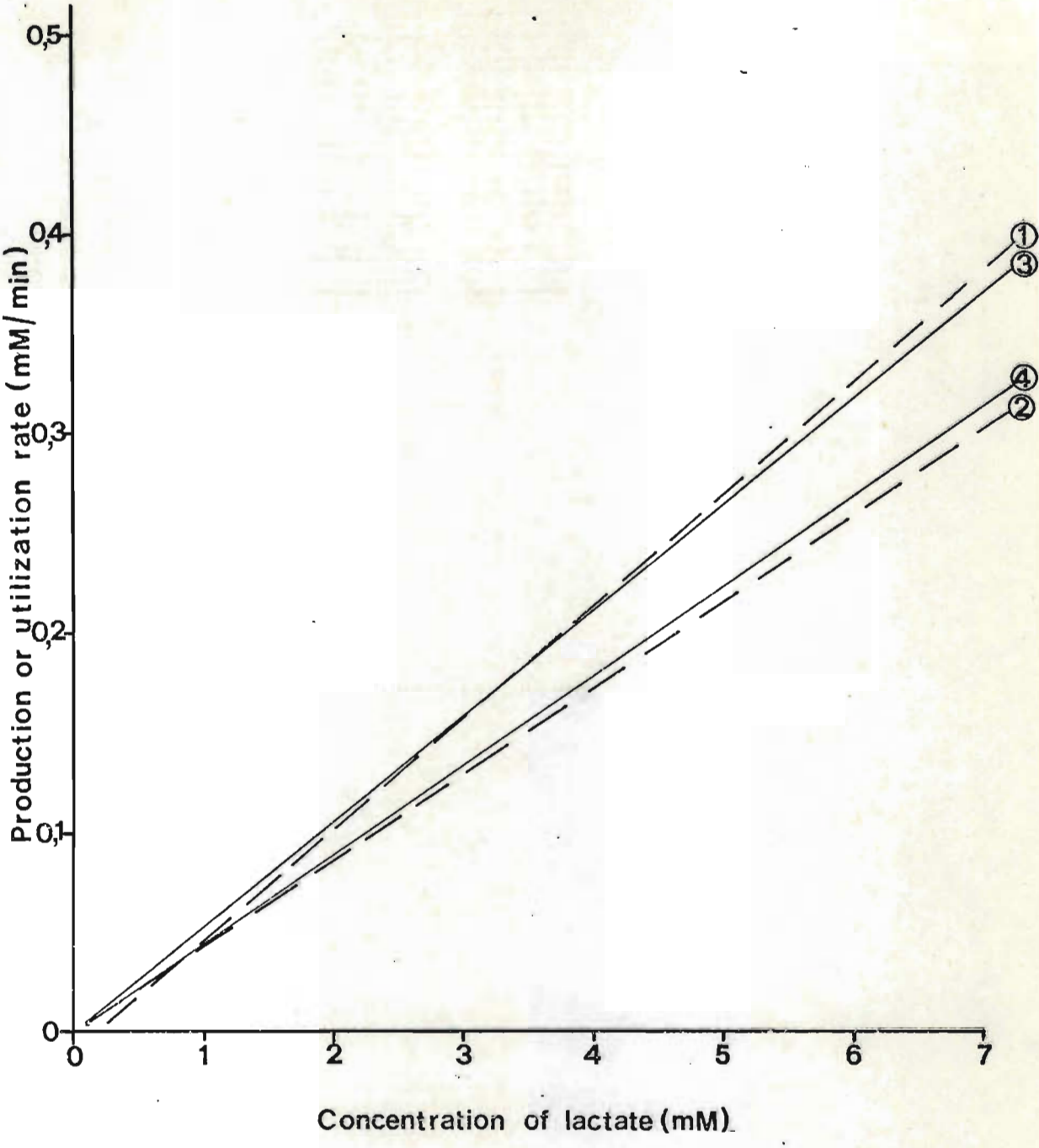

Fig.8.5. Linear regression lines calculated from the data for D- and L-lactate concentration and the production or utilization rates of each isomer in the rumen of sheep A30 fed a high concentrate diet. The regression lines are as follows:
①D-lactate concentration vs production rate of D-lactic acid;
②D-lactate concentration vs utilization rate of D-lactic acid;
③L-lactate concentration vs production rate of L-lactic acid;
④L-lactate concentration vs utilization rate of L-lactic acid.

Table 8.7. The correlation coefficients and their statistical significance for the correlation between D- and L-lactic acid concentration and their production or utilization rates in the rumen of Sheep A30 and A18 fed the High concentrate diet

Sheep	Correlation between Lactate isomer:	Rate of:	Correlation coefficient	Statistical significance[1]
A30	D-	Production	0,945	ΦΦ
	D-	Utilization	0,913	ΦΦ
	L-	Production	0,902	ΦΦ
	L-	Utilization	0,923	ΦΦ
A18	D-	Production	0,686	Φ
	D-	Utilization	0,969	ΦΦ
	L-	Production	0,155	NS
	L-	Utilization	0,824	ΦΦ

[1] ΦΦ = P>0,99, Φ = P>0,95, NS = not significant

The turnover of lactic acid in the rumen

The turnover of lactic acid in the rumen of sheep fed different amounts of RFC was calculated from a series of continuous infusion and single injection experiments. Figures 8.6 and 8.7, illustrating the production and utilization of lactic acid, were constructed from the means of the results (Appendix Table 8.2) of three continuous infusion experiments in respect of each sheep. In Fig. 8.6 the production and utilization rates for Sheep A30 fed the High concentrate diet are plotted on the same axes. When production exceeds utilization, lactic acid accumulates. If utilization exceeds production, lactate disappears and the accumulated lactate will decrease in concentration.

The production of lactic acid in the rumen, shown graphically in Fig. 8.7 varied depending on the amount of RFC in the diet. On the diet containing 65% grain and molasses the production rate at peak was 0,631 mM/min. On the Lucerne hay diet the value at peak production was 0,164 mM/min. The peak production of lactate on the diet containing 10% molasses was spread over a wider time than the lucerne peak but was not much higher, reacing a value of 0,187 mM/min. Sheep A84 fed a diet containing 43% grain and molasses had a peak lactate production value of 0,304 mM/min which was approximately half that on the High concentrate diet. The trend on all diets was similar with a marked increase imme-

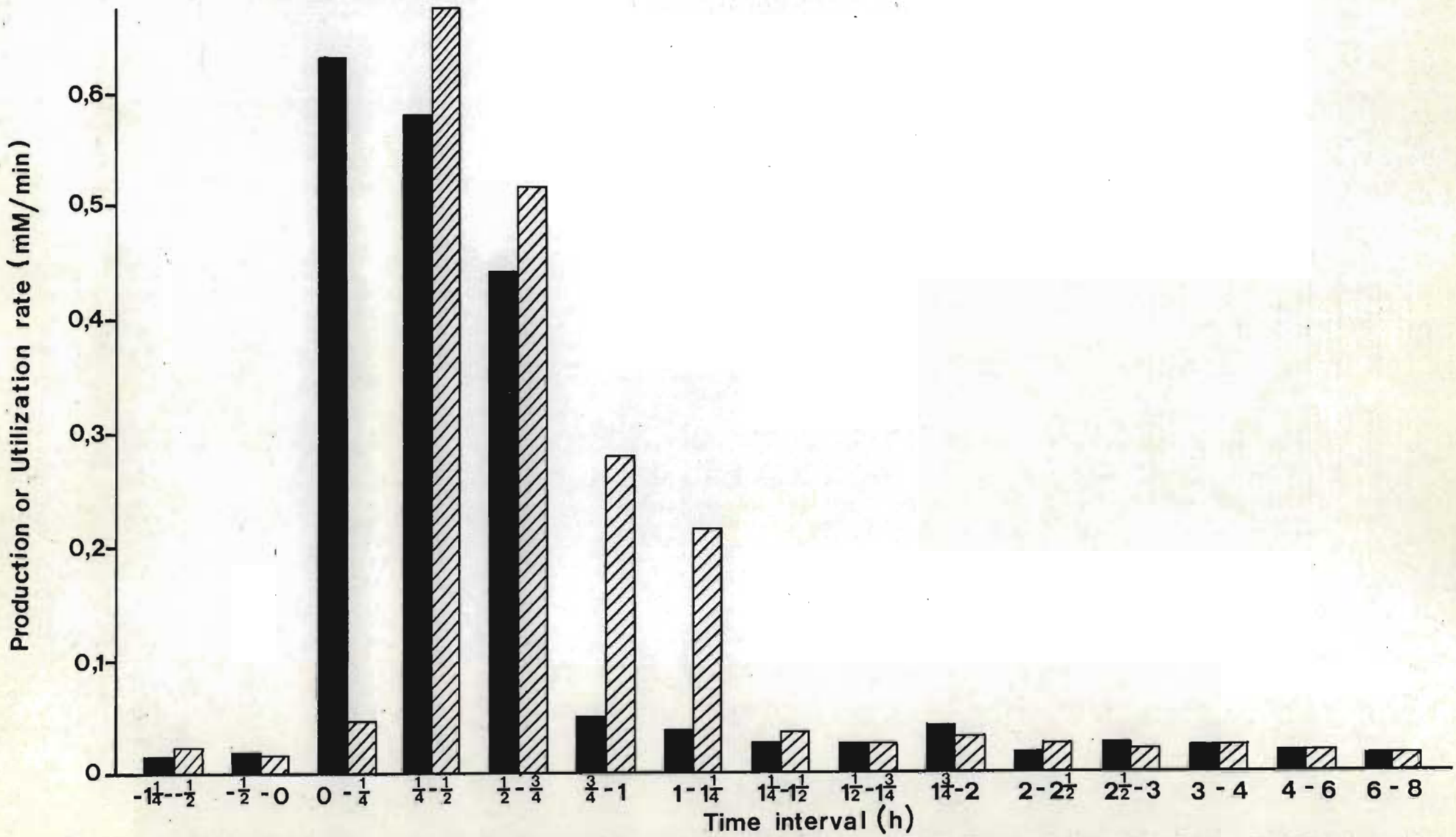

Fig.8.6. The mean production and utilization rates of DL-lactic acid in the rumen of sheep A30 fed a high concentrate diet. (■-Production rate, ▨-Utilization rate)

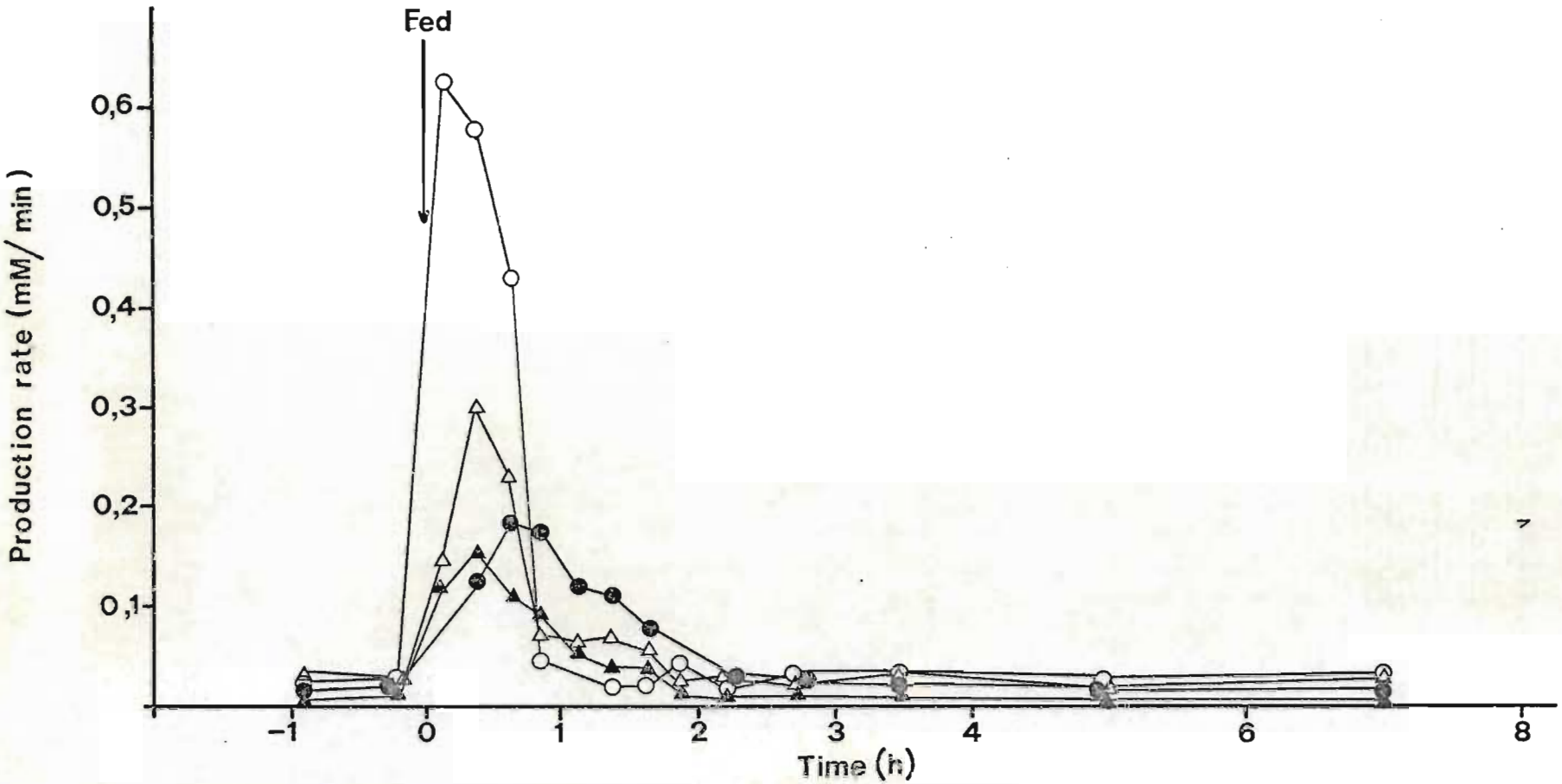

Fig.8.7. The mean production rate of DL-lactic acid in the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate (○-High concentrate diet (65% grain & molasses); △- Intermediate diet (43% grain & molasses); ●-High roughage diet (10% grain & molasses); ▲-Lucerne hay diet)

diately after the feed and a return to the basal level *ca* 2 h after the feed. The basal levels showed the same order as the peak production values but differences were much smaller. The basal levels on all diets ranged from 0,01-0,02 mM/min. This value was at least 10-300 fold less than peak production depending on diet. Utilization rates were high on diets containing added RFC and at times when production was high although with a small time lag. The time lag was probably less than the 15 min interval used to sample at peak lactate fermentation.

Linear regression lines were fitted to the values for DL-lactate production rate and concentration, concentration and utilization rate and production rate and utilization rate for each of the diets. The overall correlation coefficients for all diets in each of these three categories were also calculated. The results are presented in Table 8.8

Table 8.8. Correlation coefficients and their statistical significance for correlations between production rate and concentration, concentration and utilization, and production rate and utilization rate of DL-lactic acid in the rumen of sheep fed diets differing in amounts of readily fermentable carbohydrate

Correlation between	Diets	Correlation coefficient	Statistical significance[1]
Production rate and concentration of DL-lactic acid	High concentrate	0,978	ΦΦ
	Intermediate	0,992	ΦΦ
	High roughage	0,981	ΦΦ
	Lucerne hay	0,971	ΦΦ
	All diets	0,967	ΦΦ
Concentration and utilization rate of DL-lactic acid	High concentrate	0,704	ΦΦ
	Intermediate	0,908	ΦΦ
	High roughage	0,931	ΦΦ
	Lucerne hay	0,879	ΦΦ
	All diets	0,749	ΦΦ
Production rate and utilization rate of DL-lactic acid	High concentrate	0,676	ΦΦ
	Intermediate	0,882	ΦΦ
	High roughage	0,960	ΦΦ
	Lucerne hay	0,921	ΦΦ
	All diets	0,746	ΦΦ

[1] ΦΦ = P>0,99

with their statistical significance. All correlations were significant at the 99% level although the highest correlations were found between the production rate of DL-lactic acid and its concentration in the rumen of all diets with the values of r between 0,97-0,99. In order to plot the values for all diets in Fig. 8.8 only the linear regression lines obtained from the data for production and concentration of DL-lactic acid are given.

The turnover of lactic acid in the rumen of the sheep fed diets containing different amounts of RFC was also determined in two single injection experiments using DL-(2-^{3}H)-lactate. The results in Table 8.9 are a summary of the data given in Appendix Table 8.3. In general the rates of production and utilization were lower than during the continuous infusion experiments especially on the High concentrate diet. However, the concentration of lactic acid was also lower during the single injection experiments than during the continuous infusion experiments. On the High concentrate diet peak lactate concentration was 3,61 mM and peak production rate was 0,117 mM/min in contrast to the continuous infusion experiments where the values were 9,30 mM and 0,631 mM/min respectively. In the single injection experiments the peak utilization rate corresponded to the peak in lactate concentration whereas in the continuous infusion experiments peak lactate concentration corresponded to peak production rate. Thus although there was probably a lower production rate of lactate during the single injection experiments, this method of determining turnover is not as reliable as the continuous infusion method most probably because of mixing and sampling problems in the rumen. These problems are probably worse on the roughage diets.

Contribution of lactate to the production of individual volatile fatty acids

In the preliminary experiment the contribution of lactate to the production of the individual VFA was calculated in an indirect way. The activity of ^{14}C in each of the acids at various times after feeding was determined and used to calculate the percentage activity in each acid. From the calculated turnover rate in Table 8.3, the production of the individual VFA through lactate was calculated. The results are presented in Table 8.10. The production rate of total VFA was calculated by the zero time-rate method (page 95) on the values obtained from the *in vitro* fermentation of ingesta obtained at the same sampling times.

/Table 8.10. .....

Table 8.9. The mean production and utilization rates of DL-lactic acid in the rumen as determined by the single injection technique

Time after feeding (h)	Sheep A30 High concentrate diet			Sheep A84 Intermediate diet			Sheep A28 High roughage diet			Sheep A21 Lucerne hay diet		
	Conc of lactate (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of lactate (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of lactate (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of lactate (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)
0	0,63	-	-	0,65	-	-	0,48	-	-	0,44	-	-
¼	1,48	0,117	0,000	1,98	0,081	0,052	1,07	0,090	0,000	0,88	0,036	0,031
½	3,23	0,104	0,079	2,41	0,040	0,111	2,42	0,043	0,096	0,95	0,019	0,006
¾	3,61	0,068	0,103	1,35	0,020	0,039	1,62	0,012	0,030	1,14	0,010	0,005
1	3,09	0,028	0,059	1,06	0,010	0,015	1,35	0,004	0,016	1,06	0,006	0,011
1¼	2,62	0,012	0,066	0,99	0,005	0,016	1,17	0,008	0,021	0,98	0,007	0,012
1½	1,81	0,001	0,033	0,82	0,005	0,000	0,97	0,012	0,023	0,90	0,001	0,004
1¾	1,33	0,004	0,026	0,89	0,003	0,013	0,81	0,006	0,012	0,85	0,005	0,011
2	1,00	0,002	0,011	0,74	0,001	0,007	0,72	0,001	0,004	0,76	0,004	0,009
2½	0,72	0,002	0,002	0,57	0,002	0,003	0,63	0,003	0,007	0,61	0,001	0,001
3	0,70	0,001	0,003	0,53	0,000	0,001	0,52	0,001	0,001	0,60	0,001	0,002
4	0,59	0,002	0,003	0,48	0,001	0,000	0,52	0,000	0,000	0,54	0,001	0,002
6	0,55	0,001	0,001	0,60	0,002	0,002	0,57	0,001	0,001	0,47	0,000	0,000
8	0,53			0,62			0,59			0,43		

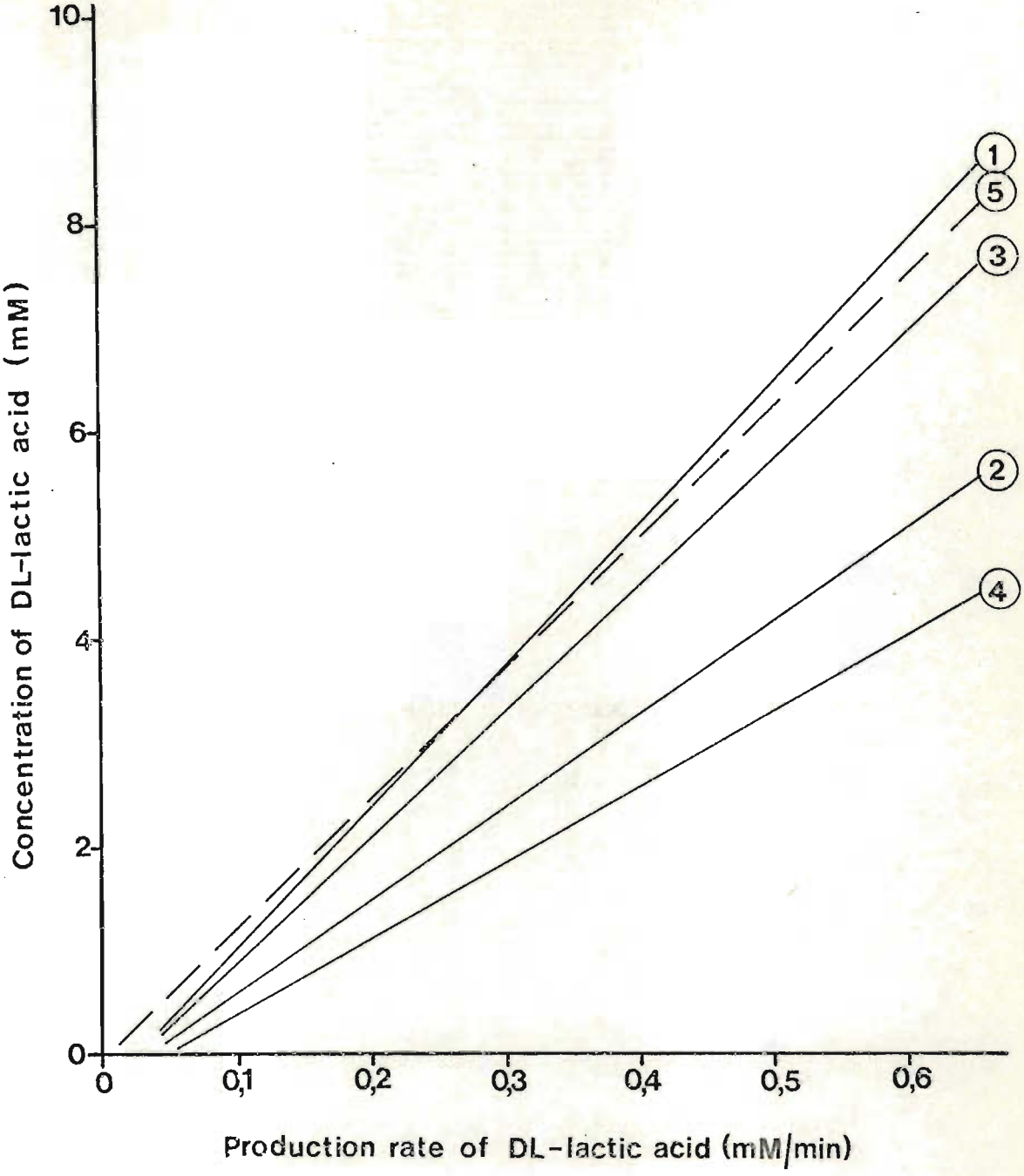

Fig.8.8. Linear regression lines representing the correlation between production rate and concentration of DL-lactic acid in the rumen of sheep fed different diets. The regression lines are as follows: ① High concentrate diet ; ② Intermediate diet ; ③ High roughage diet; ④ Lucerne hay diet and ⑤ values for all diets combined (broken line)

Table 8.10. Production of individual VFA from DL-(2-^{14}C)-lactate in the rumen of Sheep A30 during the preliminary experiment (C_2 = acetic, C_3 = propionic and C_4 = butyric acids)

Time after feeding (h)	Activity (dpm of ^{14}C) in individual VFA			Percentage activity in individual VFA			Production of individual VFA through ^{14}C-lactate (mM/min)		
	C_2	C_3	C_4	C_2	C_3	C_4	C_2	C_3	C_4
1	311	273	142	42,8	37,6	19,6	0,139	0,122	0,064
1½	247	214	110	43,3	37,5	19,3	0,255	0,221	0,114
2	243	188	97	46,0	35,6	18,4	0,012	0,010	0,005
3	258	205	93	46,4	36,9	16,7	0,013	0,011	0,005
4	224	198	91	43,7	38,6	17,7	0,004	0,004	0,002

The production rates of the individual VFA were calculated from the molar proportions of the acids which were found to be 62,2% C_2, 20,6% C_3 and 17,3% C_4. This calculation also assumes that the concentrations of the individual VFA are directly proportional to the production rate. The ratio of production of the individual VFA through lactate, to that estimated from the production rate of total VFA, is a measure of the proportion formed via lactate as an intermediate and the results are given in Table 8.11.

Table 8.11. Percentage of the individual VFA formed through lactate as an intermediate in the rumen of Sheep A30 during the preliminary experiment

Time after feeding (h)	Production rate of total VFA (mM/min)	Production rate of individual VFA (mM/min)			Percentage of individual VFA formed via lactate		
		C_2	C_3	C_4	C_2	C_3	C_4
1	1,64	1,02	0,34	0,28	13,6	35,9	11,4
1½	1,55	0,96	0,32	0,27	26,6	69,1	21,1
2	1,22	0,76	0,25	0,21	1,6	4,0	1,2
3	1,24	0,77	0,26	0,21	1,7	4,2	1,2
4	0,92	0,57	0,19	0,16	0,7	2,1	0,6

The contribution of lactate to the production of the individual VFA was calculated in the same way for samples taken ¼-½, 1-1½ and 1¾-2½ h after feeding during the series of continuous infusion experiments. The mean percentage of the individual VFA formed through lactate for the

different diets is given in Table 8.12. The full table of values used in the calculation of Table 8.10 is given in Appendix Table 8.4.

Table 8.12. Mean percentage of the individual VFA formed through lactate on the different diets during the continuous infusion experiments and estimated by the indirect method

Diet	Individual VFA	Time after feeding (h) $\frac{1}{4}$ - $\frac{1}{2}$	1 - $1\frac{1}{2}$	$1\frac{3}{4}$ - $2\frac{1}{2}$
High concentrate diet	C_2	40,5	16,2	1,7
	C_3	75,2	35,4	4,0
	C_4	33,8	14,0	1,6
Intermediate diet	C_2	20,5	6,8	1,9
	C_3	33,3	11,7	3,1
	C_4	16,2	4,1	1,0
High roughage diet	C_2	12,2	12,5	1,8
	C_3	28,0	25,9	3,3
	C_4	11,8	9,6	1,3
Lucerne hay diet	C_2	-$^{\Phi}$	17,6	5,6
	C_3	-	12,7	6,1
	C_4	-	10,0	5,4

$^{\Phi}$- denotes not determined

The contribution of lactate to the formation of the individual VFA was also calculated in a more direct way as $SA_{acetate}/SA_{lactate}$ for acetate and similarly for propionate and butyrate. A summary of the results given in Appendix Table 8.5 is presented in Table 8.13. The method using ratios of specific activities gave results which were lower than the indirect method. At peak lactate fermentation on the High concentrate diet the estimation was 16,1 to 21,9 percentage units greater for the different VFA using the indirect method. The agreement between the two methods was good for the Intermediate and High roughage diets and also on all diets at the other time intervals considered. It was also possible to estimate the contribution of lactate to the individual VFA $\frac{1}{2}$ h before feeding and 6 h after feeding by the direct method.

/Table 8.13. .....

Table 8.13. The mean percentage of the individual VFA formed through lactate on the different diets during the continuous infusion experiment and calculated from the ratio of specific activity in lactate to that in the individual VFA

Diet	Individual VFA	½ h before feeding	Time after feeding (h) ¼ - ½	1 - 1½	1¾ - 2½	6
High concentrate diet	C_2	1,9	23,9	23,9	1,7	1,0
	C_3	2,8	53,3	32,3	4,6	1,1
	C_4	1,0	17,7	11,0	1,5	1,7
Intermediate diet	C_2	2,5	19,5	13,1	9,2	1,7
	C_3	3,0	35,3	22,5	14,0	2,8
	C_4	2,0	15,9	7,7	4,2	0,9
High roughage diet	C_2	1,4	14,6	12,4	8,7	1,8
	C_3	2,5	28,1	26,7	14,3	2,0
	C_4	0,9	11,4	10,4	6,5	1,3
Lucerne hay diet	C_2	1,8	-$^{\Phi}$	10,5	1,0	1,2
	C_3	1,6	-	6,9	1,9	0,9
	C_4	1,4	-	6,5	2,3	1,2

$^{\Phi}$- denotes not determined

From the results in Table 8,13 it can be seen that at peak lactate fermentation ¼-½ h after feeding 23,9% of acetate, 53,3% of propionate and 17,7% of butyrate were formed through lactate on the High concentrate diet. The corresponding values were 19,5%, 35,3% and 15,9% on the Intermediate diet. On the High roughage diet the values were 14,6%, 28,1% and 11,4% respectively. On the Lucerne hay diet the values were lowest. At the 1-1½ h time interval the values were between 30% to 50% of the peak values, but by 1¾-2½ h after feeding they had decreased to less than half of the peak values. Values 6 h after feeding were actually slightly lower than those found ½ h before feeding. Values ½ h before feeding and 6 h after feeding were approximately 5% TO 10% of those found at peak fermentation.

The pattern was similar on all three diets containing added RFC where lactate made a larger contribution to the formation of propionate than to acetate. This difference was most marked at peak fermentation.

/On the Lucerne .....

On the Lucerne hay diet this pattern was reversed and the contribution of lactate to the formation of acetate was larger than to propionate. In all cases, except 6 h after feeding, the contribution of lactate to the formation of acetate was greater than to butyrate.

Lactate load experiments

Lactate load experiments were repeated twice on each of 4 sheep fed the different diets. Typical lactate disappearance curves for D- and L-lactic acid are presented in Fig. 8.9 for sheep A18 fed the Intermediate diet and sheep A31 fed the High roughage diet. Two hours after dosing the concentration of lactic acid had stabilized at between 2,2 and 3,8 mM of each isomer of lactic acid. In order to determine the rate of disappearance a linear regression line was fitted to the data (Appendix Table 8.6) over the first two hours using the method of least squares to obtain the best fit. The results obtained for the rates of disappearance of D-, L- and total (sum of D- and L-) lactic acid are given in Table 8.14. The proportion of D-lactate in the load

Table 8.14. The rate of disappearance of a lactate load dosed into the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate

Diet	Lactate isomer	Rate of disappearance (mM/h)	(mM/min)
High concentrate diet	D-	7,75	0,13
	L-	12,14	0,20
	Total	19,89	0,33
Intermediate diet	D-	11,43	0,19
	L-	17,14	0,29
	Total	28,57	0,48
High roughage diet	D-	18,91	0,32
	L-	18,33	0,31
	Total	37,24	0,62
Lucerne hay diet	D-	16,60	0,28
	L-	16,67	0,28
	Total	33,26	0,56

dosed was 41,6% i.e. L-lactate 58,4%. Thus concentrations of L-lactate were higher and also disappeared faster on the High concentrate and Intermediate diets. On the High roughage and Lucerne hay diets the rates of disappearance of the two isomers were the

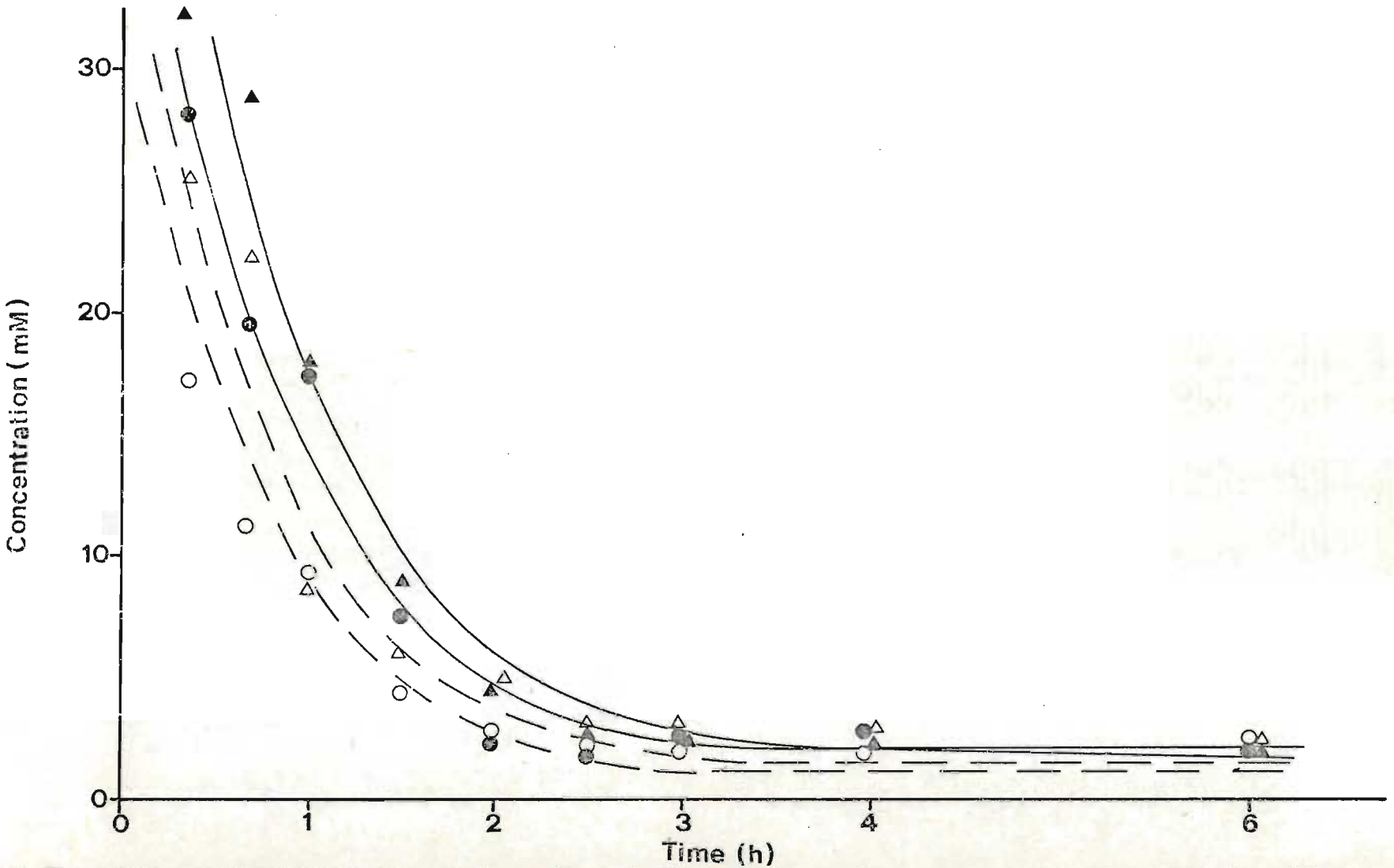

Fig.8.9. The rate of disappearance of D-and L-lactic acid from the rumen of sheep fed the intermediate and high roughage diets. (L-lactate —●— D-lactate --○-- } Intermediate diet ; L-lactate —▲— D-lactate --△-- } High roughage diet.)

same. The rates of disappearance were higher on the High roughage diet than on any of the other diets although they were not much higher than the Intermediate and Lucerne hay diets. The rates of disappearance were lowest on the High concentrate diet which contained 65% grain and molasses.

The rates of disappearance obtained in the lactate load experiments were of the same order as the utilization rates determined during the continuous infusion experiments. On the High concentrate diet the rate of disappearance for the lactate load experiment was half that determined in the continuous infusion experiments. For the other diets this trend was reversed with higher estimates (2-4 times) being found in the lactate load experiments than utilization rates in the continuous infusion experiments.

CONCLUSIONS

The main findings are summarized in point form below although the results will be discussed in more detail in Chapter 12.

1. The turnover of lactic acid was determined in the rumen of sheep adapted to diets differing in amount of RFC. Preliminary experiments on sheep fed the High concentrate diets showed that mathematical equations were available which allow the estimation of production and utilization rates which are in non-steady state. These equations are applicable to both continuous infusion and single injection type experiments.
2. The rates of production and utilization of the two isomers of lactic acid were determined. The rates tended to be higher for L-lactic acid in the two sheep fed the High concentrate diet whereas on the other three diets the rates were very similar. The concentration of each isomer reflected the production rate of the isomer. Thus on the diets fed in the present experiment the rates of production and tuilization were essentially the same for both isomers of lactic acid.
3. The turnover of DL-lactic acid in the rumen using the continuous infusion technique was highest on the sheep fed the diet containing 65,0% grain and molasses. At peak lactate fermentation $\frac{1}{4}$ - $\frac{1}{2}$ h after feeding the production rate was 0,631 mM/min. On the two roughage diets the peak production rates were 0,164-0,187 mM/min while on the Intermediate diet containing 43% grain and molasses the value was 0,304 mM/min. Production rates tended to

return to the basal levels of 0,01-0,02 mM/min *ca* 2 h after feeding. Unitlization rates followed the same trend as the production rates although with a short time lag. The correlation between lactate production and lactate concentration was high (r = 0,967).

4. Turnover rates measured using the single injection technique were not as reliable with fluctuations in values probably due to mixing and sampling problems. However, the production and utilization tended to be lower than for the continuous infusion experiments. This was related to lower concentrations of lactic acid in the rumen.

5. The contribution of lactate as an intermediate in the formation of VFA was estimated by two methods. In the indirect method the production of the individual VFA was estimated from a total VFA production rate and the molar proportions of the individual VFA. The production of the individual VFA through lactate was estimated from the percentage label in the individual VFA and the production rate of lactic acid. The ratio of the two estimates gave a measure of the contribution of lactate to the production of acetate, propionate and butyrate. In the direct method the ratio of specific activities of lactate to acetate, propionate or butyrate was used. The indirect method gave results which agreed reasonably well with the direct method except at peak fermentation on the High concentrate diet. From the results obtained using the direct method the contribution of lactate to the production of the individual VFA was dependent not only on amount of RFC but also with time after feeding. At peak fermentation $\frac{1}{4}$-$\frac{1}{2}$ h after feeding the values ranged from 14,6-23,9%, 28,1-53,3% and 11,4-17,7% for acetate, propionate and butyrate respectively depending on diet. For the Lucerne hay diet the values were lower than the High roughage diet which contained 10% grain and molasses.

6. Lactate load experiments were performed on a different group of sheep but under identical conditions of feeding and management. The lactate load contained 58,4% of L-lactate and hence L-lactate concentrations were higher in the rumen. On the High concentrate and Intermediate diets L-lactate disappeared slightly faster than D-lactate whereas on the other two diets the rates for the two isomers were the same. The rates of disappearance obtained in the lactate load experiments were of the same order as the utilization rates determined during the continuous infusion experiments.

CHAPTER 9. TURNOVER OF FREE GLUCOSE IN THE RUMEN IN RELATION TO LACTATE TURNOVER

INTRODUCTION

Although some work has been done on the *in vitro* disappearance of glucose added to ruminal ingesta, no work has been done on the ruminal turnover of free glucose *in vivo*. A number of studies have been published concerning the utilization of soluble carbohydrates by mixed populations of organisms from the rumen (Baldwin *et al*, 1963; Sutton, 1968, 1969: Wallnöfer *et al*, 1966) but in all cases the amount of carbohydrate added was much greater than the size of the carbohydrate pool which usually occurs within the rumen. Under these conditions the fermentation may become abnormal and does not necessarily reflect the fate of carbon arising from the degradation of plant polysaccharides in the rumen of animals fed under normal conditions. Slyter (1976) postulated that at ruminal pH's between 5-6 the rate of glucose release from polymers increases while the utilization rate decreases. The latter condition leads to an accumulation of free glucose in the rumen, allowing proliferation of lactic acid-producing bacteria and the increased production of lactic acid. These factors would induce lactic acidosis.

There is thus a close interrelationship between the turnover of free glucose in the rumen and that of lactic acid. In an attempt to study this relationship, simultaneous determinations were made *in vivo* of the turnover of free glucose and lactic acid in the rumen of sheep adapted to diets containing different amounts of RFC using a double label continuous infusion technique. Measurements were also made of lactate concentration *in vitro* when flasks containing ingesta were dosed with amounts of starch and glucose which were in the same proportion as the amount of RFC consumed by the animal. This was done in order to relate glucose concentration to concentrations of lactic acid produced, and the D-isomer in particular.

METHODS

Determination of specific activity of glucose

The clarified rumen fluid (CRF) samples obtained from the rumen of sheep during the simultaneous infusion of DL-(2-^{14}C)-lactate and

D-(6-^{3}H)-glucose reported in Chapter 8 were used. Glass columns containing a 15 x 1 cm resin bed of Dowex 1-X8 (Cl^- form) were prepared. Flow rate was adjusted to *ca* 1 ml/min by mixing washed Dowex resins in the ratio of 4 parts 50-100 mesh to 1 part 100-200 mesh (Schmidt, Smith & Young, 1975). Samples (3 ml) containing 22,5 mg (125 *u*mol) of carrier glucose were applied to the columns followed by 30 ml of water (pH 7,00). The first 3 ml of eluate was discarded while the following 30 ml was collected in 3x10 ml fractions directly into scintillation vials. These fractions contained the glucose (Fig. 9.1). Then 50 ml of 1,0 M-NaCl was run through the columns to elute the organic acids. For the initial calibration of the columns, collection of fractions was continued, but these fractions were discarded in the case of the CRF samples. The columns were then re-equilibrated with 30 ml of H_2O and used repeatedly for 5 samples before replacing the ion-exchange resin.

The concentration of glucose in the CRF samples was determined by a specific enzymic method using hexokinase and glucose-6-phosphate dehydrogenase (Roche).

The 10 ml fractions eluted from the columns directly into the scintillation vials were treated further by adding 10 ml of Instagel (Packard) and were placed in a Searle Mk III liquid scintillation spectrometer. The samples were allowed to stand in the spectrometer overnight before counting. Counting efficiencies were 83% for ^{14}C, used in the calibration solutions, and 16-20% for ^{3}H, in the CRF samples obtained from the turnover experiment.

Procedure for *in vitro* experiments

Two sheep were used as a source of ingesta. Sheep A63 received 2x750 g of Diet E and Sheep A52 the same amount of Diet B (refer to Table 5.1 for composition of the diets) which was all consumed. Representative samples of whole ruminal ingesta were removed from the rumen before feeding, using a scoop and 500 g of ingesta weighed into each of three prewarmed 1,5 l round-bottomed flasks at the sheep pens. An equal volume (100 ml) of warm anaerobic diluent containing dithiothreitol (see Appendix Table 5.1 for composition) was added to each flask. A sample was then taken from the rumen (0 h) and the sheep fed their respective diets. Samples were taken from the rumen ½, 1, 2 and 3½ h after the feed, using a sampling tube.

/The flasks .....

The flasks were taken to the laboratory and placed in a shaking water bath at 39°C while gassing continuously with 98% CO_2/2%H_2. The flasks were allowed to stabilize for 5 min before the zero-time sample was taken and the flasks dosed as follows: (1) 33 g finely ground food (Diet E or Diet B); (2) 17 g soluble starch for Diet E or 27 g soluble starch for Diet B plus diluent to make up to 33 g; (3) 17 g glucose for Diet E or 27 g glucose for Diet B plus diluent to make up to 33 g. The flasks were sampled ½, 1, 2 and 3½ h after dosing. Saturated Na_2CO_3 was added to maintain pH as close to initial pH as possible.

All samples were strained through 2 layers of cleesecloth and frozen in dry ice/acetone and subsequently analyzed for total VFA by steam distillation, free glucose by the GOD-Perid method (Boehringer) and D- and L-lactic acid using Method 1 (Chapter 2).

RESULTS

Separation of glucose from organic acids and recovery

The separation of ^{14}C-labelled glucose from acetate, propionate,

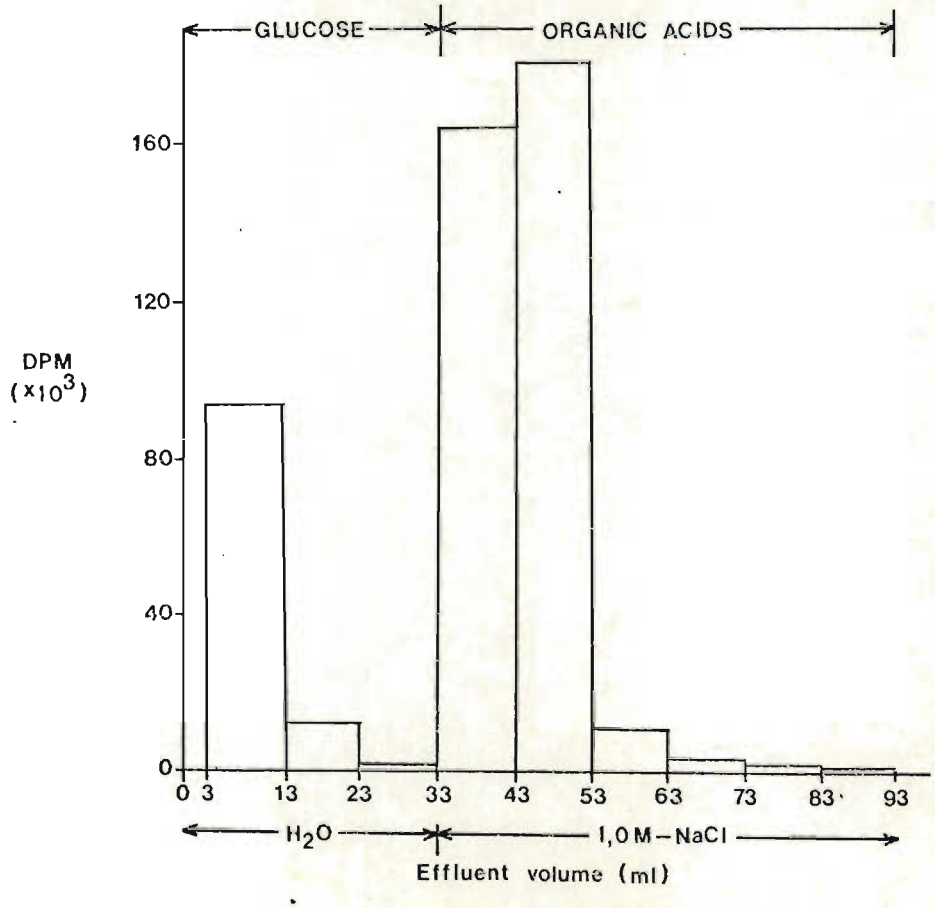

Fig. 9.1. Separation of glucose from acetate, propionate, butyrate and lactate for determination of glucose specific activity on Dowex 1-X8 (Cl- form)

/butyrate .....

butyrate and lactate (all with ^{14}C-label) achieved in the initial calibrations of the columns is shown in Fig. 9.1. Glucose was eluted with 3x10 ml fractions of water after discarding the first 3 ml (approximate void volume). The organic acids were then eluted with 50 ml of 1,0 M-NaCl. The mean recovery from 6 columns of total ^{14}C-radioactivity added to the columns was 98,2 ± 1,1%. The same 6 columns were then used again to check the recovery of ^{14}C-glucose when added alone. The mean recovery of ^{14}C-glucose was 96,3 ± 1,2% (n = 12).

The turnover of free glucose in the rumen

The turnover of free glucose in the rumen of the sheep fed the diets differing in amount of RFC was determined simultaneously with the turnover of lactic acid during the continuous infusion experiments. The mean results from two experiments are given in Table 9.1 (see Appendix Table 9.1 for full table of values). The turnover of free glucose in the rumen of the sheep fed the High concentrate diet in relation to glucose concentration (mM) and specific activity (dpm/mM) is illustrated in Fig. 9.2. Glucose concentration reached a peak value of 9,34 mM, ¾ h after feeding, while glucose turnover rate reached a peak of 0,57 mM/min only 1¼ h after feeding. As expected the glucose concentration curve and the specific activity curve were mirror images of each other.

The turnover of free glucose measured simply as infusion rate divided by specific activity, for the sheep fed diets differing in amount of RFC, is presented in Fig. 9.3. The turnover rates increased markedly immediately after feeding on the High concentrate diet but this effect was less marked on the other three diets. Turnover rates had decreased to basal levels *ca* 2-3 h after feeding. Turnover rates were lowest on the Lucerne hay diet and highest on the High concentrate diet. Values on the other two diets were between these two extremes with the values on the Intermediate diet being slightly higher but with more of a peak than the High roughage diet.

The values for production and utilization rates calculated from the equation of Shipley & Clarke (1972) in which non-steady state conditions are assumed are more suitable for comparison of rates between the different diets. On the High concentrate diet peak values of glucose production and utilization rates occurred ¾-1¼ h after feeding and were 0,59 and 0,63 mM/min respectively. The rates had returned to the basal level of 0,2-0,3 mM/min *ca* 3 h after the feed.

/Thus

Table 9.1. The mean concentration, production and utilization rate of free glucose in the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate as determined during continuous infusion of D-(6-^{3}H)-glucose

Time after feeding (h)	Sheep A30 High concentrate diet			Sheep A84 Intermediate diet			Sheep A28 High roughage diet			Sheep A21 Lucerne hay diet		
	Conc of glucose (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of glucose (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of glucose (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)	Conc of glucose (mM)	Prodn rate (mM/min)	Utilizn rate (mM/min)
-1½	6,21	0,26	0,27	2,05	0,15	0,11	1,89	0,09	0,09	0,67	0,03	0,04
-½	6,52	0,31	0,30	3,65	0,17	0,16	1,91	0,10	0,10	0,52	0,03	0,03
0	6,88	0,50	0,43	3,55	0,17	0,19	2,01	0,16	0,12	0,49	0,08	0,03
¼	7,96	0,56	0,53	3,87	0,24	0,17	2,64	0,12	0,12	1,20	0,09	0,08
½	8,52	0,53	0,48	4,97	0,22	0,23	2,67	0,13	0,14	1,41	0,06	0,08
¾	9,34	0,54	0,63	4,86	0,18	0,19	2,50	0,15	0,13	1,18	0,05	0,06
1	7,97	0,59	0,63	4,68	0,17	0,17	2,77	0,12	0,14	0,97	0,10	0,07
1¼	7,48	0,45	0,47	4,61	0,12	0,12	2,51	0,16	0,14	1,41	0,08	0,09
1½	7,25	0,36	0,35	4,67	0,15	0,18	2,88	0,15	0,17	1,12	0,08	0,07
1¾	7,38	0,40	0,41	4,23	0,12	0,14	2,62	0,12	0,14	1,32	0,05	0,06
2	7,25	0,36	0,36	4,03	0,14	0,15	2,29	0,12	0,12	1,07	0,04	0,04
2½	7,09	0,35	0,36	3,76	0,13	0,13	2,28	0,10	0,11	0,97	0,04	0,04
3	6,81	0,30	0,32	3,65	0,12	0,12	2,21	0,10	0,10	0,83	0,03	0,03
4	5,58	0,21	0,22	3,71	0,10	0,10	2,15	0,10	0,10	0,82	0,03	0,03
6	3,88			2,90			2,22			0,58		

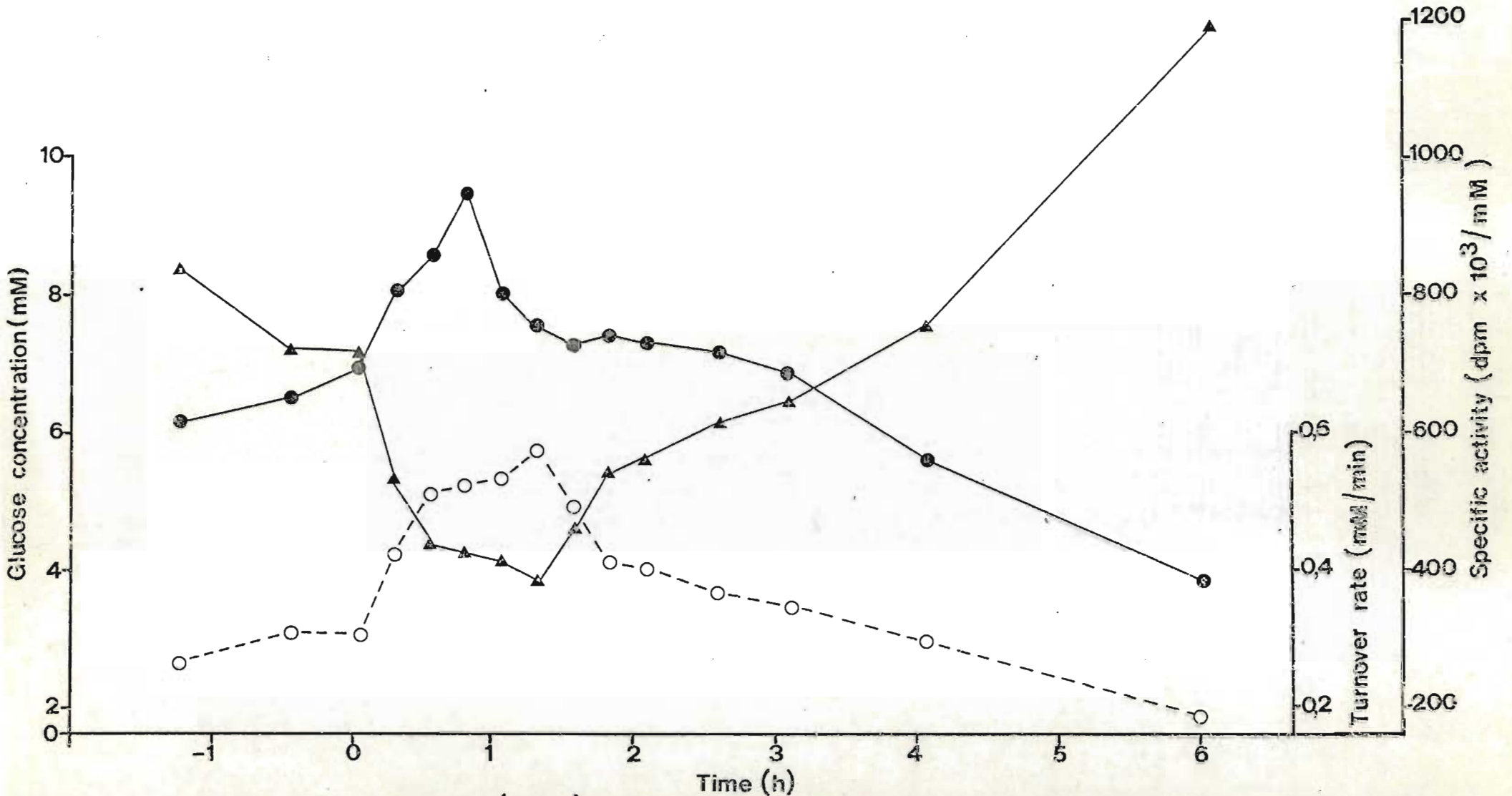

Fig.9.2.The turnover of free glucose (--○--) in the rumen of sheep A 30 fed a high concentrate diet in relation to glucose concentration (—●—) and specific activity (—▲—)

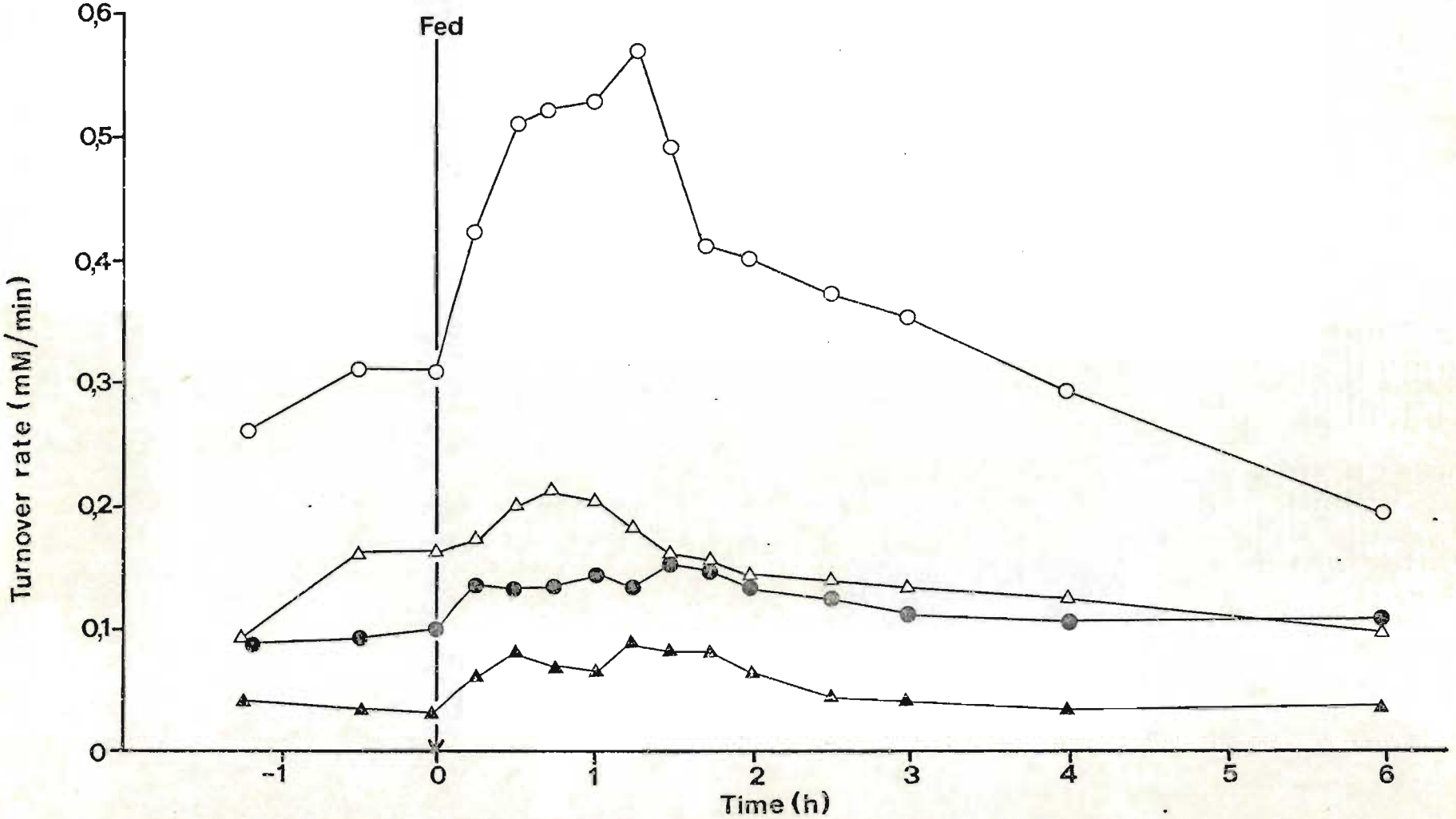

Fig.9.3. The turnover of free glucose in the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate. ((—○—) High concentrate diet; (—△—) Intermediate diet; (—●—) High roughage diet; (—▲—) Lucerne hay diet.)

Thus for this diet peak rates were only 2-3 times the basal rates. The peak values on the Intermediate diet occurred $\frac{1}{4}$-$\frac{3}{4}$ h after feeding and were 0,24 and 0,23 mM/min for production and utilization rates respectively. The basal levels on this diet were 0,10-0,15 mM/min. On the High roughage diet the basal rates were similar to those on the Intermediate diet with peak values of 0,16 and 0,17 mM/min for production rate and utilization rate respectively. Thus the increased rates at peak were less than twice the basal levels for the High roughage diet. Basal production and utilization rates were *ca* 0,03-0,05 mM/min on the Lucerne hay diet and were approximately double at peak fermentation. As the content of grain and molasses in the diet increased the basal rate of glucose production and utilization increased, the basal rate in the High concentrate diet being 5 times that on the Lucerne hay diet.

Relationship between turnover of free glucose and lactate turnover in the rumen

Correlations were determined between the rate of glucose utilization and the production rate of lactic acid determined simultaneously using the continuous infusion technique with the glucose (^{3}H) and lactic acid (^{14}C) labelled differently. The values obtained for the correlation coefficients and their statistical significance are presented in Table 9.2.

Table 9.2. The correlation coefficients between the rate of glucose utilization (mM/min) and the production rate of DL-lactic acid (mM/min) and their statistical significance on diets differing in amount of readily fermentable carbohydrate

Diet	Correlation coefficient	Statistical[1] significance
High concentrate	0,679	Φ
Intermediate	0,629	Φ
High roughage	0,592	Φ
Lucerne hay	0,471	NS
All diets combined	0,512	ΦΦ

[1] ΦΦ = P>0,99; Φ = P >0,95; NS = not significant

/The correlation .....

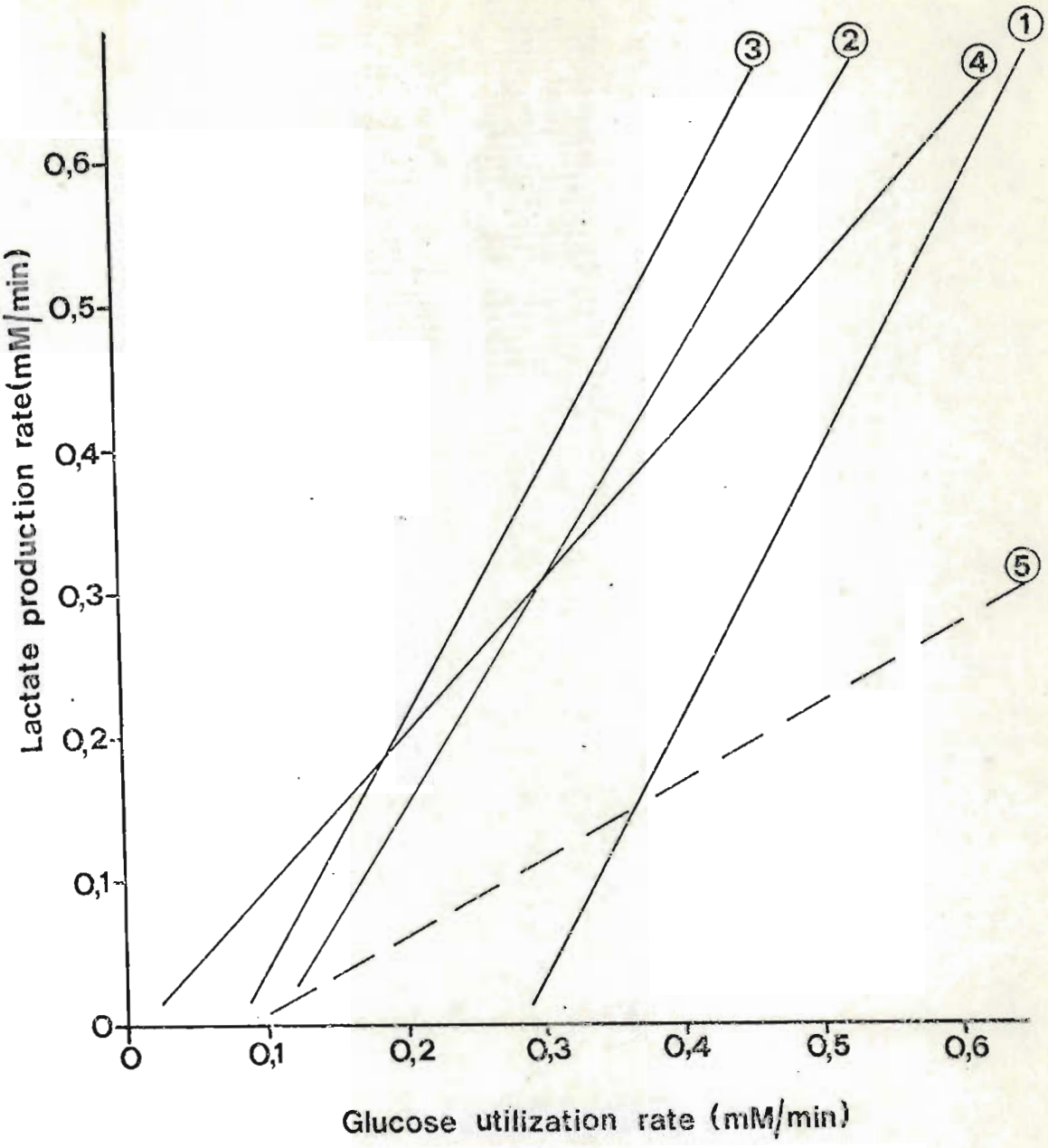

Fig.9.4. Linear regression lines for correlation between glucose utilization rate (mM/min) and lactate production rate (mM/min) in the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate. The regression lines are from ① High concentrate diet; ② Intermediate diet; ③ High roughage diet; ④ Lucerne hay diet and ⑤ all diets combined (broken line)

The correlation coefficient on the Lucerne hay diet was not significant while on the other three diets r was significant at the 95% level. When the data from all diets was combined the correlation became highly significant (P>0,99). The linear regression lines obtained by correlating the data are presented in Fig. 9.4. The regression lines from the diets containing grain and molasses all have similar slope and intersect the x-axis closer to the origin as the content of grain and molasses decrease.

In vitro experiments - the effect of dosing glucose and starch on the concentration of glucose and lactic acid

The amount of food dosed for the *in vitro* flasks was calculated assuming a 7,5 l rumen and that the sheep were fed 500 g twice daily. Thus the amount of food in 500 g of ingesta is equal to $^{0,5}/_{7,5}$ x 500 = 33 g if all the food is consumed quickly. Diet E is approximately 50% RFC and hence 33 g contains the equivalent of 17 g of starch or glucose. Diet B is approximately 80% RFC and hence 33 g contains the equivalent of 27 g of starch or glucose.

The effect of the different treatments on the concentration of D- and L-lactate and free glucose is shown in Fig. 9.5. For Diet E dosing the flask with 33 g of the same diet gave a similar pattern to that found *in vivo*. For Diet B dosing with 27 g starch gave an almost identical pattern of D- and L-lactic acid and free glucose. However, the most interesting results were obtained when the flasks were dosed with glucose. Free glucose concentrations were very high, 57,5 mM for Diet E and 56 mM for Diet B, and started to decrease after *ca* 1 h of incubation. The concentration of D-lactic acid increased to 10,7 mM for the 50% RFC diet and to 16,0 mM for the 80% RFC diet. The concentration of L-lactic acid only increased to 2,3 and 2,9 mM respectively i.e. 4-6 fold lower than the respective D-lactate concentrations.

CONCLUSIONS

The most important results are summarized below.

1. The turnover of free glucose in the rumen of sheep fed diets differing in amount of RFC was measured simultaneously with lactate turnover using a double label continuous infusion technique.

2./ .....

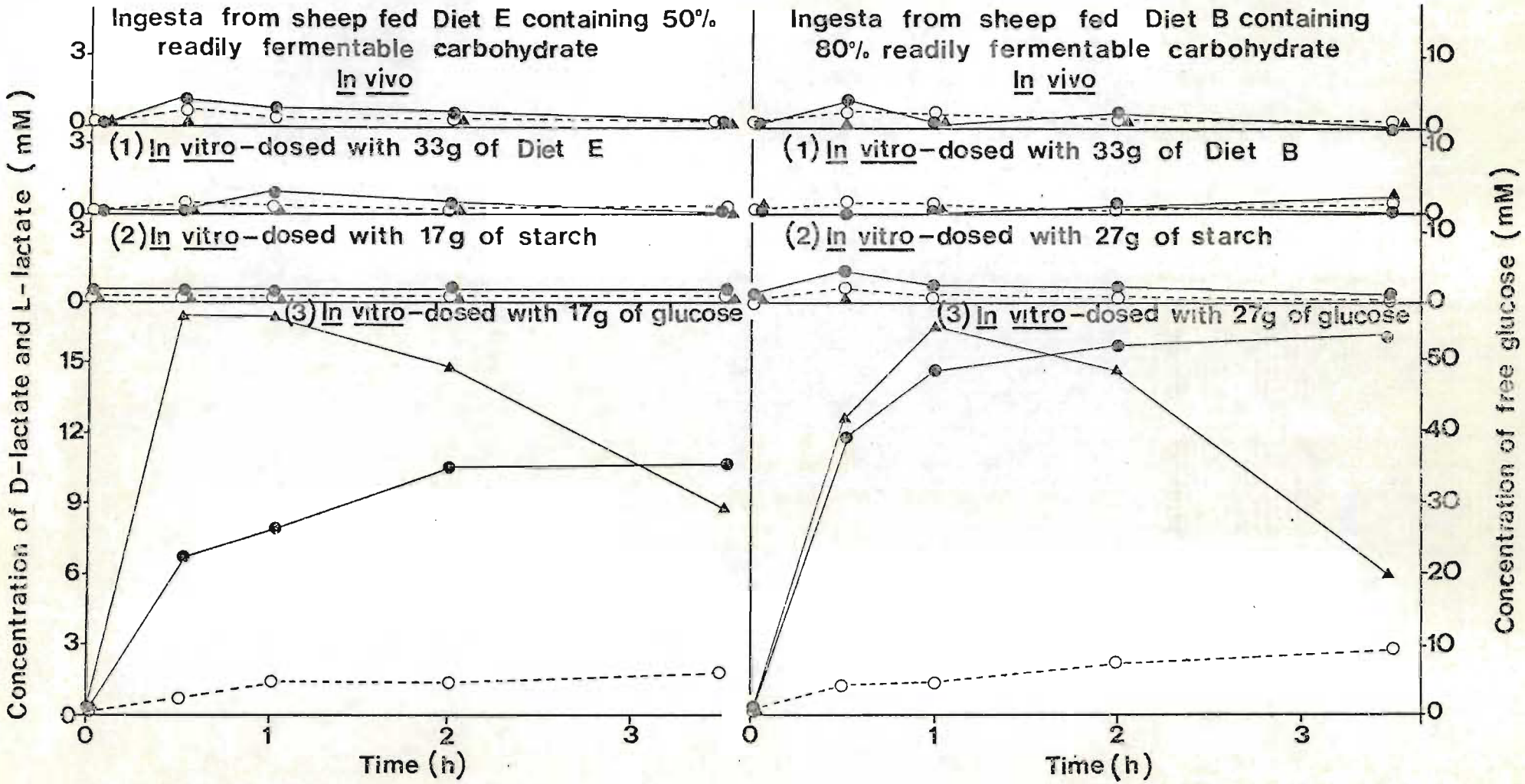

Fig.9.5.The concentration of D-lactate (—●—), L-lactate (--○--), and free glucose (—▲—) in an in vitro system dosed with amounts of the diet, starch or glucose which were proportional to those fed to the animals

2. On the High concentrate diet peak values for the rates of production (0,59 mM/min) and utilization (0,63 mM/min) occurred ¾-1¼ h after feeding with the peak of production preceeding that of utilization. The rates had returned to the basal rates *ca* 3 h after the feed which were ½-^{1}/3 of peak values. A similar pattern was found on the other three diets except on the two roughage diets when the peak values did not increase markedly. The peak values for production and utilization rates on the Intermediate diet were 0,24 and 0,23 mM/min, on the High roughage diet they were 0,16 and 0,17 mM/min and on the Lucerne hay diet they were 0,10 and 0,09 mM/min respectively.
3. Correlation coefficients were calculated between glucose utilization rate and the concomitant production of lactic acid in the rumen. Correlation coefficients of the linear regression lines for the High concentrate, Intermediate and High roughage diets were significant at the 95% level, and not significant for the Lucerne hay diet. When the data for all diets was combined the correlation was highly significant ($P>0,99$).
4. When the flasks of ruminal ingesta in the *in vitro* experiment were dosed with an amount of glucose which was proportional to the amount of RFC consumed by the donor sheep the concentration of free glucose reached a concentration of 56-57,5 mM and D-lactic acid increased to 10,7 mM for the 50% RFC diet and 16,0 mM for the 80% RFC diet. The concentration of L-lactic acid was 4-6 fold lower than the respective D-lactic acid concentrations.
5. The implications of these findings in relation to lactate turnover and concentration are discussed in Chapter 12.

CHAPTER 10. THE MICROBIAL FLORA PRESENT DURING THE TURNOVER EXPERIMENT AND PATHWAYS OF LACTIC ACID UTILIZATION

INTRODUCTION

A considerable amount of work has been done on the microbiology of sheep and cattle fully adapted to moderate or high grain-starch diets (Giesecke *et al*, 1966; Slyter *et al*, 1968; Eadie *et al*, 1970; Latham *et al*, 1971, 1972, 1974; Ogimoto & Giesecke, 1974) as well as on animals following an excessive intake of large amounts of RFC (Krogh, 1961, 1963; Allison *et al*, 1964, 1975; Mann, 1970), conditions which are likely to produce accumulations of lactic acid in the rumen. On the other hand the *in vitro* turnover of lactate by ingesta from ruminants fed these diets has been determined (Jayasuriya & Hungate, 1959; Nakamura & Takahashi, 1971), but the findings are of limited value since no concomitant microbiological study was made. The microbial flora producing and utilizing lactic acid are the primary determinants influencing lactic acid turnover in the rumen. Thus it is essential that these micro-organisms are studied during the *in vivo* determination of lactic acid turnover and as yet this has not been done. Furthermore, while *in vitro* studies on pure cultures of rumen organisms give valuable information on lactic acid metabolism they do not reflect the complex interplay of competition for substrates and beneficial interactions that exist in the rumen. Strains of lactate-utilizing bacteria have been shown to convert lactate to propionate through succinate (randomizing pathway) or acrylate (non-randomizing or direct reductive pathway) (Baldwin, 1965). *In vitro* studies with ^{14}C-labelled lactate have shown that the non-randomizing pathway increases in importance when the rumen fluid was obtained from animals fed concentrates (Baldwin *et al*, 1962, 1963; Wallnöfer *et al*, 1966).

In view of this the numbers and types of bacteria which ferment glucose and starch with the production of lactic acid and the bacteria utilizing the lactic acid produced were studied in fully adapted sheep on 3 occasions when *in vivo* lactate turnover experiments were performed. Counts of total culturable bacteria and ciliate protozoa which play an important regulatory role in the rumen fermentation (see Chapter 5) were determined at the same time. On one of the 3 occasions the labelled propionate formed from DL-(2-^{14}C)-lactate was degraded in order to determine the relative importance of the randomizing and the direct

reductive pathways in the utilization of ruminal lactate. Since *Megasphaera elsdenii* is the only bacterium among the predominating lactate-utilizers in the rumen which metabolizes lactate via the direct reductive pathway (Ladd & Walker, 1959), knowledge concerning the contribution of this pathway would also be an indication of the importance of this organism as a lactate-utilizer under normal feeding conditions.

METHODS

Microbiology

Animals and management

The animals, their management and diets are given in Methods (Chapter 8, page 88). Microbiological counts were performed during weeks 1, 3 and 5 of the experiment on all 4 sheep (see Table 8.2).

Sampling

Samples were taken 2 h after the first feed to the day using the procedure described in Chapter 5 (page 39).

Processing

This was the same as that reported in Chapter 5 (page 40) except that the sample was diluted 10-fold in the laboratory with anaerobic diluent, swirled gently to mix and poured straight into a wide-mouthed McCartney bottle. This procedure was rapid, eliminated 2 transfers of ingesta necessary for processing in the Stomacher and gave counts which were the same or higher than processed samples (see Table 5.2 and Table 10.1).

Table 10.1. Mean count ($x10^{-7}$/g ingesta) of different functional groups of bacteria in samples of ruminal ingesta from sheep fed a High concentrate diet (Diet B) or Lucerne hay and processed using the Stomacher or Minimal treatments

Diet	Functional group of bacteria	Counts on different treatments Minimal	Stomacher
Lucerne hay	Total culturable	180	200
	Glucolytic	150	150
	Amylolytic	30	30
	Lactate-utilizers	12	17
High concentrate	Total culturable	1 390	1 110
	Glucolytic	1 040	750
	Amylolytic	460	430

Protozoal counts

These were made in the same way as described in Chapter 5 (page 40) except that there was no reduction in numbers due to mechanical processing (Table 5.2). A differential count was also made according to size using an eyepiece micrometer and the following criteria. The holotrichs which ferment soluble sugars and have cilia covering the whole exterior surface of the organisms were divided into large (100-140x60-90 um i.e. *Isotricha* sp) or medium (60-80x40-50 um i.e. *Dasytricha* sp). The entodiniomorphs which ingest starch granules and have localized bands of cilia or syncilia were divided into large (120-150x70-90 um and larger i.e. *Polyplastron*, *Ophryoscolex*, *Eudiplodinium*), medium (70-100x50-80 um i.e. *Diplodinium*, *Ostracodinium*, *Epidinium*), and small (50-80x30-50 um i.e. *Entodinium*).

Bacterial counts

The procedures used were the same as those detailed in Chapter 5 with one minor modification namely replacement of the soluble starch in the medium for counting amylolytic bacteria with a native starch, (Maizena, Corn Products Co., Durban, South Africa). This gave an opaque medium in which the zones of amylolysis (or clearings) were more clearly defined and could be counted accurately after incubation for 3-5 days. Glucolytic bacteria, not included in the earlier experiments, were counted by the same methods using a medium similar to that for counting amylolytic bacteria except that the starch was replaced by glucose (Merck). Counts were made after 1 and 3 days of incubation.

Identification of amylolytic, glucolytic and lactate-utilizing bacteria

The bacterial isolates from the different media were obtained as described in Chapter 3 (page 23). All transfers were made in anaerobic diluent containing dithiothreitol. Smears made after each transfer were examined for Gram reaction, cell morphology and size. The isolates obtained from the media for counting the amylolytic and glucolytic bacteria were all tested for ability to ferment starch and glucose and for D- and L-lactic acid production. The isolates from the medium for counting the lactate-utilizing bacteria were tested for D- and L-lactic acid utilization, ability to ferment glucose, fructose, and glycerol and for triolein hydrolysis. End products of carbohydrate fermentation were determined for all isolates on the glucose containing fermentation

/medium .....

medium and also for the glucolytic and amylolytic isolates on the starch containing medium. The bacteria were identified on the basis of the above tests, together with cell size and morphology, and Gram reaction, according to the Anaerobe Laboratory Manual (Holdeman & Moore, 1975) and Bergey's Manual of Determinative Bacteriology (1974).

The media used in the fermentation tests had the same percentage composition as described previously (Appendix Tables 5.1 and 5.2) but were prepared in a different manner to improve anaerobiosis. Each batch of medium was made up in a 500 ml amount and already contained the test carbohydrate before dispensing i.e. in the same way as the media for counting organisms. End products were determined by gas chromatography as described previously (page 43).

Degradation of propionic acid

This was carried out to determine the position and amount of ^{14}C-label in isolated propionate and hence the relative importance of the 2 pathways of lactate utilization. The succinate pathway results in randomization of labelled carbon between C_2 and C_3 of propionate if (2-^{14}C)-lactate is used as substrate whereas the acrylate pathway results in direct transfer of label to C_2 of propionate.

Separation of volatile fatty acids

Propionic acid was separated from the other VFA using the same method as was used for the separation of glucose, lactate and VFA in the preliminary lactate turnover experiment mentioned in Chapter 8. The method, based on that of Harlow & Morman (1964), is described briefly here. Glass columns containing a 100x1 cm resin bed of AG 50W-X12, 200-400 mesh, H^+ form (Bio Rad, Richmond, California) were prepared. Water (pH 7,00) was used as eluant at a flow rate of 1 ml/min. The separation obtained is shown in Fig. 10.1. The method is slow requiring *ca* 4 h for a run but gave good separation of up to 5 mmoles of acid in 1 ml applied to the column. Constant flow rates were maintained using LKB Varioperpex pumps (LKB Produkter, Stockholm, Sweden) connected to 2 columns and 1,0 ml fractions were collected in an ISCO Model 328 Linear Fraction Collector (Instrumentation Specialties Company, Lincoln, Nebraska, USA) with an automatic "shut-off" valve to the pumps on completion of the run. In this way it was possible to run 4 samples on the 2 columns in one day. The fractions containing the individual VFA were pooled, titrated to pH 9,00 as described in Chapter 8, and evaporated to dryness.

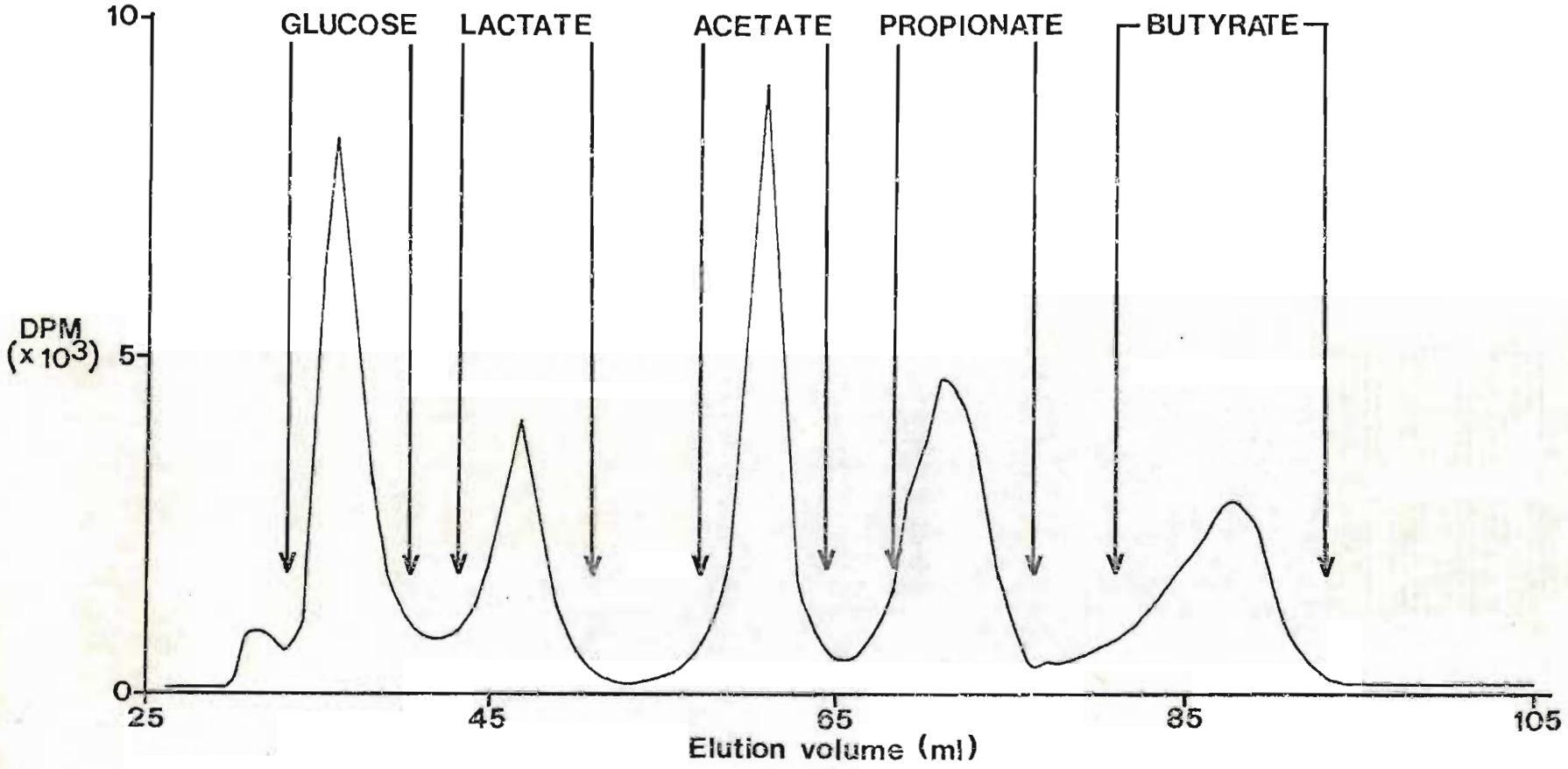

Fig.10.1. Seperation of a standard mixture containing ^{14}C-labelled glucose, lactate, acetate, propionate and butyrate on a column of AG 50W-X12, H^+ form (100 x 1cm) using water to elute the compounds at a flow rate of 1ml per min. The fractions collected for determination of radioactivity are indicated by arrows.

Procedure for Schmidt degradation of propionic acid

This was carried out according to the method of Sakami (1955) with the following modifications. The specific activity from the initial decarboxylation i.e. C_1 was not determined since it was not relevant to the study. The specific activity of the CO_2 released after degradation to the sodium acetate was collected in 0,25 M-NaOH, one portion counted for radioactivity and the remainder titrated. The methylamine was not oxidized to CO_2 but was distilled and trapped in 1M-HCl. A portion was counted for radioactivity and the remainder titrated. The ethylamine from the initial degradation was treated in the same way. The specific activities reported do not represent the specific activities in the original rumen fluid samples since carrier propionate was added to the samples and only a cut from the propionic acid peak eluted from the column was taken. Cross-contamination between carbons using this procedure is less than 1,2% (Baldwin *et al*, 1962; Prins & Van der Meer, 1976).

The contribution of the acrylate pathway to the formation of propionate from (2-^{14}C)-lactate was calculated from the specific activities of the individual carbons of propionate as follows:

$$\% \text{ acrylate pathway} = \frac{SA_{c2} - SA_{c3}}{SA_{c2} + SA_{c3}} \times 100$$

(Baldwin *et al*, 1963).

RESULTS

Ruminal pH and total VFA concentrations

The values for the mean ruminal pH and total VFA concentrations in the rumen of the sheep fed diets differing in amount of RFC are presented in Figs. 10.2 and 10.3. Each point on the curves represents the mean of 3 determinations each made on a separate occasion. Ruminal pH was lowest on the High concentrate diet and was below pH 6,00 for almost the entire sampling period (Fig. 10.2). Values on the Intermediate and High roughage diets were similar but tended to be lower on the High roughage diet especially between 3-8 h after the feed. Values on the Lucerne hay diet were highest although showing the greatest change over the sampling period. All sheep consumed their food very rapidly except for the animal fed the Intermediate diet which took *ca* 3-4 h to eat 750 g of the food. There was no dietary buffer in the Lucerne hay diet to prevent the rapid fall in ruminal pH.

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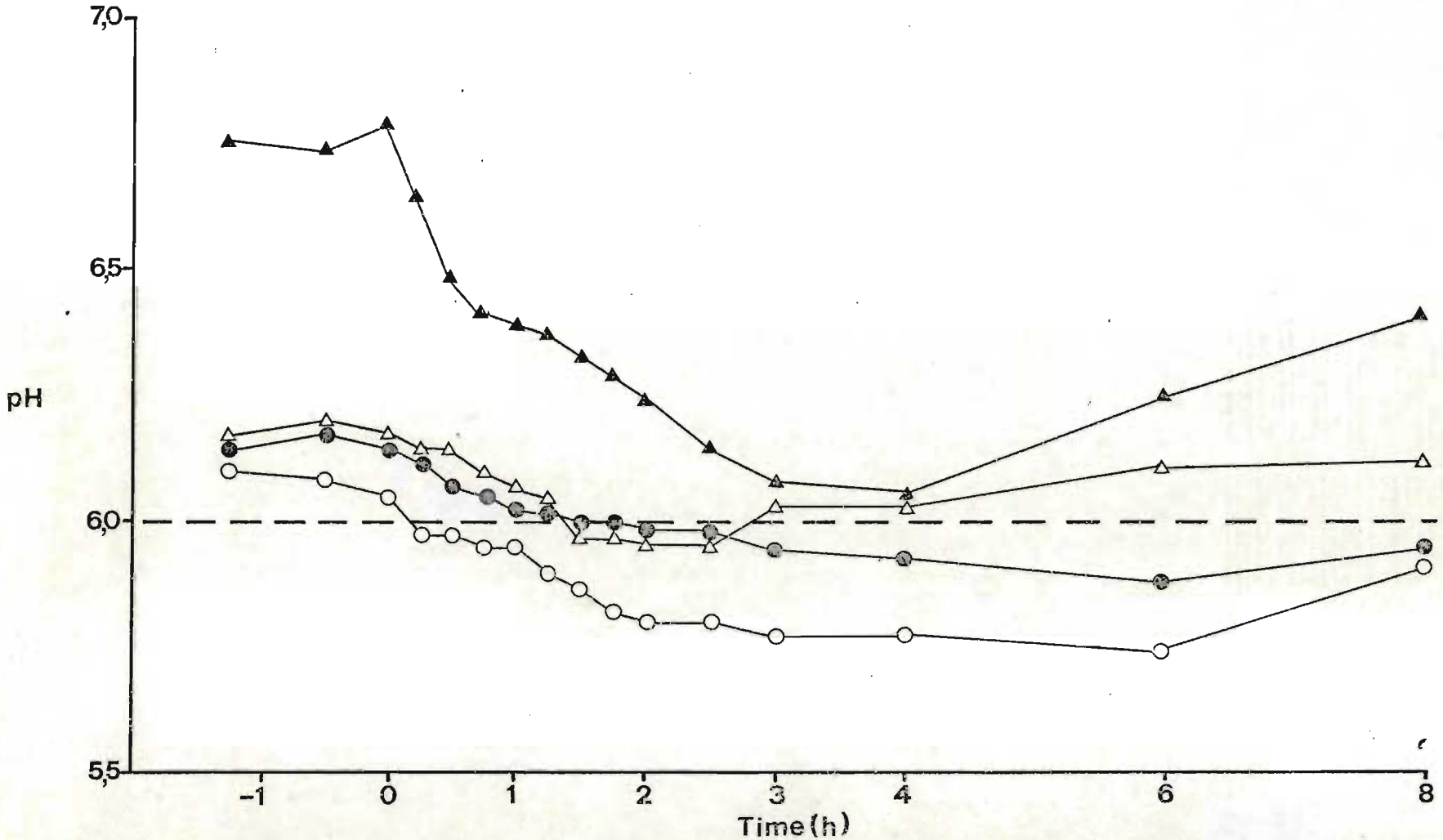

Fig.10.2. Mean pH values in the rumen of sheep fed diets containing different amounts of readily fermentable carbohydrate. Each point represent the mean of determinations made on 3 separate occasions. ((○)High concentrate; (△) Intermediate; (●)High roughage and (▲) Lucerne hay diets)

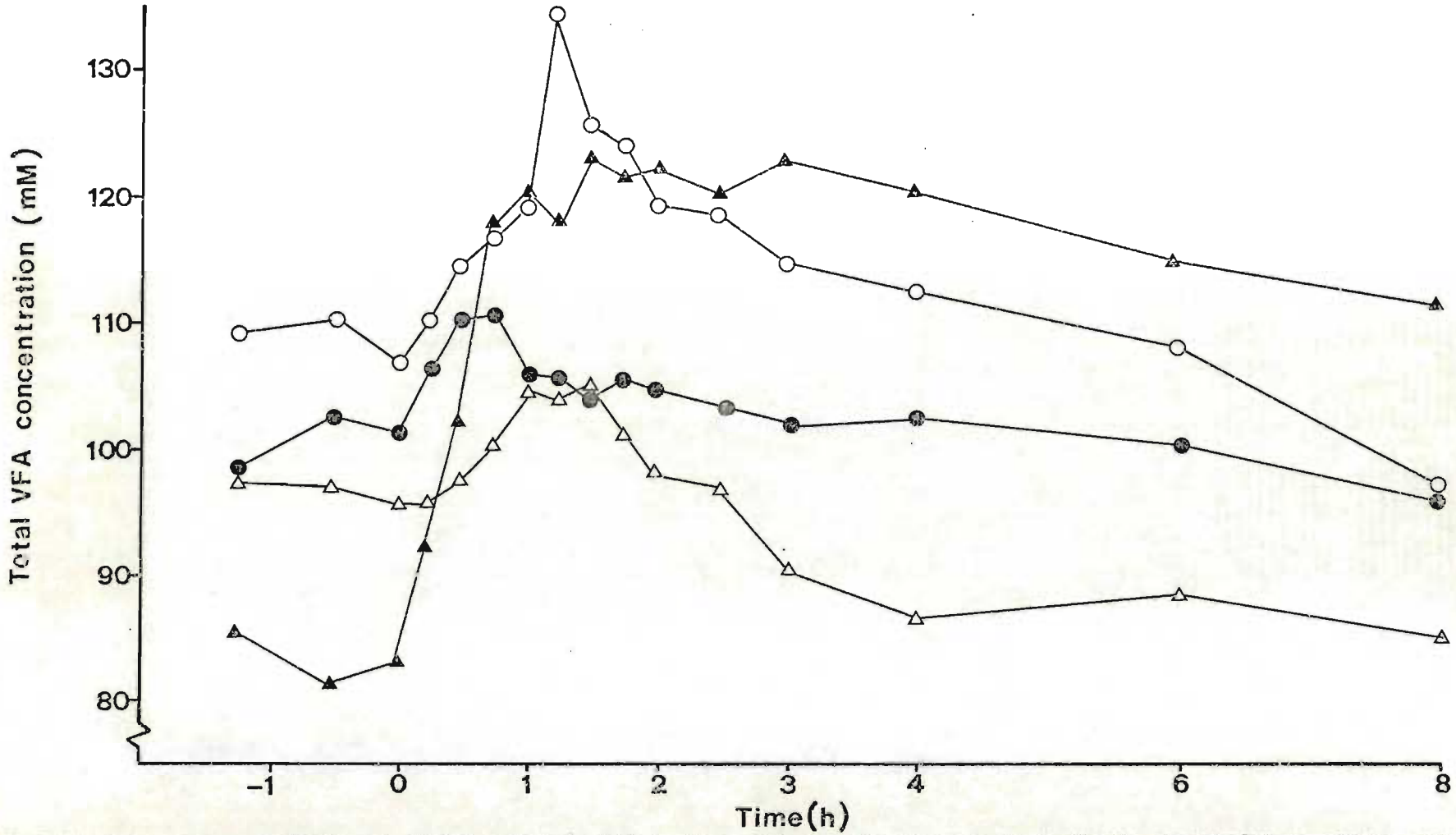

Fig.10.3. Mean concentration of Total VFA (mM) in the rumen of sheep fed diets containing different amount of readily fermentable carbohydrate. Each point represents the mean of determinations made on 3 separate occasion.((○) High concentrate; (△) Intermediate; (●) High roughage and (▲) Lucerne hay diets)

The trends in mean total VFA concentration (Fig. 10.3) on the three diets containing the dietary buffer were similar and were highest on the High concentrate diet. The values for the other two diets were similar but were higher on the High roughage than on the Intermediate diet. Concentration of Total VFA increased rapidly after feeding the Lucerne hay diet and reached a peak 1-2 h after feeding and then started to decrease slowly.

Numbers of bacteria and ciliate protozoa in the rumen

The numbers of total culturable, glucolytic, amylolytic and lactate-utilizing bacteria and of ciliate protozoa are given in Table 10.2. Numbers of micro-organisms in all groups were highest on the High concentrate diet. Counts for the Lucerne hay and High roughage diets were very similar

Table 10.2. Numbers of total culturable, glucolytic, amylolytic and lactate-utilizing bacteria ($x10^{-7}$/g ingesta) and of ciliate protozoa ($x10^{-5}$/g ingesta) on diets differing in amount of readily fermentable carbohydrate. Each value in the table is the mean of counts made on three occasions

Group of micro-organisms	Diets			
	High concentrate	Intermediate	High roughage	Lucerne hay
Total culturable bacteria	2 900	860	570	510
Glucolytic bacteria	1 220	200	230	280
Amylolytic bacteria	460	110	32	39
Lactate-utilizing bacteria	78	16	4,0	3,2
Ciliate protozoa	16	6,4	2,7	2,1

with the numbers of total culturable and lactate-utilizing bacteria being slightly higher on the High roughage than on the Lucerne hay and for the glucolytic and amylolytic bacteria the position was reversed. Counts of glucolytic bacteria ($200x10^{7}$/g ingesta) were actually lower on the Intermediate diet than on the two roughage diets just discussed. If the ratios of the numbers of lactate-producing (amylolytic + glucolytic) to lactate-

/utilizing .....

utilizing bacteria are calculated they are *ca* 20 for the High concentrate and Intermediate diets but 66 and 100 for the High roughage and Lucerne hay diets respectively. The numbers of micro-organisms present in the rumen of the sheep fed different amounts of carbohydrate are similar to the numbers of organisms present on comparable diets during the Stepwise Adaptation Experiment (Chapter 5). However, glucolytic bacteria were not included in the micro-organisms studied during the latter experiment.

The numbers of the different functional gorups of bacteria as a percentage of the number of total culturable bacteria ($x10^7$/g ingesta) are shown in Table 10.3. The glucolytic bacteria formed a major portion

Table 10.3. Numbers of the different functional groups of bacteria as a percentage of the number of total culturable bacteria. All counts were made after 5 days of incubation except the glucolytic bacteria which were counted after 3 days of incubation

Bacterial group	Bacterial counts on different diets			
	High concentrate	Intermediate	High roughage	Lucerne hay
Total culturable bacterial count ($x10^{-7}$/g ingesta)	2 150	800	490	450
	As a percentage of the total culturable count			
Glucolytic bacteria	57	25	47	62
Amylolytic bacteria	21	14	6	9
Lactate-utilizing bacteria	5	3	2	2
Contribution of the 3 groups to total	83	42	55	73

of the bacterial flora on all diets especially the Lucerne hay and High concentrate diets. The lactate-utilizing bacteria comprised 2-5% of the total culturable bacteria when determined in this manner, but this must be regarded as an underestimate since the medium for enumeration of these bacteria does not contain rumen fluid like the media for enumeration of the total culturable, glucolytic and amylolytic bacteria. If the glucolytic bacteria had been incubated longer they would obviously have increased

/in proportion. .....

proportion. These counts when added together form from 42-83% of the total bacterial flora as opposed to corresponding counts in the Stepwise Adaptation Experiment (see Appendix Table 5.5) where they were always less than 32% when averaged for the 4 sheep and usually 8-15%.

Predominant types of lactate-producing and lactate-utilizing bacteria

The predominant types of glucolytic, amylolytic and lactate-utilizing bacteria were identified and the results are given in Table 10.4. The proportions of the predominant genera in the different functional groups of bacteria differed considerably although in most cases the same organisms were present in the rumen on all the diets. Thus in the case of the amylolytic bacteria *Bacteroides* accounted for 44-59% of predominant bacteria and *Butyrivibrio* for 12-33% considering all four diets. Lactobacilli were only present amongst the glucolytic and amylolytic bacteria on the High concentrate diet. Eubacteria tended to replace the lactobacilli on the Intermediate and High roughage diets and neither group was present in the rumen of the Lucerne hay fed sheep. *Megasphaera* formed 40% of the predominant glucolytic bacteria on the High concentrate diet but did not appear among the predominant glucolytic bacteria on the other diets. On the Lucerne hay diet *Borrelia* formed 11% of the predominant amylolytic bacteria. This identification was based on morphology alone since on second transfer they had already been overgrown by contaminating organisms.

The predominant lactate-utilizing bacteria on all diets were *Propionibacter* and *Selenomonas* and accounted for 58-83% of the isolates on all four diets. *Veillonella* formed a small portion (6-11%) of the bacteria on all diets except the High roughage diet on which they were not found. *Anaerovibrio* constituted 11-19% of the predominant bacteria on the diets containing added RFC but were not present on the Lucerne hay diet. *Megasphaera* was present on all diets and accounted for 21% of the predominant lactate-utilizing bacteria on the High concentrate diet. These findings are compared with the predominant types of amylolytic and lactate-utilizing bacteria during the Stepwise Adaptation Experiment in the discussion (Chapter 12).

Pathway of lactic acid utilization

The relative importance of the two pathways of lactic acid utilization was determined by analysing the specific activities of the individual carbons (C_2 and C_3) of propionate isolated from samples of ruminal ingesta obtained during the continuous infusion of DL-(2-^{14}C)-lactate. The results

/are presented .....

Table 10.4. The predominant types of glucolytic, amylolytic and lactate-utilizing bacteria present in the rumen of sheep fed diets containing different amounts of readily fermentable carbohydrate

Genera	Percentage of bacteria on different diets			
	High concentrate	Intermediate	High roughage	Lucerne hay
Glucolytic bacteria:				
Bacteroides	20	20	63	57
Butyrivibrio	10	40	12	43
Selenomonas	-	20	-	-
Megasphaera	40	-	-	-
Eubacterium	-	20	25	-
Lactobacillus	30	-	-	-
Amylolytic bacteria:				
Bacteroides	59	44	50	58
Butyrivibrio	12	33	19	32
Selenomonas	-	11	-	-
Eubacterium	-	11	31	-
Lactobacillus	29	-	-	-
Borrelia	-	-	-	11
Lactate-utilizing bacteria:				
Veillonella	11	6	-	8
Megasphaera	21	6	15	8
Anaerovibrio	11	19	15	-
Selenomonas	21	50	8	8
Propionibacter	37	19	62	75

are presented in Table 10.5. The acrylate pathway accounted for 17,5-29,5% of the conversion of lactate to propionate in the rumen of the sheep fed the High concentrate diet. The values on the other three diets were similar ranging from 8,8-17,3%. The contribution on the High roughage diet was slightly larger than on the Intermediate diet. The percentage contribution of the acrylate pathway was greater 6-8 h after feeding than at peak lactate fermention ½-1 h after feeding. The difference was greatest on the High concentrate diet increasing from 17,5 to 29,5 percentage units. The increase on the other diets was 2,9-3,3 percentage units. These results agree with those obtained for the proportions of *Megasphaera* among the predominant lactate-utilizing bacteria which were greatest on the High concentrate diet and similar on the Intermediate and Lucerne hay diets.

Table 10.5. Contribution of the acrylate pathway in the conversion of DL-(2-^{14}C)-lactate to propionate in the rumen of sheep at different times after feeding diets differing in amount of readily fermentable carbohydrate

Diet	Conversion via acrylate pathway (%)	
	½-1 h after feeding	6-8 h after feeding
High concentrate	17,5	29,5
Intermediate	9,6	12,9
High roughage	13,0	17,3
Lucerne hay	8,8	11,7

CONCLUSIONS

The main findings of the chapter are summarized below:

1. Microbiological counts were performed on 3 occasions during the lactate turnover experiments reported in Chapter 8 in order to relate rates of production and utilization of lactic acid in the rumen with the numbers and types of micro-organisms which produce and utilize lactic acid.
2. The pattern of ruminal pH and total VFA concentrations were similar for the sheep fed diets containing a buffer but differed from the Lucerne hay diet. The pH values were highest on the Lucerne hay diet, intermediate on the High roughage and Intermediate diets and lowest on the High concentrate diets. The

/opposite .....

opposite trend was shown for the total VFA concentrations.

3. The numbers of all groups of micro-organisms were highest on the High concentrate diet and lowest on the two roughage diets. The numbers of glucolytic bacteria were 25-62% of the total culturable bacterial count and 2-7 fold higher than the amylolytic count. The numbers of lactate-utilizing bacteria were *ca* 20-fold higher on the High concentrate diet than on the two roughage diets and *ca* 5-fold higher than on the Intermediate diet.

4. Although the same organisms were usually present among the predominant glucolytic and amylolytic bacteria their proportions differed considerably. *Bacteroides* and *Butyrivibrio* formed a large proportion of both the amylolytic and glucolytic flora. On the High concentrate diet the Lactobacilli and *Megasphaera* formed 30% and 40% of the glucolytic bacteria respectively. Lactobacilli were present only on the High concentrate diet and were replaced by *Eubacterium* on the Intermediate and High roughage diets.

5. *Propionibacter* and *Selenomonas* formed a large proportion of the lactate-utilizing isolates on all diets. *Anaerovibrio* formed 11-19% of the predominant bacteria in the rumen of the sheep fed the diets with added RFC whereas *Megasphaera* formed 6-21% of the lactate-utilizing bacteria on all diets.

6. The contribution of the acrylate pathway to the conversion of lactate to propionate was greatest on the High concentrate diet and tended to decrease as the amount of RFC in the diet decreased. The contribution also increased with time after feeding and was thus 17,5% ½-1 h after feeding and 29,5% 6-8 h after feeding the High concentrate diet. These results agree with the proportions of *Megasphaera* found among the predominant lactate-utilizing bacteria.

7. The results obtained for the numbers and types of lactate-producing and lactate-utilizing bacteria will be discussed in Chapter 12 in relation to the turnover of lactic acid and free glucose determined simultaneously (see Chapters 8 & 9) and the microbiological findings of the Stepwise Adaptation Experiment (see Chapter 5).

CHAPTER 11. PRODUCTION AND UTILIZATION OF LACTIC ACID BY PURE CULTURES OF RUMEN BACTERIA

INTRODUCTION

Initially it was planned to determine the rates of production and utilization of lactic acid by pure cultures of rumen bacteria isolated from the rumen of sheep during the Stepwise Adaptation Experiment and the Turnover Experiment. However, as substrate utilization and end product formation are largely dependent on growth rate (Wolin, 1975) the use of continuous culture techniques, which more closely approximate *in vivo* fermentation than closed vessel incubations, were required. This was beyond the scope of the present study. Instead the qualitative findings from pure culture studies on the production and utilization of D- and L-lactic acid are presented in this chapter.

A further aspect of lactate metabolism was also studied. Whanger & Matrone (1970) showed that feeding sheep sulphur-deficient purified diets with urea as the sole source of N resulted in the production of ruminal lactic acid, a large proportion of which was the D-isomer. When provided with supplemental S, lactate accumulation was greatly reduced. Furthermore, when ingesta was incubated with (2-^{14}C)-lactate it was found that propionate was labelled as though 30% was formed through acrylate in the sulphur fed sheep but little was formed via this pathway by the microorganisms from the S-deficient sheep. Thus it was thought that lactate-utilizing bacteria, and *Megasphaera elsdenii* in particular, since it metabolizes lactate via acrylate, could couple lactate utilization with sulphate reduction in the same way as the dissimilatory sulphate-reducing bacteria (Peck, 1970) i.e.

$$4 \text{ Lactate} \longrightarrow 4 \text{ Pyruvate} + 4H_2$$

$$4H_2 + SO_4^{=} \longrightarrow S^{=} + 4H_2O$$

This possibility was investigated by incubating cultures of lactate-utilizing bacteria with $Na_2{}^{35}SO_4$ and lactate as the only energy source and determining production of $H_2{}^{35}S$.

/METHODS .....

METHODS

Production and utilization of lactic acid by pure cultures

The methods used to determine the qualitative production and utilization of D- and L-lactic acid have been described previously (Chapters 5 and 10). Culture supernatant was analysed for appearance or disappearance of the two isomers of lactic acid as compared to bottles of media inoculated with sterile anaerobic diluent by the specific enzymic methods reported in Chapter 2.

Sulphate reduction by lactate-utilizing bacteria

The isolates were washed off maintenance slopes with diluent containing dithiothreitol (Appendix Table 5.1) and 0,5 ml of inoculum added to a McCartney bottle containing liquid fermentation medium with 1,0% Na-DL-lactate. This medium contained thioglycollate and dithiothreitol as reducing agents, since poor growth and a low survival rate was obtained in respect of many of the lactate-utilizing isolates when cysteine hydrochloride alone was used to reduce the medium (see Appendix Table 11.1 for composition and preparation of the medium). An aliquot (1,0 ml) of filter sterilized $Na_2{}^{35}SO_4$ of high specific activity (>5 m Ci/*ug* S; Radiochemical Centre, Amersham, England) was added and the bottles incubated for 7 days at 39°C. After incubation 0,5 ml carrier Na_2S (*ca* 25 mg) was added to each bottle and allowed to equilibrate for 60 min. A 5,0 ml portion of the fermentation medium was removed from the bottle using a syringe and placed in a test tube containing 2 drops of octan-2-ol. The tube was stoppered rapidly and 5,0 ml of 5N-H_3PO_4 added through a funnel and tube inserted through the rubber stopper. The tube was gassed with N_2, increasing the rate to 28,4 l per h slowly where it was maintained for 15 min. The $H_2{}^{35}S$ evolved was trapped in a 50 ml volumetric flask containing 5 ml of Zn acetate/Na acetate solution (50 g of $(CH_3COO)_2$ $Zn.2H_2O$ + 12,5 g of $CH_3COONa.3H_2O$ in 1 l) and 30 ml of water. After gassing, the delivery tube was removed with the collection flask and washed down with deionized water. The flask was made up to volume, mixed thoroughly, and duplicate 10 ml aliquots pipetted into scintillation vials. An equal volume of Instagel (Packard) was added and the vials placed in the Packard Tri-Carb Liquid Scintillation Counter overnight before counting. The channels ratio method was used for quench correction. Quench curves were prepared using a certified standard of ^{35}S obtained from the Radiochemical Centre

/(Amersham, ...

(Amersham, England). The remaining fermentation medium was tested for lactate disappearance as described earlier (Chapter 2).

RESULTS

Production of lactic acid by pure cultures of rumen bacteria

The production of D- and L-lactic acid by pure cultures of glucolytic and amylolytic bacteria is given in Table 11.1. From the results it can be seen that most of the isolates produced DL- or only L-lactic acid. Only 13 isolates produced D-lactic acid alone and all were *Bacteroides*. Of the isolates producing DL-lactic acid 49% were *Bacteroides* and 23% were *Butyrivibrio*. The Lactobacilli comprised 26% of the isolates producing only L-lactic acid and 18% of the isolates producing DL-lactic acid. *Streptococcus bovis* comprised 7% of the isolates producing L-lactic acid alone and did not produce D- or DL-lactic acid. Of the total number of isolates tested 7,5%, 33,5% and 59% produced D-lactate only, L-lactate only or DL-lactate respectively.

Although *Bacteroides* formed a major portion of the lactic acid-producing bacteria, the quantities produced by these organisms were small (usually < 1 mM), whereas the other lactate-producers formed greater quantities (see Appendix Table 5.7). Under the experimental conditions described some of the Lactobacilli produced final concentrations of 10-20 mM lactic acid in the culture bottles.

End-products analysis for the identification of the amylolytic and glucolytic isolates showed that the quantity of lactic acid produced was greater with glucose as substrate than with starch as substrate. This was probably related to the faster growth rate of the isolates on the glucose containing fermentation medium.

Utilization of lactic acid by pure cultures of rumen bacteria

The utilization of D- and L-lactic acid by pure cultures of lactate-utilizing bacteria is shown in Table 11.2. Most of the isolates tested utilized DL-lactic acid. A greater number of isolates were able to utilize L-lactic acid alone than D-lactic acid alone. About half (51-52%) of the isolates utilizing DL-lactic acid or only L-lactic acid were *Anaerovibrio*. *Propionibacter* accounted for 42%, 32% and 23% of the isolates which utilized only D-lactate, only L-lactate or DL-lactate respectively. Except for *Veillonella*, the other four genera of lactate-

/utilizing .....

Table 11.1. Percentage of the different genera of glycolytic and amylolytic bacteria producing D-lactate only, L-lactate only or DL-lactate. The total number of isolates tested was 173

Genera of glucolytic and amylolytic bacteria	Numbers and percentage of isolates producing:								
	D-lactate only			L-lactate only			DL-lactate		
	No of isolates giving positive reactions	% of total isolates tested	% of D-lactate producers	No of isolates giving positive reactions	% of total isolates tested	% of L-lactate producers	No of isolates giving positive reactions	% of total isolates tested	% of DL-lactate producers
Bacteroides	13	8	100	30	17	52	50	29	49
Butyrivibrio	-	-	-	5	3	9	23	13	23
Selenomonas	-	-	-	1	1	2	3	2	3
Lactobacillus	-	-	-	15	9	26	18	10	18
Eubacterium	-	-	-	3	2	5	8	5	8
Streptococcus	-	-	-	4	2	7	-	-	-

Table 11.2. Percentage of the different genera of lactate-utilizing bacteria with ability to utilize D-lactate only, L-lactate only or DL-lactate. The total number of isolates tested was 166

Genera of lactate-utilizing bacteria	Numbers and percentage of isolates utilizing:								
	D-lactate only			L-lactate only			DL-lactate		
	No of isolates giving positive reaction	% of total isolates tested	% of D-lactate utilizers	No of isolates giving positive reaction	% of total isolates tested	% of L-lactate utilizers	No of isolates giving positive reaction	% of total isolates tested	% of DL-lactate utilizers
Veillonella	1	1	5	-	-	-	4	2	4
Megasphaera	2	1	11	6	4	14	9	5	9
Selenomonas	5	3	26	1	1	2	13	8	13
Anaerovibrio	3	2	16	23	14	52	53	32	51
Propionibacter	8	5	42	14	8	32	24	14	23

utilizing bacteria were all distributed over the three different categories. Although a lactate-utilizer, depending on strain *Selenomonas* is able to ferment glucose and starch with the formation of lactic acid. *Megasphaera* is also able to ferment glucose but does not produce lactic acid on this substrate.

Sulphate reduction by lactate-utilizing bacteria

Some time was spent on this aspect of the work which yielded negative results. Initially all isolates were tested on the medium containing cysteine.HCl as reducing agent, but showed no production of $H_2{}^{35}S$ from $^{35}SO_4^{=}$ despite recoveries of 95% for $H_2{}^{35}S$ in the apparatus described under "Methods". These results were not considered satisfactory since growth and survival of isolates was poor. Therefore another approach was tried which still used the "closed system" of McCartney bottles for incubation. Isolates of sulphate-reducing bacteria were obtained from several sources. Two known sulphate-reducing bacteria were obtained, namely *Desulfotomaculum ruminis* (NCIB 8452) isolated by Coleman (1960) and a *Desulfovibrio* sp (ATCC 27882) isolated by Huisingh, McNeil & Matrone (1974), from the respective Type Culture Collections. Sulphate-reducing bacteria were also isolated from the 10^{-4} and 10^{-5} dilutions of ingesta from the rumen of sheep at Onderstepoort using a medium similar to that used for the sulphate-reduction tests (Appendix Table 11.1) except that it contained 2% agar.

Using dithiothreitol to poise the medium at low redox potential, the sulphate reduction tests were then repeated on the 2 type cultures, 8 of the above sulphate-reducers and 15 isolates of lactate-utilizing bacteria from the Stepwise Adaptation Experiment (Chapter 5). The results (Table 11.3) showed conclusively that the sulphate-reducing isolates converted 28-40% of the total activity of $^{35}SO_4^{=}$ into $H_2{}^{35}S$, while none of the 15 isolates obtained from the Stepwise Adaptation Experiment showed this ability despite the fact that all isolates utilized lactic acid during the sulphate reduction test. Thus the 15 isolates from the Stepwise Adaptation Experiment were not dissimilatory sulphate-reducers.

CONCLUSIONS

The main findings are summarized below.

1. Most of the glucolytic and amylolytic bacteria were found to produce DL- or L-lactic acid. Although *Bacteroides* formed a major portion of all the isolates they only produced small

/quantities .....

Table 11.3 Production of $H_2{}^{35}S$ from $^{35}SO_4$ and lactate utilization by isolates of bacteria during tests for sulphate reduction. Total counts of $Na_2{}^{35}SO_4$ added to each bottle of medium was 199 638 dpm

Isolate	Production of $H_2{}^{35}S$ dpm in $H_2{}^{35}S$	% of total	Utilization of DL-lactate (mM)
Type cultures:			
NCIB 8452	78 223	39,2	40,6
ATCC 27882	47 198	23,6	25,7
Sulphate-reducers:			
F19	79 297	39,7	41,7
F39	75 719	37,9	37,2
F42	58 365	29,2	31,4
F44	66 791	33,5	35,8
F48	56 138	28,1	30,6
F49	71 324	35,7	29,1
F50	62 573	31,3	34,9
F57	69 638	34,9	39,1
Lactate-utilizers:			
L7	Φ-	-	35,3
L23	-	-	95,5
L30	-	-	78,1
L37	-	-	94,2
L77	-	-	90,7
L80	-	-	97,9
L88	-	-	58,7
L121	-	-	100,4
L143	-	-	95,3
L146	-	-	88,5
L150	-	-	85,1
L151	-	-	92,4
L166	-	-	78,3
L172	-	-	95,6
L176	-	-	90,2

Φ Counts obtained were of the same order as the controls without added cells

quantities of lactic acid which were normally <1 mM. The other types of bacteria, especially the Lactobacilli produced large amounts of lactic acid. The amount of lactic acid produced was greater on glucose than with starch as substrate probably related to faster growth rates on glucose containing fermentation medium.

2. The lactate-utilizing bacteria were found to utilize D-, L- or DL-lactic acid except for *Veillonella* which did not utilize L-lactic acid. *Anaerovibrio* and *Propionibacter* accounted for 58%, 84% and 74% of the isolates utilizing D-, L- or DL-lactic acid respectively.

3. Sulphate reduction tests were carried out on 15 isolates of lactate-utilizing bacteria from the Stepwise Adaptation Experiment, 2 type cultures of sulphate-reducing bacteria and 8 isolates of sulphate-reducing organisms obtained from the rumen of sheep at Onderstepoort. The results showed conclusively that none of the 15 lactate-utilizing isolates was a dissimilatory sulphate-reducer. Thus these organisms were not able to link lactate utilization with sulphate reduction in order to derive energy as proposed in the introduction.

CHAPTER 12. DISCUSSION OF PART III

Before discussing the results obtained some attention will be paid to the equations used in the calculation of the rates of production and utilization of lactic acid. By definition turnover rate is input rate-output rate of unlabelled material in a steady state system. By infusing tracer at a constant measured rate (R) into a single pool and analysing activity at equilibrium (SA_E) or at plateau value the input or production rate (PR) of unlabelled material can be calculated as $SA_E = {}^{R}/_{PR}$ or $PR = {}^{R}/_{SA_E}$ (Shipley & Clarke, 1972). If the system is in steady state the input rate (PR) is also the output rate or utilization rate (UR) i.e. it is the turnover rate for the system. The term steady state means a stationary state for unlabelled tracee and the constancy applies to all rates, rate constants and the amount of unlabelled material. If any of these change during the period of observation the condition is non-steady state. Non-steady state is most commonly envisaged as changing pool size caused by unequal rates of input and output. Shipley & Clarke (1972) have derived an equation for calculation of input rate where a single pool receives a constant infusion of tracer and where input and output rates are unequal but constant. The equation is

$$PR = \frac{R - Q(t)(d(SA)/dt)}{SA(t)}$$

which states that input rate may be evaluated by dividing the instantaneous SA into the difference between infusion rate and the product of then existing concentration Q and instantaneous slope of the SA-time curve. Output rate is calculated as

$$UR = PR - ({}^{dQ}/_{dt})$$

where the last term is the slope of the curve for concentration of tracee versus time and is simply the change in Q between any two points divided by the associated time interval i.e.

$$UR = PR - {}^{(Q_2 - Q_1)}/_{(t_2 - t_1)}$$

However, if in addition to being unequal, input and output rates are not constant i.e. they vary as a function of time, an approximation of inflow rate can be made from observed curves for Q and SA provided the time intervals are sufficiently small. The equivalent slope (d(SA)/dt) is assumed to be that of the straight line between SA_1 and SA_2 at times

/t_1 and t_2 .....

t_1 and t_2 respectively and is calculated as $(SA_2-SA_1)/(t_2-t_1)$. The value taken for Q(t) is the midpoint in time between t_1 and t_2 i.e. $(Q_1+Q_2)/2$. A similar mean is used for the denominator SA(t). Thus input rate may be approximated by the equation

$$PR = \frac{R-((Q_1+Q_2)/2)((SA_2-SA_1)/(t_2-t_1))}{(SA_1+SA_2)/2}$$

and if calculated values are to be meaningful when flow rates are changing rapidly the time intervals must be small.

Because random error of the difference between close set data points is potentially large a smoothed curve provides better reference values (Shipley & Clarke, 1972). In connection with errors in calculated rates arising from errors in the value of SA , Hetenyi & Norwich (1974) have shown that the difference between two successive samples $SA_1-SA_2 = \Delta SA$ is made up of two components: SA_p which is the actual difference due to physiological factors and SA_e which is the difference due to experimental errors. Thus $\Delta SA=\Delta SA_p+\Delta SA_e$, and if $t_2-t_1=\Delta t$ becomes smaller ΔSA_p becomes smaller but ΔSA_e does not change. Therefore the shorter Δt becomes the more ΔSA reflects random error (ΔSA_e) in the determination of SA. Thus as the experimenter takes samples more frequently in order to gain more precise information about input and output rates, the more imprecise the calculated values become.

From the foregoing discussion it is evident that the equations for the calculation of input (production) and output (utilization) rate, assuming that the rates are unequal and vary as a function of time, are well suited for calculation of the rates of production and utilization of lactic acid *in vivo*. The same equations may be converted to those applicable for a single dose by placing R=0. Perhaps the most serious limitation is the assumption of a single pool, having no reversible connections with other pools, as such a situation is unlikely to exist. Recirculation of label is unlikely to have caused an underestimation of the rates since the label in lactate is converted into metabolic sink products (VFA) in the rumen.

A major problem associated with the use of labelled materials in the study of rumen metabolism is the limitation imposed by the rate and uniformity of mixing of infused material and consequently of obtaining representative samples of ingesta. Attempts to improve mixing by using pumps to circulate ruminal fluid for the estimation of VFA production have not afforded much improvement and have given results comparable to

/those .....

those obtained without mixing pumps (Leng, Corbett & Brett, 1968). Furthermore, the use of circulation pumps has also been shown to influence the molar proportion of the individual VFA (Sutherland, Ellis, Reid & Murray, 1962; Whitelaw, Hyldgaard-Jensen, Reid & Kay, 1970) probably due to diffusion of oxygen into the circulating fluid and subsequent alteration in fermentation. Therefore in the present experiment no attempt was made to use mixing pumps but relied on the natural mixing movements of the rumen.

The results obtained by different workers for lactate turnover are compared to the results obtained in the present experiment in Tables 12.1(a) and 12.1(b). There is generally very good agreement between the results of Satter & Esdale (1968), Nakamura & Takahashi (1971) and those in the present experiment for comparable diets. The value for peak fermentation, ¼ h after feeding the High concentrate diet was 0,502 mM/min which was very similar to the value of 0,567 mM/min fed the concentrate/alfalfa/lactate salt diet in the experiment of Satter & Esdale (1968). In contrast the pool size of lactic acid was determined enzymically by assaying for L-lactic acid by Jayasuriya & Hungate (1959) and thus lactate turnover would be underestimated by an amount equivalent to the D-lactate contribution. It has been shown that D-lactic acid concentration in the rumen can be greater than L-lactic acid on occasion. On the other hand, lactate pool size was calculated accurately in the experiments of Nakamura & Takahashi (1971) using the isotope dilution technique on samples of fluid obtained from the rumen at the same time as ingesta samples for the *in vitro* incubations.

The *in vivo* experiments (Chapter 8) where sheep were dosed with a lactate load (*ca* 35 g of Na-DL-lactate) were very interesting. In an experiment of Kunkle *et al* (1976a) the disappearance *in vivo* was faster on the roughage diets + lactate than on the all-concentrate diet and this was contrary to the *in vitro* findings. Similar results were obtained in the present experiments. Since absorption of lactate from the rumen is not likely to play a role, the explanation could be that greater salivation due to the fibrous roughage in the diet markedly increases dilution of lactic acid in the rumen and increases the flow of liquid out of the rumen. These results demonstrate that extrapolation of results obtained using unphysiological concentrations of metabolites or

/Table 12.1(a) .....

Table 12.1(a). Comparison of calculated lactate turnover rates in the literature with those obtained in the present studies

Reference	Experimental details and animals	Composition and amount of diet fed	Time after feeding (h)	Turnover rate (mM/min)
Jayasuriya & Hungate (1959)	*In vitro* isotope expt Ingesta from	Alfalfa hay	Average	0,004
		High grain ration	3	0,048
		High grain ration	1	0,096
Satter & Esdale (1969)	*In vitro* expt unlabelled lactate Ingesta from cattle	Alfalfa hay *ad libitum*	3½ - 4	0,120
		2,5:1 concentrate/alfalfa hay mixture fed *ad libitum*	3½ - 4	0,158
		3,6 kg concentrate + corn silage *ad libitum*	3½ - 4	0,300
		3,9 kg concentrate + 2,3 kg alfalfa + *ca* 585 g sodium and calcium lactate	3½ - 4	0,567
Nakamura & Takahashi (1971)	*In vitro* isotope expt Ingesta from sheep	800 g Orchard grass hay	½	0,031
			1½	0,063
			4	0,086
		500 g Orchard grass hay + 500 g concentrate	½	0,232
			1½	0,096
			4	0,121
Kunkle, Fetter & Preston (1976a)	*In vitro* unlabelled lactate Ingesta from sheep	1 300 g Orchard grass hay	3	0,036
		1 300 g Orchard grass hay + 97,5 g lactic acid syrup	3	0,081
		1 300 g Orchard grass hay + 195 g lactic acid syrup	3	0,105
		950 g All concentrate diet containing 88% shelled corn	3	0,235
	In vivo expt Sheep dosed 37,5-50 g of lactate	1 300 g Orchard grass hay	3 - 7	0,282
		1 300 g Orchard grass hay + 97,5 g lactic acid syrup	3 - 7	0,892
		1 300 g Orchard grass hay + 195 g lactic acid syrup	3 - 7	0,917
		950 g All concentrate diet containing 88% shelled corn	3 - 7	0,573

Table 12.1(b). Comparison of calculated lactate turnover rates in the literature with those obtained in the present studies

Reference	Experimental details and animals	Composition and amount of diet	Time after feeding (h)	Turnover rate (mM/min)
Present studies	*In vivo* expt constant infusion of ^{14}C-lactate into rumen of sheep fed twice daily	1 500 g Lucerne hay containing *ca* 7,5% RFC	½ (peak)	0,163
			1	0,093
			4	0,013
		1 500 g High roughage diet containing 67% maize stalks & 10% molasses	1 (peak)	0,171
			1¾	0,085
			4	0,016
		1 500 g Intermediate diet containing 35% maize stalks & 43% molasses + maize grain	½ (peak)	0,296
			1	0,119
			4	0,019
		1 500 g High concentrate diet containing 15% maize stalks & 65% molasses + maize grain	¼ (peak)	0,502
			1	0,215
			4	0,023
	In vivo expt Sheep dosed *ca* 35 g Na-DL-lactate	1 500 g Lucerne hay	0 - 2	0,56
		1 500 g High roughage diet	0 - 2	0,62
		1 500 g Intermediate diet	0 - 2	0,48
		1 500 g High concentrate diet	0 - 2	0,33

substrates can give erroneous results and care should be taken with their interpretation.

For comparitive purposes the results presented in Table 12.1 were turnover rates calculated from infusion rate divided by specific activity. Although similar results were obtained using the more appropriate equations applicable to non-steady conditions, the calculations of production and utilization rates showed several interesting features.

The pattern of production and utilization which is not easily seen on the High concentrate diet shows a low production rate before feeding which is balanced by utilization rate and hence no lactate accumulates. Immediately after feeding the rate of production increases up to 30-fold and remains elevated for *ca* 1 h after the feed. Utilization rate follows the same pattern but with a time lag and for a time (between ½ - 1½ h after the feed) actually exceeds production rate. This removes the lactate which has accumulated. Thus the peak in production precedes

/that ....

that in utilization although it could well be over a shorter interval than the 15 min between samples used in the present experiment. The production and utilization rates had decreased to basal levels *ca* 1½ h after the feed. The same pattern existed for the two roughage diets although the peaks were lower and lasted longer returning to basal level *ca* 2-2½ h after the feed.

In the present experiments the concentration of L-isomer usually exceeded that of the D-isomer as a result of greater production of the L-isomer (page 100). When production rate of the L-isomer was greater than that of the D-isomer, the utilization rate showed the same trend. Presumably when D-lactic acid concentration is greater than L-lactic acid concentration this is because of greater production of the D-isomer and not to any difference in the rate of utilization of the two isomers.

The results for the contribution of lactate to the formation of the individual VFA show that lactate was not an important intermediate except for the period 0-2 h after the feed. On the 3 diets containing added RFC the contribution of lactate to the formation of total VFA was approximately 25%, 21% and 15% for the High concentrate, Intermediate and High roughage diets respectivley over a quarter of the feeding cycle. During the remaining portion of the feeding cycle the contribution was approximately 1-2% for all diets. This means that roughly 8, 6½, 5 and 2½% of the total VFA was formed through lactate as an intermediate on the High concentrate, Intermediate, High roughage and Lucerne hay diets respectively. These estimates are similar to those of Jayasuriya & Hungate (1959) namely 8 - 17% of the substrate fermented in grain fed animals but only 1% for hay fed animals.

The contribution from lactate was greatest in the formation of propionate, then acetate and least in butyrate for the 3 diets containing added RFC. For the Lucerne hay diet however, the contribution of lactate to the formation of acetate was greater than that to propionate. This is in apparent contrast to the percentage distribution of label observed with the 3 diets containing added RFC where 45 - 64% of label was found in acetate, 23 - 33% in propionate and 13 - 23% in butyrate. For acetate the distribution of label was lowest on the High concentrate diet and highest on Lucerne hay diet and the opposite

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for butyrate. Since the production rate of acetic acid (2,88 mmol/min) is approximately 3 times greater than the production rate of propionate (0,87 mmol/min) with the concentrations of these acids in the rumen differing by a similar factor (Leng, 1970) label from lactate would have to be distributed in a similar ratio if lactate is to contribute equally to the formation of the two acids. When large amounts of acetate are formed from lactate, ruminal flora obviously play a role but pH also has an important influence. Thus it was found that the rate of production of acetic acid was more dependent on pH than was propionic acid (Satter & Esdale, 1968). Acetate production was maximal at pH 7,4 and butyrate at 6,2 whereas propionate production was largely unaffected within this range. At pH 5,5 propionic acid was reduced to 14% of the maximum rate (Bruno & Moore, 1962).

Acetate is not necessarily the terminal end product from lactate since it can be used to synthesize butyrate Interconversion of label between acetate and butyrate results in an underestimation of entry rate for these acids by 6 - 15 % whereas very little interconversion of label occurs between propionate and acetate or butyrate (Leng, 1970; Van der Walt & Briel, 1976). Depending on the type of ration it has been shown that lactate fed into the rumen or produced during ruminal fermentation can lower the molar percentage of acetate and increase the molar percentage of propionate and butyrate (Elsden, 1945; Hueter, Shaw & Doetsch, 1956; Waldo & Schultz, 1956, 1960; Ekern & Reid, 1963; Montgomery, Schultz & Baumgardt, 1963). Satter & Esdale (1968) provided evidence to explain these findings. Thus when lactate is oxidized to pyruvate, the formation of butyrate from extracellular acetate may be obligatory in that an electron sink must be provided for the 2 hydrogens generated in the lactate oxidation in order to maintain an oxidation-reduction balance. This explanation would also apply to the results in the present experiment for the diets containing added RFC.

The results obtained by Walker & Monk (1971) clearly demonstrate a very large effect of substrate concentration on the distribution of glucose carbon among the VFA. When only trace amount of glucose was added to rumen contents (about 1% the pool size), glucose carbon appeared in acetate and propionate in the ration 24:1. On the other hand, when sufficient glucose was added to increase the pool size 300-fold glucose carbon appeared in acetate and propionate in the ratio 2,4:1. A high concentration of glucose leads to rapid generation of large amounts of reducing power the disposal of which is reflected in greater propionate

/production. ....

production. Similarly when high concentrations of glucose are used lactate accumulates. Thus there is a shift in the position of the rate limiting step in the fermentation, from hydrolysis of polysaccharide to the dissimilation of pyruvic acid. Instead of being converted to VFA, pyruvate acts as H-acceptor in the re-oxidation of the reduced pyridine nucleotide coenzymes generated by the rapid breakdown of RFC (Walker, 1968). When trace amounts of glucose were added to ingesta very little ^{14}C label entered propionate suggesting that this acid was largely formed from substrates whose carbon did not equilibrate with that in free glucose or with that in intermediates of free glucose metabolism (Walker & Monk, 1971).

The glucose turnover rates measured in the present experiments are highest on the High concentrate diet and lowest on the Lucerne hay diet. This is consistent with more rapid fermentation on diets containing large amounts of RFC (Nakamura, Kanegasaki & Takahashi, 1971). Significant correlations were found between glucose utilization rate and lactate production rate for the three diets containing added RFC. Each of the three regression lines (Fig. 9.4) had a similar slope but the point of intersection moved closer to zero on the x-axis (glucose production rate) as RFC in the diet decreased. This suggests that a certain basal rate of glucose utilization exists for each diet, with its own particular microflora, below which no lactate accumulates and above which fermentation becomes rapid enough to cause lactate accumulation. Although a better fit may have been obtained using a higher order curve, the data indicate that the higher the glucose production above basal the greater the lactic acid production.

The *in vitro* experiment supports the hypothesis (Ryan, 1964a; Slyter, 1976) that large accumulations of free glucose give rise to an accumulation of predominantly D-lactate. However under normal feeding conditions the accumulation of free glucose is never as high as this and in the present experiments D-lactate did not often predominate over the L-isomer.

The numbers of bacteria producing and utilizing lactic acid in the rumen of the sheep on the different diets used for the turnover experiments agreed well with those found with comparative diets in the Stepwise Adaptation Experiment. However the types of predominating bacteria were

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different. During the turnover experiment *Bacteroides* formed a large percentage of the amylolytic and glucolytic flora on all diets. During the Stepwise Adaptation Experiment however, the proportion of *Bacteroides* decreased on the final High concentrate diet although this occurred only during the period of instability. *Lactobacillus* were present in the rumen of the sheep fed the High concentrate diet during the turnover experiment, however in the Adaptation Experiment they formed a greater proportion of the flora on the final High concentrate diet.

Among the predominant lactate-utilizing bacteria the most important differences were that *Anaerovibrio* was never as predominant in the turnover experiments while *Veillonella* occurred even on the High concentrate diet despite its reported acid-sensitivity (see Chapter 5). During the turnover experiments *Selenomonas* formed an important portion of the predominant flora in the rumen of the sheep fed the Intermediate diet and occurred amongst the predominating glucolytic, amylolytic and lactate-utilizing bacteria. On the High concentrate diet *Megasphaera* occurred among the predominating glucolytic and lactate-utilizing bacteria which corresponded with the greatest contribution of the acrylate pathway to propionate formation. *Borrelia* sp are known to be very difficult to isolate and cultivate *in vitro* but they have been shown to ferment glucose and starch with the production of some lactic acid (Bryant, 1959).

An explanation was sought for the sporadic appearance of *Streptococcus bovis* in the adaptation experiment and the absence of this species in the turnover experiment. This organism has a high maximum specific growth rate and under favourable conditions has a doubling time of as little as 9 min (Brüggemann & Giesecke, 1965). The most probably explanation is that despite the capacity to double rapidly it has a very high substrate saturation constant (Schwartz & Gilchrist, 1975) so that under the conditions in the present experiments the concentration of free glucose was never high enough for rapid growth except maybe for a short time after feeding.

During the turnover experiment *Megasphaera* was present among the predominant flora whereas it appeared speradically during the first Adaptation Experiment probably due to the lack of sufficient substrate, i.e. lactate, in that experiment. However any lactate-utilizing bacteria which can ferment other substrates such as glucose or starch in addition

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to lactate would be at an advantage in the rumen under normal feeding conditions where there is insufficient lactate to sustain growth of large numbers of lactate-utilizing bacteria. Thus organisms like *Megasphaera*, *Selenomonas* and *Propionibacteria* would be at an advantage to *Anaerovibrio* and *Veillonella* which do not ferment glucose but a great deal more information will have to be obtained on growth rates on multiple substrates with and without competition from other organisms before an aswer can be given to this complex problem. In this respect Hishinuma *et al* (1968) found that, on glucose plus lactate, *S. Ruminantium* utilized glucose preferentially at high concentation and that lactate utilization occurred only after a lag period during which glucose was exhausted. At low glucose concentration lactate utilization occurred with no lag.

Although *S. bovis* produces only L-lactic acid, as shown in the present experiment and also by Slyter, Bond, Rumsey & Weaver (1974), the presence of concentrations of L-lactic acid which were greater than D-lactic acid in the present experiments can be explained by the presence of organisms which produce DL-lactic acid such as the Lactobacilli, *Butyrivibrio* and *Selenomonas*. Some of the species of lactobacilli such as *L. ruminis* produce 95% L- and 5% D-lactic acid while *L. vitulinus* produces the D-isomer only (Sharpe, Latham, Garvie, Zirngibl & Kandler, 1973) and this characteristic is of value in their identification. Ogimoto & Giesecke (1974) have shown that *Megasphaera elsdenii* utilizes D- and L-lactic acid at about equal rates whereas *Veillonella* utilized 93% of L-lactic acid and only 23% of D-lactic acid.

The degradation studies on labelled propionate showed that the randomizing pathway was quantitatively much more important than the acrylate pathway in the fermentation of lactate accounting for 70,5-91,2% of the lactate fermented. In confirming the findings of other workers (Baldwin *et al*, 1962; Wallnöfer *et al*, 1966; Prins & Van der Meer, 1976) the contribution of the acrylate pathway increased when RFC was included in the diet although the values obtained were quantitatively much lower in the present experiment. The values obtained by Baldwin *et al* (1962) could be an overestimate since their incubations were run under a nitrogen atmosphere whereas it is known that those organisms metabolizing lactate via succinate require relatively high concentrations of CO_2 for rapid initiation of growth and optimal growth rates while

/*M. elsdenii* .....

M. elsdenii would not be affected. The production of propionate from lactate 4-8 h after feeding is very much lower than 0-2 h after feeding so quantitatively the contribution of the acrylate pathway is small at this time.

The increase in the contribution of acrylate with time after feeding is possibly due to a change in substrate utilization by *M. elsdenii*. Shortly after feeding this organism can ferment the free glucose present which then decreases to a level at which *M. elsdenii* cannot compete and so it might switch to lactate utilization at the later time after feeding. This assumes preferential glucose utilization and a competitive advantage in the utilization of lactate because of rapid growth rate.

The finding that the lactate-utilizing bacteria were unable to couple sulphate reduction with lactate utilization as do the dissimilatory sulphate reducers, showed that this mechanism was unlikely to account for the decrease in lactate accumulation when sheep fed the S-deficient purified diet where provided with supplemental S. It is possible that the lactate-utilizing bacteria on the S-deficient purified diet with urea as sole source of N where growth-limited by the lack of suitable sulfur containing amino acids which are known to be stimulatory (see Chapter 3). An "open system" in which the H_2S is removed as it is produced by gassing, in order to remove the possibility of end product inhibition, would be better than the closed system used in the present experiments. The lactate analyses show that the sulphate-reducers were not growth limited by lack of lactate since they only utilized approximately $^1/3$ of that provided. Furthermore not all the sulphate was utilized thus supporting the idea of end product inhibition. Sufficient lactate was added to enable complete conversion of all sulphate to sulphide. Based on known pathways, rumen micro-organisms can obtain their essential S supplies: (a) entirely from cysteine directly; (b) partially from methionine directly; (c) partially or wholly from inorganic sulphates by assimilatory reduction; (d) partially or wholly from sulphide accumulating from dissimilatory reduction of inorganic sulphates; or (e) from sulphide originating from cysteine or other organic substances (Moir, 1969). The lactate-utilizers could fit into one or more of the above categories. The numbers of sulphate-reducers found in the rumen of sheep on various diets were 10^4-10^5 per g of ingesta

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and were outnumbered by a factor of 1 000 - 10 000 by the lactate-utilizers determined simultaneously. Gutierrez (1953) obtained similar numbers of lactate-utilizing, sulphate-reducing bacteria in the rumen of cattle which were classified as *Desulfovibrio* sp. This number of organisms is sufficient to account for the free sulphide pool found in rumen contents (Huisingh, McNeil & Matrone, 1974).

The results reported in this part of the thesis reveal that even on high roughage diets there is a variable production of lactic acid and lactate accumulation was dependent on the relative rates of production and utilization. Thus shortly after feeding production exceeded utilization and lactate accumulated transiently. Thereafter utilization exceeded production and the concentration of lactate decreased until low basal levels were reached when production was balanced by utilization. The rate of production was influenced mainly by the numbers and types of bacteria capable of producing lactic acid as well as the concentration and turnover of free glucose. The utilization of lactic acid was dependent on the production of an adequate amount of growth substrate to stimulate the growth of the lactate-utilizing bacteria. When one of the isomers of lactic acid predominated in the rumen this was due to a greater production rate of that isomer and not to a lower utilization rate which was concentration dependent.

The contribution of lactate to the formation of the individual VFA was greatest for propionate and least for butyrate on the 3 diets containing added RFC whereas on the Lucerne hay diet the contribution of lactate was greatest to the formation of acetate, then propionate and least in butyrate. Lactate was not an important intermediate on these diets since no more than 8% of the total VFA was formed through lactate on the High concentrate diet and less on the other diets. Under the conditions prevailing in these experiments the randomizing pathway was quantitatively more important in lactate fermentation than the direct reductive pathway but it is recognized that these mechanisms may not operate in the same manner under conditions where large intakes of more easily fermented carbohydrate exist.

SUMMARY

Preliminary studies were performed to establish and test methods for the analysis of D- and L-lactic acid and for the enumeration and isolation of lactate-utilizing bacteria from the rumen. Good reproducibility was obtained for the enzymic analysis of the two isomers of lactic acid, using steriospecific D- and L-LDH, in ruminal fluid and culture supernatant. A highly specific growth medium without rumen fluid was developed which supported the growth of all species of lactate-utilizing bacteria known to occur in the rumen. Numbers of lactate-utilizing bacteria on this medium which contained 2,0% Na-DL-lactate and 2,0% Trypticase/0,2% Yeast extract were lower than those on a medium containing 0,35% Na-DL-lactate and 0,2% Trypticase/0,05% Yeast extract. However, the medium containing the low concentration of components did not support the growth of lactate-utilizing cocci and in addition specificity and survival of isolates was higher on the medium containing the high concentration of componants. An exploratory experiment established that under "normal" feeding conditions to be used throughout the studies in the thesis, lactic acid accumulated transiently immediately after a feed but had returned to the low basal concentration *ca* 2 h after the feed. The amount of readily fermentable carbohydrate in the diet and the rate of food consumption were shown to influence the concentration of lactic acid in the rumen.

A Stepwise Adaptation Experiment was carried out at restricted food intake in order to study some of the critical factors involved in adaptation to high concentrate diets. The sheep were changed at weekly intervals through diets containing 10, 24, 44, 60 to a final diet containing 71% ground maize and molasses. The numbers of ciliate protozoa in the rumen increased in proportion to the amount of readily fermentable carbohydrate fed, up to and including the 60% grain and molasses diet, while the numbers of total culturable bacteria remained essentially constant. However, the proportions of amylolytic and lactate-utilizing bacteria increased, and there was an orderly shift from acid-sensitive to more acid-tolerant genera particularly amongst the lactate-utilizers in response to the gradual fall in ruminal pH. Up to this stage the protozoa controlled the rate of fermentation by engulfing starch grains and bacteria, aided by the inclusion of 3% Ca CO_3 as dietary buffer. Lactic acid appeared transiently and did not increase as the adaptation progressed.

/The day .....

The day after the final diet was fed ruminal pH decreased and within 7 days the protozoal numbers had decreased by 50 - 80% while the numbers of total culturable bacteria increased sharply to a peak ($2\ 825 \times 10^7$ per g of ingesta). Conditions in the rumen became unstable and sheep A72 refused all food for one day. The lowest ratio (2,1) of acetate/propionate was found after 21 days on the final diet, when acid-tolerant species of lactate-utilizing bacteria multiplied rapidly in response to the increased production of ruminal lactic acid, and the ratio of amylolytics to lactate-utilizers decreased from a mean of 10,7 to 3,6. This controlled the increase in lactic acid and decrease in ruminal pH, allowing the ciliate protozoa to proliferate and regain control of the fermentation.

Among the amylolytic bacteria, *Bacteroides* tended to predominate during adaptation but was superseded by *Lactobacillus* and *Eubacterium* on the final diet containing 71% grain and molasses. *Butyrivibrio* were present among the predominant amylolytic bacteria throughout the experiment. *Anaerovibrio* was found to be the predominant lactate-utilizer although *Propionibacter* were also present throughout the study. *Veillonella* and *Selenomonas* had disappeared from the predominant lactate-utilizing bacteria by the time the 24 and 44% grain and molasses diets had been fed respectively.

A value was calculated which took into account the length of time that pH stayed below a certain critical value (chosen as pH 6,00) since this would have most effect on growth rate and hence on the proportions of predominating ruminal bacteria. This value was given the unit of "pH_6-hours". Highly significant correlations were obtained between the mean values for "pH_6-hours" and the percentage proportions of acid-sensitive *Veillonella + Selenomonas* and acid-tolerant *Lactobacillus + Eubacterium*.

A subsequent stepwise adaptation experiment was carried out. It was designed to be more in line with intensive feeding practice where food is offered *ad libitum* and animals are kept on the final High concentrate diet for 100 - 150 days. In view of the fact that the crude protein requirements of ruminants increase as the energy content of the diet increases, and that on a few occasions in the first Adaptation Experiment concentrations of NH_3-N were marginal for growth of ruminal bacteria and could thus have influenced the adaptation, the crude protein

/content .....

content of all diets was increased to 15%. Ruminal pH decreased as the adaptation progressed and on the final High concentrate was below 6,00 for almost the whole 24 h period on day 98 and day 119. Despite the fact that minimal ruminal NH_3-N concentrations were only slightly higher than those found in the first Adaptation Experiment, and that the sheep remained on the final diet at *ad libitum* intake for 120 days, total lactate concentration never exceeded 10 mM indicating that a balanced ruminal flora was maintained throughout the experiment.

The turnover of lactic acid in the rumen was determined *in vivo* with sheep fully adapted to a series of diets containing different amounts of readily fermentable carbohydrate. Simultaneous determinations were also made of the turnover of free glucose in the rumen and of the micro-organisms that were present during the experimental period. The turnover of lactic acid in the rumen using the continuous infusion technique was highest in the rumen of the sheep fed the diet containing 65% grain and molasses. At peak lactate fermentation, ¼ - ½ h after feeding production rate was 0,631 mM/min. On the 2 roughage diets the peak production rates were 0,164-0,187 mM/min, while on the Intermediate diet containing 43% grain and molasses the value was 0,304 mM/min. Production rates returned to low basal values (0,01-0,02 mM/min) *ca* 2 h after feeding. The values for utilization rates followed the same trend as production rates but with a short time lag. The correlation between lactate production and lactate concentration was highly significant (r=0,967). The concentration of D- and L-lactic acid was a reflection of their relative production rates, and on the diets fed in the present experiment, the rates of production and utilization were essentially the same for both isomers of lactic acid.

The contribution of lactate to the production of the individual volatile fatty acids was dependent not only on the amount of readily fermentable carbohydrate in the diet but also with time after feeding. At peak lactate turnover, ¼ - ½ h after feeding, the values ranged from 14,6 - 23,9% for acetate, 28,1 - 53,3% for propionate and 11,4 - 17,7% for butyrate on the diets containing added readily fermentable carbohydrate. These values decreased to 1 - 3% 6 h after feeding. The values on the Lucerne hay diet at peak fermentation, 1 - 1½ h after feeding, were 10,5%, 6,9% and 6,5% for acetate, propionate and butyrate respectively.

The randomizing or succinate pathway was quantitatively more important than the direct reductive or acrylate pathway. The contribution of the acrylate pathway to the formation of propionate from lactate

/was greatest .....

was greatest on the High concentrate diet and decreased as the amount of readily fermentable carbohydrate in the diet decreased. The contribution also increased with time after feeding and was thus 17,5% ½ - 1 h and 29,5% 6 - 8 h after feeding the High concentrate diet. Although the acrylate pathway increased in importance with time after feeding, the contribution of lactate as a whole to propionate formation 6 - 8 h after feeding was reduced to *ca* 2%.

The *in vivo* turnover of free glucose was measured simultaneously with lactate turnover using a double label, continuous infusion technique. On the High concentrate diet peak values for the rates of production (0,59 mM/min) and utilization (0,63 mM/min) occured ¾ - 1¼ h after feeding with the peak in production preceeding that in utilization. The rates had returned to the basal rates, *ca* 3 h after feeding, which were $^{1}/3$ to ½ of peak values. A similar pattern was found for the other 3 diets except that peak values did not increase markedly on the 2 roughage diets. The peak values for production and utilization rates on the Intermediate diet were 0,24 and 0,23 mM/min, on the High roughage diet they were 0,16 and 0,17 mM/min and on the Lucerne hay diet they were 0,10 and 0,09 mM/min respectively Correlations between glucose utilization rate and the production rate of lactic acid were significant at the 95% level for the 3 diets containing readily fermentable carbohydrates.

When the flasks of ruminal ingesta used for an *in vitro* experiment were dosed with an amount of glucose which was proportional to the amount of readily fermentable carbohydrate consumed by the donor sheep, the free glucose reached a concentration of 56 - 57,5 mM/min and D-lactic acid increased to 10,7 - 16,0 mM. The concentration of L-lactic acid was 4 - 6 times lower than the corresponding D-lactic acid concentration. These results show that under conditions where large accumulations of free glucose occur the production rate of D-lactate is high. These conditions occur in the rumen after excessive intake of readily fermentable carbohydrate. In the present *in vivo* experiments this was not the case.

The numbers of micro-organisms present in the rumen of the sheep fed different amounts of readily fermentable carbohydrate were similar to the number of organisms present on comparable diets during the Stepwise Adaptation Experiment. However, glucolytic bacteria were not included in the micro-organisms studied in the Adaptation Experiment. The numbers of glucolytic bacteria were 25 - 62% of the total culturable bacterial count and 2 - 7 fold higher than the amylolytic count. The numbers of lactate-utilizing bacteria were *ca* 20-fold higher on the High concentrate diet than on the 2 roughage diets and *ca* 5-fold higher than on the Intermediate diet. Although

the same organisms were usually present among the amylolytic and glucolytic bacteria their proportions differed considerably. *Bacteroides* and *Butyrivibrio* formed a large proportion of these two functional groups of bacteria. On the High concentrate diet *Lactobacillus* spp and *Megasphaera* formed 30% and 40% of the glucolytic bacteria respectively. The lactobacilli were only present on the Intermediate diet and were replaced by *Eubacterium* on the Intermeidate and High roughage diets. *Propionibacter* and *Selenomonas* formed a large proportion of the lactate-utilizing bacteria on all diets. *Anaerovibrio* formed 11 - 19% of the predominant lactate-utilizers in the rumen of sheep fed the diets with added readily fermentable carbohydrate whereas *Megasphaera* formed 6 - 21% of the lactate-utilizers on all diets. The large proportion of *Megasphaera*, the only organism metabolizing lactate via the acrylate pathway in the rumen, corresponded with the greatest contribution of the acrylate pathway to propionate formation on the High concentrate diet. The proportions of the predominant bacteria in the rumen of sheep adapted to the series of diets differing in amount of readily fermentable carbohydrate were different to those found on comparable diets during the Stepwise Adaptation Experiment, especially amongst the lactate-utilizers.

Most of the glucolytic and amylolytic bacteria were found to produce DL- or L-lactic acid. Although *Bacteroides* formed a major portion of the isolates in these two functional groups of organisms they only produced small quantities of lactic acid (<1 mM) in comparison to the other lactate producers such as the Lactobacilli and *Butyrivibrio*. Lactic acid production was greater on fermentation medium containing glucose than on medium containing starch, most probably due to faster growth rate on glucose-containing medium. The lactate-utilizing bacteria were found to utilize mostly DL-lactic acid although many isolates were found to utilize D-lactic acid only or L-lactic acid only. This confirmed the earlier finding that the concentrations of D- and L-lactic acid were dependent on their relative rates of production by bacteria in the rumen rather than their utilization rates, at least under "normal" feeding conditions as used throughout these studies. It was found that the utilization of lactic acid by species of lactate-utilizing bacteria was not coupled with the reduction of sulphate which acts as a hydrogen sink. This is in contrast to the results obtained with sulphate reducing bacteria.

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A P P E N D I X

Appendix Table 2.1. The mean, SD and CV (n=5) for ΔE values of D- and L-lactate in Method 1 using hydrazine to trap pyruvate

Conc (mM)	ΔE for D-lactate Mean	SD	CV	ΔE for L-lactate Mean	SD	CV
0,5	0,020	0,001	8,2	0,148	0,007	5,1
1,0	0,041	0,002	6,5	0,248	0,014	5,7
2,5	0,086	0,008	9,9	0,405	0,022	5,5
5,0	0,157	0,005	3,7	0,518	0,007	1,5
7,5	0,217	0,006	2,9	0,579	0,011	2,0
10,0	0,260	0,009	3,7	0,657	0,015	2,4

Appendix Table 2.2. The mean, SD and CV (n=5) for ΔE values of D- and L-lactate in Method 2 using GPT to trap pyruvate

Conc (mM)	ΔE for D-lactate Mean	SD	CV	ΔE for L-lactate Mean	SD	CV
0,5	0,081	0,001	1,7	0,098	0,003	3,1
1,0	0,128	0,004	3,1	0,192	0,005	2,6
2,5	0,317	0,012	3,8	0,482	0,014	2,9
5,0	0,619	0,011	1,8	0,873	0,019	2,3
7,5	0,818	0,008	1,0	1,157	0,021	1,8
10,0	1,037	0,015	1,4	1,399	0,030	2,1

Appendix Table 3.1. Composition of media with and without ruminal fluid (RF) for enumerating total culturable and lactate-utilizing bacteria. All quantities are per 100 ml of medium

Component		Supplier	Total culturable medium		Lactate utilizing medium				
			with RF	without RF	LH	LL	LM	LA	LB
Agar	(g)	Difco	2	2	2	2	2	2	2
Na-DL-Lactate (10% soln)	(ml)	BDH	-	-	20	3,5	10	20	3,5
Maltose	(g)	Merck	0,05	0,05	-	-	-	-	-
Cellobiose	(g)	Fluka	0,05	0,05	-	-	-	-	-
Soluble Starch	(g)	Merck	0,05	0,05	-	-	-	-	-
Xylan	(g)	Fluka	0,05	0,05	-	-	-	-	-
Trypticase	(g)	BBL	-	0,2	2	0,2	1	0,2	2
Yeast extract	(g)	Difco	-	0,05	0,02	0,05	0,1	0,05	0,2
Clarified ruminal fluid	(ml)	-	40	-	-	-	-	-	-
VFA solution	(ml)	-	-	1	1	1	1	1	1
Trace element soln	(ml)	-	-	2	2	2	2	2	2
Haemin soln (50 mg/1)	(ml)	Fluka	-	2	2	2	2	2	2
Mineral soln 1	(ml)	-	7,5	7,5	7,5	7,5	7,5	7,5	7,5
Mineral soln 2	(ml)	-	7,5	7,5	7,5	7,5	7,5	7,5	7,5
Indigo carmine (0,05% soln)	(ml)	Matheson, Coleman & Bell	1	1	1	1	1	1	1
Deionized water	(ml)	-							

Notes:

1. After melting 7 ml of 9,1% $NaHCO_3$ soln was added to all media.
2. After sterilization 0,2 ml of 25% (w/v) soln of glucose which had been filter sterilized was added to the total media.
3. VFA soln was according to Caldwell & Bryant (1966).
4. Trace element soln was according to Kogut & Podoski (1953).
5. Mineral solns 1 and 2 were according to Bryant & Robinson (1961).
6. Cysteine-Na2S reducing reagent was added to bottles of counting medium (0,2 ml per 8,8 ml medium) immediately before use or 10 ml added to medium if maintenance slopes were made. The reducing reagent was made up as described by Van Gylswyk (1970).
7. The gas phase was 98% CO_2/2% H_2. Final pH of all media was 6,8.
8. Anaerobic diluent contained 3,75 ml each of mineral soln 1 and 2, 1,0 ml of indigo carmine, 70 ml of $NaHCO_3$ soln and 0,05 g cysteine-HCl. The diluent was dispensed into bottles gassed with 98% CO_2/2% H_2 and heat sterilized.
9. Maintenance slopes of the LH and LL media were made by dispensing the media containing $NaHCO_3$ and reducing agent (see note 6) in 5 ml amounts into gassed bottles and allowing the molten medium to solidify into a slope.

Appendix Table 5.1. Composition and preparation of media for carbohydrate fermentation tests used in the identification of isolates

The carbohydrates tested were all added to the same basal medium. The following components were mixed prior to dissolving in 250 ml Erlenmeyer flasks and stoppered:

Solution 1 contained 3 g K_2HPO_4, 4 g $NaHCO_3$ and 30 mg Indigo carmine

Solution 2 contained 3 g KH_2PO_4, 6 g NaCl, 6 g $(NH_4)_2SO_4$, 0,6 g $CaCl_2.2H_2O$ and 1,23 g $MgSO_4.7H_2O$.

An 8 l flask fitted with a stopper and tubes for gassing and dispensing was partially filled with 3,9 l of water and poiled to expel O_2. While allowing the water to cool it was gassed with 88% N_2/10% CO_2/2% H_2. When cool the above two solutions, 500 ml of clarified rumen fluid (see text for preparation, page 23), 50 g Trypticase, 10 g yeast extract and 100 ml of Cysteine.HCl-Na_2S reducing agent were added. The pH was adjusted to 6,80, gassing stopped and the volume made up to 5 l. The basal medium was dispensed in 5 ml amounts into 1 oz McCartney bottles pregassed with 88/10/2 gas mixture. The 1 oz bottles were heat sterilized.

The respective carbohydrate substrates were weighed out in 2,5 g amounts and made up to 25 ml with deaerated water. Each solution was then passed through a sterile millipore filter (25 mm filter, GS with 0,22 μm pre size. Millipore Corp., Bedford, Mass., USA) into sterile 4 oz McCartney bottles pregassed with nitrogen. Cellulose, starch, xylan and aesculin were heat sterilized. Immediately before the fermentation tests 0,5 ml amounts of test carbohydrate solution were added to the basal medium using sterile Cornwall syringes.

The maintenance slopes were taken from the dry-ice cabinets, allowed to thaw and washed off onto fresh slopes with 10 ml of anaerobic diluent. The fresh slopes were incubated for 2-3 days and all fluid containing the growing cells transferred to a bottle of anaerobic diluent. The anaerobic diluent (9 ml amounts) was prepared as described previously but also contained 0,2 ml of a Dithiothreitol solution (1,0 g dithiothreitol (Miles Serevac) made up in 100 ml of boiled water and heat sterilized in a N_2-gassed, 4 oz McCartney bottle) added just before use. The test media were inoculated with 0,25 ml of the cell suspension in anaerobic diluent using Cornwall syringes. The carbohydrates tested are listed in Appendix Tables 5.6 and 5.8.

Appendix Table 5.2(a). Composition and preparation of biochemical test media based on the Anaerobe Laboratory Manual. The final volume of medium was 250 ml

Composition	Unit	Urea medium	Nitrate medium	PY-Arginine medium	PY-Threonine medium	PY-Hippurate medium
Peptone (Difco)	g	2,5	0,25	2,5	2,5	2,5
Yeast extract (Difco)	g	1,0	0,25	2,5	2,5	2,5
Urea (Merck)	g	5,0	-	-	-	-
Glucose (Merck)	g	0,25	0,25	-	-	-
Lactate (10% soln) (BDH)	ml	2,5	2,5	-	-	-
Vit K-Heme soln[1]	ml	2,5	-	2,5	2,5	2,5
VFA soln[1]	ml	5,0	-	-	-	-
Trace element soln[2]	ml	10,0	-	-	-	-
Salts soln[1]	ml	25,0	10,0	25,0	25,0	25,0
Indigo carmine soln[2]	ml	2,5	-	2,5	2,5	2,5
Cysteine/Na_2S soln[2]	ml	5,0	-	5,0	5,0	5,0
Deionized water	ml	197,5	207,5	215,0	215,0	215,0
Casein hydrolysate (Merck)	g	-	1,0	-	-	-
Sodium pyruvate (Boehringer)	g	-	2,75	-	-	-
Clarified rumen fluid[2]	ml	-	25,0	-	-	-
$NaNO_3$ (Merck)	g	-	0,5	-	-	-
Dithiothreitol[2]	ml	-	5,0	-	-	-
Arginine (Merck)	g	-	-	2,5	-	-
Threonine (Merck)	g	-	-	-	0,75	-
Hippurate (Merck)	g	-	-	-	-	2,5
Gas phase	-	98% N_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2
Sterilization$^{\Phi}$	-	Filter	Filter	Heat	Heat	Heat

[1] As described in the Anaerobe Laboratory Manual (Holdeman & Moore, 1975)

[2] As described under counting methods in Chapter 3 and Chapter 5

$^{\Phi}$ Procedure for filter sterilized media - boiled water was used to make up the medium with the reducing agent added last. The pH was adjusted to 6,80, passed through a sterile Millipore filter and then dispensed in 5,0 ml amounts into sterile gassed 1 oz McCartney bottles

$^{\Phi}$ Procedure for heat sterilized media - all components except dithiothreitol, $NaHCO_3$ and glucose were added before melting. After melting these components were added, pH adjusted to 6,80 and then distributed in 5,0 ml amounts into gassed 1 oz McCartney bottles. These were then heat sterilized

Appendix Table 5.2(b). Composition and preparation of biochemical test media. The final volume of medium was 250 ml

Composition	Unit	Casein medium	Catalase slopes	Gelatin medium	Pyruvate medium	Lecithin medium	Tributyrin medium	Triolein medium
Agar (Difco)	g	5,0	5,0	-	-	5,0	5,0	5,0
Peptone (Difco)	g	-	-	1,5	2,5	-	-	-
Yeast extract (Difco)	g	-	0,25	1,5	0,25	-	-	-
Glucose (Merck)	g	-	0,25	0,1	-	-	-	-
Lactate (10% soln) (BDH)	ml	1,0	2,5	1,0	-	-	-	-
Indigo carmine soln[2]	ml	2,5	2,5	2,5	2,5	2,5	2,5	2,5
Cysteine/Na_2S soln[2]	ml	-	5,0	5,0	5,0	5,0	5,0	5,0
Deionized water	ml	159,0	157,5	159,0	160,0	135,0	135,0	135,0
Sodium pyruvate (Boehringer)	g	-	-	-	2,75	-	-	-
Clarified rumen fluid[2]	ml	25,0	25,0	25,0	25,0	25,0	25,0	25,0
Soluble starch (Merck)	g	0,1	0,25	0,1	-	-	-	-
Casein (Koch-Light)	g	2,5	-	-	-	-	-	-
Tryptose (Difco)	g	0,75	-	-	-	-	-	-
Mineral soln 1[2]	ml	20,0	20,0	20,0	20,0	20,0	20,0	20,0
Mineral soln 2[2]	ml	20,0	20,0	20,0	20,0	20,0	20,0	20,0
Casitone (Difco)	g	-	1,0	-	-	-	-	-
Gelatin (Merck)	g	-	-	30,0	-	-	-	-
Dithiothreitol[2]	ml	5,0	-	-	-	-	-	-
$NaHCO_3$ (9,1% w/v soln)	ml	17,5	17,5	17,5	17,5	17,5	17,5	17,5
Lecithin (Merck)[3]	ml	-	-	-	-	25,0	-	-
Tributyrin (Merck)[3]	ml	-	-	-	-	-	25,0	-
Triolein (BDH)[3]	ml	-	-	-	-	-	-	25,0
Gas phase	-	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2	98% CO_2/2% H_2
Sterilization	-	Heat	Heat	Heat	Filter	Heat	Heat	Heat

[2]As described in counting methods in Chapter 3 and Chapter 5

[3]The emulsions were prepared as follows. Lecithin (1,6g), Tributyrin (3,2 ml) and Triolein (3,2 ml) were each added to 40 ml of warm deaerated water in a conical flask. The emulsion was dispersed using a Dawe Soniprobe tuned to maximum output for 4 min. The required volume (25 ml) was added to the medium.

Appendix Table 5.3. Biochemical tests performed on growth media for identification of isolates

The composition and preparation of the different media used is given in Appendix Table 5.2. All media were inoculated with growing cells in anaerobic diluent (see Appendix Table 5.1) and incubated for 7 days. The following tests were then performed.

1. Urease test on urea medium. The medium was tested for pH and NH_3 using Nessler's reagent. A positive reaction was recorded if pH > 8 or NH_3 was present in the spot plate test as compated to uninoculated bottles.

2. Nitrate reduction on nitrate medium. The culture medium was tested for the presence of nitrite using the colour reagents described in the Anaerobe Laboratory Manual (Holdeman & Moore, 1975). The development of a red colour was recorded as positive.

3. Proteolysis in casein medium. The molten inoculated growth medium was injected into Astell roll bottles and a thin film of solidified medium obtained using a roll bottle apparatus (see page 41). The roll bottles were incubated for 7 days and casein hydrolysis detected by clearings in the opaque medium.

4. Gelatin liquefaction. The bottles of media were chilled in a refrigerator until the control bottles solidified. They were then removed to room temperature and inverted. If they failed to solidify the reaction was positive and liquefaction in less than $\frac{1}{2}$ the time required for the controls was weak positive.

5. Catalase production on slopes. The growth on the slopes was exposed to air for 30 min and the surface flooded with 3% H_2O_2. The surface was observed for continuous bubble formation to indicate a positive reaction as compared to control slopes.

6. Pyruvate utilization. This was tested using an enzymic method with L-LDH and NADH obtained from Boehringer. Utilization was calculated as disappearance of pyruvate as compared with uninoculated control bottles.

7. Arginine fermentation on PY-medium. This was tested using Nessler's reagent to detect the presence of NH_3.

8. Conversion of threonine to propionate. The PY-medium was tested for the presence of propionate using the gas chromatographic procedure described in Chapter 5 (page 43).

9./ .....

Appendix Table 5.3. (continued)

9. Hippurate hydrolysis. This was detected in the PY-medium using the acidic $FeCl_3$ test or by alkaline reaction.

10. Lipase and lecithinase activity. This was detected as clearings in agar films of the respective opaque medium obtained using the roll bottles technique described earlier for proteolysis on casein medium.

Appendix Table 5.4. Molar percent of individual VFA in the rumen fluid of sheep adapted stepwise to a high concentrate diet. The samples were taken two hours after the morning feed and were also used for the microbiological counts (C_2 = acetic, C_3 = propionic, i-C_4 = isobutyric, C_4 = butyric, i-C_5 = isovaleric, C_5 = valeric and C_6 = caproic acid)

Sheep	Diet & day	Molar percent of individual VFA							Diet & day	Molar percent of individual VFA						
		C_2	C_3	i-C_4	C_4	i-C_5	C_5	C_6		C_2	C_3	i-C_4	C_4	i-C_5	C_5	C_6
A18	A	64,8	18,4	0,9	13,7	0,7	0,9	0,4								
A58	17	61,9	20,8	0,7	14,1	0,5	0,9	0,3								
A72		60,7	19,8	1,0	15,7	0,7	0,9	0,3								
A18		62,7	18,7	0,9	15,9	0,5	1,0	0,3		62,0	20,3	0,9	14,6	0,7	0,9	0,2
A57	AE	65,2	17,9	1,0	14,5	0,5	0,7	0,1	AE	63,4	19,2	1,1	14,0	1,0	0,8	0,1
A58	1	61,4	23,0	0,6	13,2	0,5	0,7	0,1	7	62,6	20,7	1,0	13,1	1,0	0,7	0,0
A72		64,6	19,5	1,0	13,0	0,5	0,7	0,1		66,1	18,6	0,9	12,1	0,8	0,8	0,3
A18		62,5	18,1	0,8	17,1	0,6	0,8	0,2		60,5	20,0	1,9	15,4	0,9	0,8	0,4
A57	E	65,7	16,6	0,9	14,1	0,7	0,8	0,6	E	55,7	23,4	1,4	15,3	1,5	1,2	0,5
A58	1	67,6	17,0	0,9	11,4	0,9	0,3	0,2	7	54,0	20,7	1,7	17,1	2,5	1,9	1,4
A72		62,7	22,1	1,1	12,7	0,6	0,8	0,5		60,7	17,2	1,3	16,1	1,7	1,4	0,8
A18		57,6	24,9	1,0	14,5	0,9	0,9	0,2		53,2	27,3	1,0	15,9	1,1	1,1	0,2
A57	EB	58,9	24,2	0,9	13,0	0,8	1,0	0,6	EB	61,5	17,2	1,2	15,9	1,6	1,2	0,6
A58	1	52,6	24,4	1,0	17,8	1,4	1,6	0,7	7	57,3	23,3	1,2	13,5	2,7	1,3	0,3
A72		63,5	15,9	0,9	15,7	1,0	1,0	0,5		57,5	19,5	1,2	17,8	1,3	1,1	0,5
A18		51,2	28,5	1,1	16,2	1,1	1,1	0,2		44,9	26,3	1,2	22,9	1,6	1,9	0,8
A57	B	62,1	17,6	1,0	15,1	1,5	1,1	0,4	B	53,7	17,6	1,5	20,4	3,1	1,7	0,9
A58	1	56,3	21,1	1,0	16,7	2,4	1,2	0,4	7	56,2	21,9	0,8	13,2	5,1	1,5	0,5
A72		58,4	19,9	1,4	15,9	1,3	1,0	0,3		50,0	26,6	1,0	18,6	1,0	1,1	0,3
A18	B	50,0	28,1	0,9	17,9	1,2	1,3	0,3	B	57,9	27,7	0,8	11,6	0,9	0,8	0,0
A72	21	46,1	17,2	2,0	23,6	3,6	2,3	1,7	40	51,8	25,7	1,1	15,9	1,5	1,1	0,2
A18		53,3	25,5	0,8	17,0	1,5	1,5	0,3								
A57	B	50,6	22,6	1,2	21,7	2,1	1,2	0,2								
A58	54	55,5	20,0	1,3	17,5	3,1	1,3	0,9								
A72		55,2	21,6	1,0	18,6	1,6	1,1	0,2								

Appendix Table 5.5 The number of amylolytic and lactate-utilizing bacteria as a percentage of the total culturable bacterial count

Diets		A	AE		E		EB		B				
Grain & molasses (%)		10	24		44		60		71				
Days on diet		17	1	7	1	7	1	7	1	7	21	40	54
	A18	1,4	3,2	3,8	13,2	8,5	5,8	6,1	7,4	9,7	22,9	30,8	24,2
Amylolytic	A57	0,3	2,8	1,3	7,8	8,9	11,8	5,8	14,1	4,8	-	-	29,8
bacteria	A58	1,7	5,4	3,7	32,7	1,6	34,1	31,7	38,5	0,9	-	-	6,2
	A72	4,1	16,8	12,1	8,3	2,6	3,8	10,4	10,8	30,6	12,9	7,8	2,3
	A18	0,2	0,9	0,4	0,3	1,9	0,7	0,3	0,6	9,7	22,9	2,4	36,9
Lactate-	A57	0,1	0,4	0,7	0,3	0,8	2,3	0,7	1,0	1,4	-	-	5,9
utilizing	A58	0,2	3,5	0,3	3,5	0,4	2,0	5,2	6,0	0,6	-	-	19,2
bacteria	A72	0,4	0,4	0,6	0,5	0,2	1,1	1,0	3,2	3,3	5,7	1,0	1,5
Contribution of	A18	1,6	4,1	4,2	13,5	10,4	6,5	6,4	8,0	19,4	45,8	33,2	61,1
both groups to	A57	0,4	3,2	2,0	8,1	9,7	14,1	6,5	15,1	6,2	-	-	35,7
total culturable	A58	1,9	8,9	4,0	36,2	2,0	36,1	36,9	44,5	1,5	-	-	25,4
count	A72	4,5	17,2	12,7	8,8	2,8	4,9	11,4	14,0	33,9	18,6	8,8	3,8

Appendix Table 5.6. Fermentation tests of selected amylolytic isolates

Carbohydrate	Amylolytic isolates										
	A10	A32	A62	A65	A75	A88	A101	A132	A152	A155	A156
Arabinose	-	w	a	-	a	a	a	a	a	-	w
Cellobiose	-	a	a	w	a	a	a	a	a	-	w
Aesculin (pH)	-	-	w	-	w	w	-	-	a	-	-
Aesculin (hyd)	-	-	+	-	+	+	-	-	+	-	-
Fructose	a	a	a	a	a	a	a	a	a	a	a
Galactose	a	a	a	a	a	a	a	a	-	a	+
Glucose	a	a	a	a	a	a	a	a	a	a	a
Glycerol	-	w	w	w	a	w	-	-	w	-	-
Glycogen	-	a	a	-	a	a	-	a	a	-	a
Inositol	-	-	-	-	-	-	a	a	-	-	a
Inulin	-	w	a	-	a	a	-	a	a	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-
Lactose	a	a	a	a	a	a	a	a	a	a	a
Maltose	a	a	a	a	a	a	a	a	a	a	a
Mannitol	-	a	a	-	-	a	-	-	a	-	w
Mannose	a	a	a	-	a	a	a	a	a	a	a
Melezitose	-	w	-	-	-	-	w	-	-	w	w
Melibiose	-	a	a	-	a	+	+	a	-	-	+
Raffinose	a	a	a	-	a	+	+	a	-	a	+
Rhamnose	a	w	a	-	a	w	a	a	a	a	a
Ribose	a	a	a	-	a	a	a	a	a	a	a
Salicin	-	a	a	-	a	a	a	a	a	-	a
Sorbitol	-	a	a	-	-	a	a	a	a	-	a
Starch (pH)	a	a	a	a	a	a	a	a	a	a	a
Starch (hyd)	+	+	+	+	+	+	+	+	+	+	+
Sucrose	a	a	a	w	a	a	a	a	a	a	a
Trehalose	a	a	a	w	w	a	a	a	a	a	a
Xylose	a	a	a	w	a	a	a	a	a	w	a

Explanation of symbols: A = strong acid, pH < 5,5; w = weak acid, pH 5,5-6,0;
- = negative reaction; + = positive reaction

Appendix Table 5.7. Biochemical tests of selected amylolytic isolates

Test	Amylolytic isolates										
	A10	A32	A62	A65	A75	A88	A101	A132	A152	A155	A156
Indole	-	+	+	+	+	+	+	+	+	+	+
AMC	a	-	-	+	-	-	w	+	-	+	+
H_2S	a	+	+	+	+	+	+	+	+	+	+
NO_3 reduction	-	+	+	w	-	+	+	-	-	+	+
Urease	-	-	-	+	-	-	-	-	-	+	-
Casein hydrolysis	-	+	-	-	+	+	-	+	-	-	-
Gelatin liquefaction	+	+	-	-	-	+	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	+	+
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	+	-
Arginine ⟶ NH_3	-	+	-	+	+	+	-	-	+	+	+
Threonine ⟶ propionate	-	+	+	+	+	+	+	+	+	+	+
Lipase	+	+	+	+	+	+	-	-	+	+	-
Lecithinase	-	-	-	-	-	-	-	-	-	-	+
End-products:											
Glucose	A S p ib b iv l	A P L s	L f a	A S p ib b iv l	A B L f p s	L f a s	L f a s	A B L f p s	F L a p	A S p ib b iv l	L f a s
Starch	A p ib b iv l s	A P l s	L f a	A S p ib b iv l	A B f p l	L a s	L a p s	A B L p s	F L a p	A S L p ib b iv ic	L f a s

Explanation of symbols: - = negative reaction; + = positive reaction; w = weak reaction
End products: upper case letters represent > 1 mM, lower case < 1 mM
Where A = acetic, P = propionic, IB = isobutyric, B = butyric, IV = isovaleric, V = valeric
IC = isocaproic, C = caproic, L = lactic and S = succinic acids

Appendix Table 5.8. Fermentation tests of selected lactic acid utilizing isolates

Carbohydrate	Lactic acid utilizing isolates													
	L7	L23	L30	L77	L80	L88	L121	L143	L146	L150	L151	L166	L172	L176
Arabinose	a	-	a	-	a	-	a	w	a	w	-	-	-	w
Cellobiose	a	-	a	-	a	-	a	w	a	w	-	-	-	w
Aesculin (pH)	w	-	-	-	w	-	w	w	w	-	w	-	-	w
Aesculin (hyd)	+	-	-	-	+	-	+	-	+	-	+	-	-	+
Fructose	a	-	a	w	a	a	a	a	a	a	a	w	-	a
Galactose	a	-	a	-	a	-	a	a	a	a	a	-	-	a
Glucose	a	-	a	-	a	-	a	a	a	a	-	w	a	a
Glycerol	-	a	-	a	w	a	w	w	-	w	w	w	-	a
Glycogen	a	-	-	-	a	-	a	-	-	-	-	-	-	w
Inositol	-	-	-	-	-	-	-	w	-	a	-	-	-	-
Inulin	w	-	-	-	-	-	-	a	a	a	-	-	-	a
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	a	-	a	-	a	w	a	a	a	a	-	w	-	a
Maltose	a	-	a	-	a	-	a	a	a	a	-	-	-	a
Mannitol	a	-	a	-	a	-	a	a	a	a	-	-	-	-
Mannose	a	-	a	-	a	-	a	a	a	a	-	w	-	a
Melezitose	a	-	-	-	-	-	-	-	w	-	-	-	-	-
Melibiose	a	-	a	-	-	-	-	a	a	a	-	-	-	-
Raffinose	a	-	a	-	-	w	-	a	a	a	w	a	w	-
Rhamnose	a	-	w	-	-	w	w	a	a	a	w	w	w	-
Ribose	a	-	a	a	a	a	a	w	a	a	a	a	w	a
Salicin	a	-	a	-	a	w	a	a	a	a	w	-	w	a
Sorbitol	a	-	w	-	a	w	a	w	a	-	w	w	w	-
Starch (pH)	a	-	-	w	a	-	a	a	a	a	-	-	-	a
Starch (hyd)	+	-	-	-	+	-	+	+	+	+	-	-	+	w
Sucrose	a	-	a	-	a	w	a	a	a	a	w	a	w	a
Trehalose	a	-	w	-	w	w	a	a	a	a	a	a	a	a
Xylose	a	-	a	-	a	w	a	a	a	a	a	w	w	-

Refer to footnote in Appendix Table 5.6 for explanation of symbols

Appendix Table 5.9. Biochemical tests of selected lactic acid utilizing isolates

Test	Lactic acid utilizing isolates													
	L7	L23	L30	L77	L80	L88	L121	L143	L146	L150	L151	L166	L172	L176
Indole	+	+	+	+	-	+	-	+	-	+	+	+	+	+
AMC	-	-	+	+	-	+	-	w	w	+	-	w	+	+
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NO_3 reduction	-	+	w	+	-	-	-	-	-	+	w	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Casein hydrolysis	+	-	+	-	+	+	+	+	-	-	+	+	+	+
Gelatin liquefaction	-	-	-	-	+	-	+	-	-	-	+	+	+	+
Catalase	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Hippurate hydrolysis	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Arginine → NH_3	-	-	-	-	+	-	+	+	-	+	+	+	+	+
Threonine → propionate	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Lipase	-	-	+	+	+	+	+	-	+	-	+	+	-	-
Lecithinase	+	-	-	+	+	-	-	-	-	-	-	-	-	-
End-products:														
Glucose	A P L s	-	A P L s	-	A B C p ib iv v ic s	-	A B C p ib iv v ic s	A P b l s	A P L s	A P b l s	-	-	A P b l s	A P b l s
Lactate	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s	A P s
Glycerol	-	-	-	A P s	-	A P s	-	-	-	-	A P s	A P s	-	-
Fructose	-	-	-	A P s	-	A P s	-	-	-	-	A P s	A P s	-	-
Ribose	-	-	-	A P s	-	A P s	-	-	-	-	A P s	A P s	-	-

Refer to footnote in Appendix Table 5.7 for explanation of symbols

Appendix Table 6.1. Maxima of D-, L- and total lactic acid (mM) and minima of ruminal pH in relation to the food intake (g) of sheep during adaptation to high protein diets

Sheep	Lucerne hay (14th day)					High roughage diet (1st day)					High roughage diet (7th day)				
	Maximum lactic acid			Minimum pH	Food intake	Maximum lactic acid			Minimum pH	Food intake	Maximum lactic acid			Minimum pH	Food intake
	Total	L-	D-			Total	L-	D-			Total	L-	D-		
K1	2,73	1,09	1,64	5,85	1 200	1,18	0,46	0,80	6,80	700	1,46	0,39	1,14	5,75	1 450
K29	0,89	0,41	0,72	5,90	1 200	1,67	0,44	1,18	7,00	300	0,98	0,44	0,66	5,80	850
A27	1,36	0,73	0,63	5,95	1 200	1,78	0,64	1,14	6,40	1 000	1,74	0,66	1,08	5,95	1 000
A35	2,04	1,02	1,02	6,35	1 200	1,72	1,24	0,48	6,50	850	1,41	0,48	0,93	5,75	1 250
A85	1,64	0,79	0,86	6,35	1 200	1,93	0,26	1,85	6,75	1 500	1,41	0,50	0,94	6,40	1 800
A52*	-	-	-	-	-	-	-	-	-	-	1,06	1,06	0,68	6,35	800

Sheep	High roughage/Intermediate diet (1st day)					High roughage/Intermediate diet (7th day)					Intermediate diet (1st day)				
K1	5,57	1,77	3,81	5,90	1 700	4,16	1,08	3,08	5,70	1 600	2,39	0,79	1,61	5,75	2 150
K29	0,42	0,22	0,39	6,10	1 400	6,18	1,43	4,75	5,50	1 550	5,61	2,15	3,90	5,60	1 850
A27	5,20	1,82	3,38	5,75	1 550	3,14	1,46	1,75	5,75	1 400	0,66	0,25	0,54	6,00	400
A35	1,84	1,11	0,85	6,10	1 650	-	-	-	-	-	-	-	-	-	-
A85	0,79	0,40	0,48	6,55	1 100	7,12	3,61	3,50	6,20	2 050	4,50	2,20	2,30	5,85	2 750
A52*	1,72	0,45	1,36	6,40	900	1,64	0,36	1,28	6,10	1 200	1,45	0,56	1,11	6,65	750

Sheep	Intermediate diet (7th day)					Intermediate/High concentrate diet (1st day)					Intermediate/High concentrate diet (7th day)				
K1	3,48	1,28	2,23	5,60	2 000	3,29	1,01	2,29	5,50	2 350	1,21	0,47	0,74	5,50	2 350
K29	2,60	0,86	1,98	5,50	1 650	4,36	1,21	3,23	5,55	2 250	9,20	7,04	2,16	5,45	1 900
A27	1,64	0,60	1,04	5,70	1 100	1,04	0,36	0,68	5,60	1 400	1,04	0,45	0,59	5,55	1 500
A35	3,35	2,28	1,07	5,70	1 500	2,63	1,01	1,62	5,45	1 450	4,34	1,80	2,54	5,70	1 400
A85	5,78	2,97	3,28	5,65	2 750	1,58	0,59	0,99	5,60	2 850	1,27	0,51	0,76	6,10	1 800
A52*	2,13	2,13	0,63	6,15	750	0,51	0,51	0,25	6,20	800	2,04	1,16	0,88	5,85	1 000

Sheep	High concentrate diet (1st day)					High concentrate diet (7th day)					High concentrate diet (22nd day)				
K1	4,07	1,20	2,87	5,60	2 250	3,65	2,20	1,44	5,55	2 050	3,73	0,98	2,75	5,30	2 200
K29	8,02	3,85	4,17	5,45	1 950	2,20	1,38	0,82	6,50	300	-	-	-	-	-
A27	0,82	0,61	0,54	5,70	1 700	2,19	0,57	1,62	5,80	1 550	-	-	-	5,40	1 650
A35	1,64	1,26	0,81	5,70	1 400	5,87	3,18	2,69	5,40	1 150	6,48	3,44	3,05	5,30	1 450
A85	3,27	1,07	2,20	5,90	2 250	2,42	1,79	0,73	5,95	2 300	2,40	0,80	1,60	5,75	2 650
A52*	1,24	0,70	0,54	5,80	1 000	1,93	1,93	0,64	5,80	1 350	6,78	6,78	0,00	5,60	1 400

Sheep	High concentrate diet (42nd day)					High concentrate diet (71st day)					High concentrate diet (98th day)				
K1	6,11	2,56	4,19	5,40	2 250	8,30	5,64	2,66	5,80	1 900	1,66	0,59	1,28	5,65	1 400
A27	1,37	0,60	0,90	5,50	850	2,18	2,18	0,44	5,80	1 500	-	-	-	-	-
A35	6,63	3,50	3,13	5,20	1 550	3,72	1,65	2,26	5,50	1 650	2,76	1,36	1,40	5,40	1 400
A85	2,49	1,69	0,89	5,80	2 400	2,20	0,82	1,38	5,90	1 950	1,06	0,52	0,64	5,65	1 950
A52*	2,93	2,42	0,85	5,50	1 150	4,04	3,45	0,98	6,00	1 350	1,58	1,58	0,54	5,45	950

Sheep	High concentrate diet (119th day)				
K1	3,64	0,83	2,81	5,50	1 400
A85	3,87	0,39	3,69	5,30	1 500
A52*	1,19	1,19	0,46	5,40	850

*Refer to Tables 6.1 and 6.2 for details of diet and the number of days on each diet for sheep A52

Appendix Table 8.1. Turnover rate of DL-lactate in the rumen of sheep A18 adapted to an Intermediate diet but fed a High concentrate diet before the dosing of DL- (2-^{14}C)-lactate

Time after feeding (h)	Conc of lactate (mM)	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	Φ Calculated rates (mM/min) Production (2)	Utilization (3)
0	0,56	-	-		
¼	10,43	9 154	87 750		
				-0,102	0,245
½	5,23	5 581	106 750		
				-0,170	0,035
¾	2,15	4 726	219 875		
				-0,009	0,036
1	1,48	3 492	236 000		
				-0,062	-0,005
1¼	0,62	3 815	615 375		
				0,003	0,000
1½	0,67	3 811	568 875		
				0,008	0,012
1¾	0,61	2 873	470 875		
				-0,012	-0,008
2	0,56	3 564	636 375		
				0,001	0,001
2½	0,54	3 390	627 750		
				0,001	0,001
3	0,54	3 139	581 250		
				0,001	-0,001
4	0,64	3 271	511 125		
				0,002	0,002
6	0,71	2 370	333 750		

Φ Using equations given in the text (page 97)

Appendix Table 8.2. Data from continuous infusion experiments used to calculate lactate turnover

Time after feeding (h)	Conc of lactate (mM)	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	Calculated rates (mM/min) Turnover (1)	Production (2)	Utilization (3)
		Sheep A30		R = 69 349		
-1¼	0,67	24 405	3 642 537	0,019		
					0,016	0,021
-½	0,46	19 295	4 194 565	0,017		
					0,018	0,017
0	0,50	19 440	3 888 000	0,018		
					0,631	0,045
¼	9,30	16 280	175 054	0,396		
					0,578	0,675
½	7,85	10 840	138 089	0,502		
					0,439	0,513
¾	6,75	10 150	150 370	0,461		
					0,049	0,280
1	3,28	10 600	323 171	0,215		
					-0,115	0,062
1¼	0,62	11 105	1 791 129	0,039		
					0,026	0,034
1½	0,50	11 210	2 242 000	0,031		
					0,025	0,026
1¾	0,48	12 300	2 562 500	0,027		
					0,040	0,030
2	0,63	12 390	1 966 667	0,035		
					0,018	0,023
2½	0,48	15 280	3 183 333	0,022		
					0,025	0,022
3	0,57	16 280	2 856 140	0,024		
					0,023	0,024
4	0,54	16 190	2 998 148	0,023		
					0,016	0,017
6	0,41	19 170	4 675 610	0,015		
					0,016	0,015
8	0,48	20 450	4 260 417	0,016		
		Sheep A84		R = 57 222		
-1¼	0.56	18 190	3 248 214	0,018		
					0,019	0,020
-½	0,50	15 450	3 090 000	0,019		
					0,017	0,016
0	0,52	17 020	3 273 077	0,017		
					0,149	0,067
¼	1,75	7 020	401 143	0,143		
					0,304	0,219
½	3,02	5 840	193 377	0,296		
					0,235	0,259
¾	2,66	6 160	231 579	0,247		
					0,070	0,165
1	1,23	5 910	480 488	0,119		
					0,059	0,086
1¼	0,83	6 410	772 289	0,074		
					0,065	0,071
1½	0,75	6 380	850 667	0,067		
					0,047	0,052
1¾	0,67	7 360	1 098 507	0,052		
					0,025	0,035
2	0,52	8 690	1 671 154	0,034		
					0,021	0,021
2½	0,53	13 040	2 460 377	0,023		
					0,016	0,016
3	0,52	16 690	3 209 615	0,018		
					0,019	0,018
4	0,56	17 250	3 080 357	0,019		
					0,016	0,016
6	0,51	18 960	3 717 647	0,015		
					0,015	0,016
8	0,50	18 510	3 702 000	0,015		

/Sheep A28 .....

Appendix Table 8.2. (continued)

Time after feeding (h)	Conc of lactate (mM)	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	Calculated rates (mM/min) Turnover (1)	Production (2)	Utilizatio (3)
		Sheep A28		R = 56 693		
-1¼	0,50	21 200	4 240 000	0,013		
					0,013	0,014
-½	0,44	19 035	4 326 136	0,013		
					0,014	0,012
0	0,48	20 170	4 202 083	0,013		
					-0,038	-0,117
¼	1,67	13 880	831 138	0,068		
					0,130	0,104
½	2,05	11 490	560 488	0,101		
					0,187	0,141
¾	2,74	10 160	370 803	0,153		
					0,180	0,205
1	2,36	7 840	332 203	0,171		
					0,124	0,147
1¼	2,01	8 190	407 463	0,139		
					0,114	0,135
1½	1,69	7 840	463 905	0,122		
					0,061	0,067
1¾	1,60	10 650	665 625	0,085		
					-0,037	0,033
2	0,55	12 060	2 192 727	0,026		
					0,020	0,020
2½	0,55	14 730	2 678 182	0,021		
					0,022	0,021
3	0,58	15 036	2 592 414	0,022		
					0,016	0,017
4	0,51	17 913	3 512 353	0,016		
					0,015	0,015
6	0,56	20 715	3 699 107	0,015		
					0,014	0,014
8	0,52	21 680	4 169 231	0,014		
		Sheep A21		R = 66 144		
-1¼	0,37	22 950	6 202 703	0,011		
					0,009	0,010
-½	0,34	23 540	6 923 529	0,010		
					0,009	0,009
0	0,35	25 160	7 188 571	0,009		
					0,107	0,052
¼	1,18	5 490	465 254	0,142		
					0,164	0,142
½	1,52	6 160	405 263	0,163		
					0,110	0,142
¾	1,03	5 770	560 194	0,118		
					0,088	0,091
1	0,98	6 980	712 245	0,093		
					0,049	0,057
1¼	0,86	9 270	1 077 907	0,061		
					0,040	0,048
1½	0,74	10 310	1 393 243	0,047		
					0,038	0,034
1¾	0,80	12 730	1 591 250	0,042		
					0,014	0,033
2	0,51	12 550	2 460 784	0,027		
					0,015	0,018
2½	0,40	15 230	3 807 500	0,017		
					0,012	0,012
3	0,39	19 280	4 943 590	0,013		
					0,011	0,011
4	0,38	22 670	5 965 789	0,011		
					0,011	0,011
6	0,39	23 590	6 048 718	0,011		
					0,010	0,011
8	0,37	23 710	6 408 108	0,010		

Appendix Table 8.3. Data from single injection experiment used to calculate lactate turnover

Sheep A30

Time after feeding (h)	Conc of lactate	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	Calculated rates (mM/min) Production (2)	Utilization (3)
0	0,63	-	-	-	-
¼	1,48	1 454	98 243	-0,082	-0,199
½	3,23	5 417	167 709	-0,104	-0,130
¾	3,61	9 649	267 285	-0,068	-0,033
1	3,09	11 213	362 880	-0,028	0,004
1¼	2,62	11 005	420 038	-0,012	0,042
1½	1,81	8 229	454 641	-0,001	0,032
1¾	1,33	6 070	456 391	-0,004	0,018
2	1,00	4 827	482 700	-0,002	0,007
2½	0,72	3 725	517 361	0,002	0,002
3	0,70	3 362	480 286	0,001	0,003
4	0,59	2 469	418 475	0,002	0,003
6	0,55	1 498	272 364	0,001	0,001
8	0,53	1 075	202 830		
		Sheep A84			
0	0,65	-	-	-	-
¼	1,98	2 576	130 101	-0,081	-0,109
½	2,41	5 523	229 170	-0,040	0,030
¾	1,35	4 285	317 407	-0,020	-0,001
1	1,06	4 340	409 434	-0,010	0,005
1¼	0,99	4 684	473 131	-0,005	0,007
1½	0,82	4 189	510 854	0,000	-0,005
1¾	0,89	4 543	510 449	-0,003	0,007
2	0,74	3 962	535 405	-0,001	0,005
2½	0,57	3 150	552 632	0,002	0,003
3	0,53	2 607	491 887	0,000	0,001
4	0,48	2 297	478 542	0,001	0,000
6	0,60	2 531	421 833	0,002	0,002
8	0,62	1 783	287 581		

/Sheep A28 .....

Appendix Table 8.3. (continued)

Time after feeding (h)	Conc of lactate (mM)	Activity (dpm/10ml RF)	Specific activity	Calculated rates (mM/min) Production (2)	Utilization (3)
		Sheep A28			
0	0,48	-	-	-	-
¼	1,07	2 681	250 561	0,007	-0,083
½	2,42	5 698	235 455	-0,043	0,010
¾	1,62	5 282	326 049	-0,012	0,006
1	1,35	4 986	369 333	-0,004	0,008
1¼	1,17	4 510	385 470	-0,008	0,005
1½	0,97	4 193	432 268	-0,012	-0,001
1¾	0,81	4 282	528 642	-0,006	0,000
2	0,72	4 258	591 389	-0,001	0,002
2½	0,63	3 825	607 143	-0,003	0,001
3	0,52	3 649	701 731	0,001	0,001
4	0,52	3 227	620 577	0,000	0,000
6	0,57	3 364	590 175	0,001	0,001
8	0,59	3 031	513 729		
		Sheep A21			
0	0,44	-	-	-	-
¼	0,88	3 166	359 773	-0,036	-0,041
½	0,95	4 301	661 692	0,019	0,006
¾	1,14	5 763	505 526	-0,010	-0,004
1	1,06	6 105	575 943	-0,006	-0,001
1¼	0,98	6 204	633 061	-0,007	-0,002
1½	0,90	6 390	710 000	-0,001	0,002
1¾	0,85	6 187	727 882	-0,005	0,001
2	0,76	6 023	792 500	-0,004	0,001
2½	0,61	5 801	950 984	0,001	0,001
3	0,60	5 392	898 667	0,001	0,002
4	0,54	4 185	775 000	-0,001	0,000
6	0,47	4 114	875 319	0,000	0,000
8	0,43	3 923	912 326		

Appendix Table 8.4. Values used to calculate the contribution of lactate to the formation of the individual VFA by the indirect method

Time after feeding (h)	Production of individual VFA through ^{14}C-lactate (mM/min)			[1]Production rate of individual VFA (mM/min)			Percentage of individual VFA formed through lactate (%)		
	C_2	C_3	C_4	C_2	C_3	C_4	C_2	C_3	C_4
				High concentrate diet					
¼ - ½	0,325	0,200	0,151	0,802	0,266	0,223	40,5	75,2	33,8
1 - 1½	0,127	0,092	0,061	0,784	0,260	0,218	16,2	35,4	14,0
1¾ - 2½	0,013	0,010	0,007	0,759	0,251	0,211	1,7	4,0	1,6
				Intermediate diet					
¼ - ½	0,152	0,058	0,049	0,743	0,174	0,154	20,5	33,3	16,2
1 - 1½	0,052	0,021	0,013	0,763	0,179	0,158	6,8	11,7	4,1
1¾ - 2½	0,013	0,005	0,003	0,680	0,160	0,141	1,9	3,1	1,0
				High roughage diet					
¼ - ½	0,074	0,042	0,026	0,605	0,150	0,110	12,2	28,0	11,8
1 - 1½	0,082	0,042	0,023	0,654	0,162	0,119	12,5	25,9	9,6
1¾ - 2½	0,011	0,005	0,003	0,605	0,150	0,110	1,8	3,3	1,3
				Lucerne hay diet					
1 - 1½	0,104	0,021	0,017	0,590	0,165	0,085	17,6	12,7	10,0
1¾ - 2½	0,036	0,011	0,010	0,639	0,179	0,092	5,6	6,1	5,4

[1]The mean molar proportions of the individual VFA were
High concentrate diet : 62,2% C_2; 20,6% C_3; 17,3% C_4
Intermediate diet : 69,4% C_2; 16,3% C_3; 14,4% C_4
High roughage diet : 70,3% C_2; 17,4% C_3; 12,8% C_4
Lucerne hay diet : 70,2% C_2; 19,7% C_3; 10,1% C_4

Appendix Table 8.5. Values used to calculate the contribution of lactate to the formation of the individual VFA by the direct method using ratios of specific activities

Time after feeding (h)	Concentration of individual VFA (mM)			Specific activity in individual VFA (dpm/mM)		
	C_2	C_3	C_4	C_2	C_3	C_4
$-\frac{1}{2}$	97,85	29,25	27,90	78 334	118 974	42 248
$\frac{1}{4} - \frac{1}{2}$	147,95	37,15	44,20	40 047	93 271	31 053
$1 - 1\frac{1}{2}$	99,75	30,45	29,45	43 759	104 434	35 654
$1\frac{3}{4} - 2\frac{1}{2}$	100,85	27,10	29,90	43 580	116 568	38 378
6	98,30	35,85	22,60	48 525	53 138	80 310
$-\frac{1}{2}$	105,45	25,00	21,95	78 331	93 200	61 959
$\frac{1}{4} - \frac{1}{2}$	97,60	20,55	19,05	78 074	141 606	63 780
$1 - 1\frac{1}{2}$	93,95	21,70	20,25	63 119	108 295	37 037
$1\frac{3}{4} - 2\frac{1}{2}$	87,05	20,40	19,35	100 976	153 431	45 737
6	87,65	23,25	23,70	62 864	104 946	32 701
$-\frac{1}{2}$	100,90	22,05	19,70	60 654	108 390	38 071
$\frac{1}{4} - \frac{1}{2}$	82,45	24,10	18,25	53 972	104 149	42 219
$1 - 1\frac{1}{2}$	94,70	22,30	16,00	50 475	108 969	42 188
$1\frac{3}{4} - 2\frac{1}{2}$	81,95	23,25	16,65	57 718	95 484	43 544
6	109,10	28,60	19,35	65 261	75 524	47 339
$-\frac{1}{2}$	89,65	21,90	13,25	121 807	110 502	100 000
$\frac{1}{4} - \frac{1}{2}$	-	-	-	-	-	-
$1 - 1\frac{1}{2}$	135,40	40,60	17,90	74 742	49 216	46 090
$1\frac{3}{4} - 2\frac{1}{2}$	134,60	44,90	17,40	51 114	47 216	57 472
6	138,70	35,50	19,40	73 035	51 268	74 227

Appendix Table 8.6. D- and L- lactic acid concentrations (mM) in the rumen of sheep fed diets differing in amount of readily fermentable carbohydrate

Time after feeding (h)	Sheep A16 High concentrate diet	Sheep A18 Intermediate diet	Sheep A31 High roughage diet	Sheep A21 Lucerne hay diet
	D-lactic acid (mM)			
0	-	-	-	-
1/3	13,1	17,4	25,8	26,0
2/3	14,0	11,4	22,4	11,6
1	11,8	9,6	8,7	7,9
1½	7,4	4,6	6,3	4,6
2	6,3	2,7	4,6	3,4
2½	5,1	2,2	3,1	3,1
3	3,9	2,3	3,6	3,8
4	2,8	2,3	2,9	2,9
6	2,7	2,9	2,5	3,0
	L-lactic acid (mM)			
0	-	-	-	-
1/3	23,7	28,5	32,5	-
2/3	25,0	19,7	29,2	22,1
1	20,8	17,8	17,8	17,5
1½	14,6	7,6	9,2	7,2
2	11,9	2,6	4,7	5,0
2½	11,1	2,3	2,7	3,8
3	8,1	2,6	2,4	2,3
4	2,9	2,9	2,6	2,3
6	2,1	2,3	2,3	2,7

Appendix Table 9.1. Values used in the calculation of glucose turnover in the rumen of sheep on different diets

Sheep A30 (High concentrate diet) R = 220 933 dpm/min

Time after feeding (h)	Conc of glucose (mM)	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	*Calculated rates (mM/min) Turnover (1)	Production (2)	Utilization (3)
-1¼	6,21	51 895	835 668	0,26		
					0,26	0,27
-½	6,52	46 715	716 488	0,31		
					0,31	0,30
0	6,88	48 820	709 593	0,31		
					0,50	0,43
¼	7,96	42 165	529 711	0,42		
					0,56	0,53
½	8,52	37 115	435 622	0,51		
					0,53	0,48
¾	9,34	39 560	423 555	0,52		
					0,54	0,63
1	7,97	33 180	416 311	0,53		
					0,59	0,63
1¼	7,48	28 750	384 358	0,57		
					0,45	0,47
1½	7,25	32 680	450 759	0,49		
					0,36	0,35
1¾	7,38	39 655	537 331	0,41		
					0,40	0,41
2	7,25	39 670	547 172	0,40		
					0,36	0,36
2½	7,09	42 920	605 360	0,37		
					0,35	0,36
3	6,81	43 160	633 774	0,35		
					0,30	0,32
4	5,58	42 185	756 004	0,29		
					0,21	0,22
6	3,88	45 910	1 183 247	0,19		
Sheep A84 (Intermediate diet)			R = 158 182 dpm/min			
-1¼	2,05	34 745	1 694 878	0,09		
					0,15	0,11
-½	3,65	36 800	1 008 219	0,16		
					0,17	0,16
0	3,55	37 115	959 044	0,16		
					0,17	0,19
¼	3,87	37 370	956 633	0,17		
					0,24	0,17
½	4,97	38 665	777 968	0,20		
					0,22	0,23
¾	4,86	36 480	750 617	0,21		
					0,18	0,19
1	4,68	37 930	810 470	0,20		
					0,17	0,17
1¼	4,61	40 205	872 126	0,18		
					0,12	0,12
1½	4,67	47 370	1 014 347	0,16		
					0,15	0,18
1¾	4,23	43 205	1 021 395	0,15		
					0,12	0,14
2	4,03	45 155	1 120 471	0,14		
					0,14	0,15
2½	3,76	42 425	1 128 324	0,14		
					0,13	0,13
3	3,65	43 195	1 183 425	0,13		
					0,12	0,12
4	3,71	47 300	1 274 933	0,12		
					0,10	0,10
6	2,90	50 575	1 743 966	0,09		

*Using the equations given in the text (page 97)

Appendix Table 9.1 (continued)

Sheep A28 (High roughage diet) R = 160 668 dpm/min

Time after feeding (h)	Conc of glucose (mM)	Activity (dpm/10ml RF)	Specific activity (dpm/mM)	Calculated rates (mM/min) Turnover (1)	Production (2)	Utilizatio (3)
$-1\frac{1}{4}$	1,89	32 150	1 701 058	0,09	0,09	0,09
$-\frac{1}{2}$	1,91	34 605	1 811 780	0,09	0,10	0,10
0	2,01	32 430	1 613 433	0,10	0,16	0,12
$\frac{1}{4}$	2,64	31 590	1 196 591	0,13	0,12	0,12
$\frac{1}{2}$	2,67	31 200	1 248 000	0,13	0,13	0,14
$\frac{3}{4}$	2,50	30 575	1 223 000	0,13	0,15	0,13
1	2,77	31 520	1 137 906	0,14	0,12	0,14
$1\frac{1}{4}$	2,51	30 910	1 231 474	0,13	0,16	0,14
$1\frac{1}{2}$	2,88	30 995	1 076 215	0,15	0,15	0,17
$1\frac{3}{4}$	2,62	28 085	1 071 947	0,15	0,12	0,14
2	2,29	28 245	1 233 406	0,13	0,12	0,12
$2\frac{1}{2}$	2,28	29 820	1 307 895	0,12	0,10	0,11
3	2,21	33 115	1 498 416	0,11	0,10	0,10
4	2,15	33 870	1 575 349	0,11	0,10	0,10
6	2,22	36 925	1 663 288	0,10		
Sheep A21 (Lucerne hay diet)			R = 177 163 dpm/min			
$-1\frac{1}{4}$	0,67	30 165	4 502 239	0,04	0,03	0,04
$-\frac{1}{2}$	0,52	29 060	5 588 462	0,03	0,03	0,03
0	0,49	27 395	5 590 816	0,03	0,08	0,03
$\frac{1}{4}$	1,20	32 870	2 739 167	0,06	0,09	0,08
$\frac{1}{2}$	1,41	30 830	2 186 525	0,08	0,06	0,08
$\frac{3}{4}$	1,18	30 180	2 557 627	0,07	0,05	0,06
1	0,97	29 540	3 045 361	0,06	0,10	0,07
$1\frac{1}{4}$	1,41	28 995	2 053 546	0,09	0,08	0,09
$1\frac{1}{2}$	1,12	25 100	2 241 071	0,08	0,08	0,07
$1\frac{3}{4}$	1,32	28 970	2 194 697	0,08	0,05	0,06
2	1,07	30 705	2 869 626	0,06	0,04	0,04
$2\frac{1}{2}$	0,97	41 785	4 307 732	0,04	0,04	0,04
3	0,83	38 260	4 609 639	0,04	0,03	0,03
4	0,82	44 050	5 371 951	0,03	0,03	0,03
6	0,58	40 000	6 896 552	0,03		

Using the equations given in the text (page 97)

Appendix Table 11.1. Composition and preparation of fermentation medium for sulphate reduction tests

The following were placed in a vacolitre bottle

Na-DL-lactate (10% w/v soln)	50	ml
Yeast extract	0,5	g
Clarified rumen fluid	50	ml
K_2HPO_4	0,25	g
NH_4Cl	0,50	g
Na_2SO_4	0,50	g
$Ca\ Cl_2.2H_2O$	0,05	g
$Mg\ SO_4.7H_2O$	1,00	g
Indigo carmine (0,05% w/v soln)	5	ml
Deionized water	335	ml

After melting the following was added

$Na\ HCO_3$ (9,1% w/v soln)	35	ml

After sterilization the following were added

Fe solution[1]	15	ml
Reducing agent[2]	5	ml
Dithiothreitol solution[3]	5	ml

The medium was dispensed in 9,0 ml amounts into sterile 1 oz McCartney bottles gassed with 98% CO_2/2% H_2. Final pH 6,7 - 6,8.

[1]Fe solution - 2,5 g $Fe\ SO_4.\ (NH_4)_2\ SO_4.6\ H_2O$ in 100 ml deionized water. Sterilized by Millipore filtration.

[2]Reducing agent - 0,5 g Na thioglycollate in 50 ml deionized water. Dispensed in 5 ml amounts into N_2 gassed 1 oz McCartney bottles and heat sterilized.

[3]Dithiothreitol solution - 1,0 g dithiothreitol in 100 ml deionized water. Dispensed in 5 ml amounts into N_2 gassed 1 oz McCartney bottles and heat sterilized.