STUDIES ON RUMEN METABOLISM

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by

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ABSTRACT

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The present study has been concerned primarily with the effects of supplementary lipid on protein and carbohydrate metabolism in the rumen and with the degree of hydrolysis and hydrogenation of lipids by rumen microorganisms. In addition, *in vivo* and *in vitro* experiments were carried out to determine the effect of the addition of soluble carbohydrates on the concentrations of fermentation end-products.

In the early part of this work, the effect of lipid on protein and carbohydrate metabolism was investigated in rumen-fistulated twin cows fed freshly-cut pasture. Linseed and whale oil were used as supplementary sources of lipid. The infusion of either oil into the rumen resulted in reductions in feed intake and in the concentrations of acetic and butyric acids. Concentrations of propionic acid remained at or above those found during the pretreatment periods. Added linseed oil also resulted in increased concentrations of ammonia in the rumen. Further investigation led to the observation that lipid depressed cellulose digestion and inhibited rumen motility.

Lipid, incubated with rumen liquor obtained from animals fed freshly-cut pasture and with ryegrass juice extract, increased the concentrations of ammonia and propionic acid but had no consistent effect on gas production or on the concentrations of acetic and butyric acids. Following the replacement of the grass juice extract with grass fibre, lipid decreased

the formation of acetic acid and the digestion of fibre, increased the formation of propionic acid but had no effect on the concentration of ammonia.

The hydrolysis and hydrogenation of grass and clover lipids and of linseed oil were followed in conjunction with some of the above *in vivo* and *in vitro* experiments. Mono- and diglycerides, in addition to fatty acids, were found in the rumen liquor after infusion of linseed oil into the rumen. The proportions of linoleic acid found in rumen liquor 6.5 hr. after infusion of oil were above normal but the proportions of linolenic acid had returned to about the prefeeding level in this time. The free fatty acids were more saturated than the unhydrolyzed triglycerides but the proportions of unsaturated fatty acids in the partially hydrolyzed glycerides were not greatly different to those of the triglycerides. Rapid hydrogenation of the constituent unsaturated fatty acids of linseed oil also occurred *in vitro*.

In the *in vivo* experiments in which starch was added to the rumen of cows fed pasture containing one of two levels of nitrogen it was found that the added carbohydrate brought about marked reductions in ammonia concentrations but had no consistent effect on total volatile fatty acid concentrations.

Incubation of each of a series of soluble carbohydrates with rumen liquor and ryegrass juice extract for periods of up to 3 hr. showed that galactose, glucose, sucrose, lactose, galacturonic acid, xylose and L-arabinose were readily fermented but that D-arabinose was not. A close relationship was found between the rate of fermentation, the degree of ammonia utilization and the production of volatile fatty acids.

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INTRODUCTION

'For at the bottom of the gullet there is a double Orifice; What is first received at the mouth descendeth into the first and greater stomack, from whence it is returned into the mouth again; and after a fuller mastication, and salivous mixture, what part thereof descendeth again, in a moist and succulent body, it slides down the softer and more permeable Orifice, into the Omasus or third stomack; and from thence conveyed into the fourth, receives its last digestion. The other dry and exuccous part after ruminating by the larger and stronger orifice beareth into the first stomack, from thence into the Reticulum, and so progressively into the other divisions.'

SIR THOMAS BROWNE, 'The Garden of Cyrus', 1658.

With the population of the world expanding at a hitherto unprecedented rate, efficiency in the production of food is becoming increasingly important. The ruminant is not as efficient a converter of feed as are most of the other classes of domestic livestock and poultry. However, it does possess the ability to digest cellulose.

Indeed, the primary function of the rumen is to provide a site for the digestion of the complex polysaccharides of pasture herbage by enzymes associated with the rumen microorganisms. As a result, energy is available to the host from a source which is largely unavailable to non-ruminants. In addition, protein, lipid and the remaining carbohydrate constituents of the feed all undergo extensive modification by the rumen microflora. An understanding of the type and the extent of these changes would, therefore, be of the greatest importance to any attempt to improve the feed efficiency of the ruminant.

Of the various groups of nutrients present in feeds, the lipids have received least attention. This has been due, in part, to the limitations of the techniques available for fat analysis and also to a lack of interest in lipids because of their relatively small contribution to dry matter intake. However, adequate techniques have recently been introduced, gas liquid chromatography being among the most useful, and the vast surplus of tallow in North America has stimulated interest in the inclusion of fat in animal rations as an inexpensive source of energy. Vegetable and animal oils and fats have also proved useful in the prevention and control of pasture bloat (Reid & Johns, 1957). The need for more information on lipid metabolism in the rumen is, therefore, evident.

Information on the relationship between the protein and carbohydrate content of the feed and the subsequent metabolism of these constituents in the rumen is also very limited. It is known, however,

that the inclusion of soluble carbohydrates improves the utilization of dietary nitrogen and that, under certain conditions, the addition of supplementary protein will improve the utilization of carbohydrate. In New Zealand, ruminants exist almost entirely on pasture herbage, in which the relative proportion of protein to carbohydrate has been found to show considerable seasonal variation (Johns, 1955a).

The present study has been concerned primarily with the effect of supplementary lipid on the concentration in the rumen of various fermentation end-products and the degree of hydrolysis and hydrogenation of lipids by rumen microorganisms. In addition, an investigation was carried out to determine the effect of the addition of soluble carbohydrates on the metabolism of protein and carbohydrate by rumen microorganisms. Before proceeding to an account of this work, brief reference to the relevant literature is made.

REVIEW OF LITERATURE

A. Metabolism of Lipids by Ruminants

1. Lipids of Pasture Species

The ether extractible portion of roughages generally constitutes between 2 - 6% of the dry matter (Morrison, 1956). This portion is composed mainly of a mixture of glycerides, free fatty acids, sterols, waxes and phospholipids. In pasture species, approximately half the total lipid consists of fatty acids (Garton, 1960; Hawke, 1963) of which most is in glyceride combination. Weenink (1959, 1961), Benson, Wintermans & Weiser (1959), Garton (1960) and Shorland (1961) have shown that, in various pasture species, most of the acetone-soluble lipids are present, not as triglycerides as was originally believed, but as galactolipids. Shorland, Weenink & Johns (1955), Weenink (1959, 1961), Garton (1960), Shorland (1961) and Hawke (1963) have all found that the unsaturated long-chain fatty acids, particularly linolenic, constitute a very high percentage of the total fatty acids of pasture species. Concentrates of vegetable origin (eg. barley, oats) have also been shown to contain triglycerides with a high proportion of unsaturated fatty acids (Dahl, 1958).

2. Digestion and Metabolism of Lipids

It has been estimated (Hawke, 1963) that a cow eating 20 lb. of dry matter/day could consume over 700 g. of lipid. This material is then modified by the microorganisms in the rumen in three main ways: (a) hydrogenation (b) hydrolysis and (c) fermentation of the

glycerol released on hydrolysis.

(a) Hydrogenation

Ruminant depot fats are recognized as being 'hard' fats and their fatty acid composition remains relatively unchanged regardless of the nature of the diet. The milk of ruminants also tends to contain a higher proportion of saturated acids than that found in the diet (Hilditch & Thompson, 1936). It had been apparent, therefore, that hydrogenation was occurring but it was not until Reiser (1951) demonstrated that linolenic acid was converted to linoleic acid by rumen contents of sheep that the rumen became implicated as the possible major site of hydrogenation. Willey, Riggs, Colby, Butler & Reiser (1952) observed that the depot fat of steers fed cottonseed oil contained more stearic and less oleic acid than that from steers which received no oil. They suggested that hydrogenation of the fatty acids occurred in the rumen, the resulting saturated fatty acids being subsequently absorbed and deposited. Reiser & Reddy (1956) examined the rumen contents of goats after slaughter, two of which had been on a diet containing 10% cottonseed oil and two on a diet containing 10% linseed oil. Lower levels of linolenic and linoleic acids were present in the ingesta than in the feed and were accompanied by increases in levels of oleic and saturated acids. In contrast, the proportion of unsaturated fatty acids in the depot fat of young calves without a functional rumen increased considerably following the

feeding of linseed oil (Hoflund, Holmberg & Sellmann, 1956a). Tove & Mochrie (1963) showed that when cottonseed oil was infused intravenously into Holstein cows there was a dramatic increase in the linoleic acid content of milk fat, indicating that hydrogenation outside the rumen was not a significant biochemical process.

A number of workers (Shorland et al., 1955; Hoflund, Holmberg & Sellmann, 1956b; Shorland, Weenink, Johns & McDonald, 1957) have shown that when linolenic acid is exposed to mixed cultures of rumen bacteria, either in vivo or in vitro, it is first reduced to the conjugated diene, then to the monoene and finally to stearic acid. Wood, Bell, Grainger & Teekell (1963), using labelled linoleic - 1 - C^{14} injected into the rumen of sheep with a ligated reticulo-rumen orifice, found that 85 - 96% of the activity was still present in the rumen after 48 hours. Of this, only 3 - 6% was the original linoleic acid. Approximately 45% had been hydrogenated to saturated acids and 33 - 50% hydrogenated to oleic or elaidic acids. Wright (1960) noted the specificity of bacterial enzymes for the more unsaturated acids. Polan, McNeill and Tove (1964) observed that stearic acid was not produced from linoleic acid until the proportion of mono-unsaturated acid present exceeded that of linoleic acid. They concluded that two systems were involved in the complete hydrogenation of linoleic acid, one specific for its conversion to the mono-unsaturated state and the other specific

for the subsequent formation of stearic acid.

Garton, Lough & Vioque (1961) found that the free fatty acids released on hydrolysis were more saturated than the corresponding triglycerides, indicating that hydrogenation proceeds more rapidly in the free state than in the combined.

Shorland *et al.*(1955) were the first to observe the formation of *trans*-isomers of the long chain fatty acids by rumen microorganisms and, in later work, Shorland *et al.*(1957) found that conversion of oleic, linoleic and linolenic to *trans*-isomers amounted to 17%, 48%, and 67%, respectively, of the acid added. Positional isomers, particularly of linolenic acid, were also formed.

Wright (1959, 1960) has shown that both bacterial and protozoal fractions of rumen contents are capable of hydrogenating the unsaturated long chain fatty acids. Detailed work on the organisms responsible is very limited but Gutierrez, Williams, Davis & Warwick (1962) using washed suspensions of two species of protozoa isolated from the rumen, *Isotricha prostoma* and *Entodinium simplex*, found that both species were capable of concentrating stearic, oleic, linoleic and palmitic acids. *I. prostoma* suspensions converted oleic acid to a substance identified chromatographically as stearic acid. A strain of the bacterial organism, *Butyvibrio fibrisolvens*, was found to effect hydrogenation of one of the two double bonds of linoleic acid (Polan *et al*, 1964).

(b) Hydrolysis

Garton, Hobson & Lough (1958), in addition to observing hydrogenation of linseed oil by rumen organisms, noted that more than 75% of the total lipid recovered at the end of the fermentation was present as free higher fatty acids. Since no lipolytic activity was found following the incubation of linseed oil with rumen contents which had previously been heated to 90° for 1 hr., or with sheep saliva, it was concluded that the microorganisms must be responsible for lipolysis. Free fatty acids in rumen contents from animals fed a variety of diets, usually represented between 50 - 60% of the total lipids. On examining the rumen contents of a sheep previously fed on a diet containing linseed oil (40 g/day) and which had been killed 7 hr. after the last feed, these workers also found that 80 - 90% of the lipid was present as free fatty acids. In more detailed *in vitro* work, Garton, Lough & Vioque (1959, 1961), Allen, Hill, Hammond & Jacobson (1959) and Wright (1961) found wide variations (15 - 90% for linseed oil) in the amounts of free fatty acids released after incubation for 22 or 24 hr. of either (a) different lipids or (b) the same lipid but incubated with rumen liquor obtained on different days or from different animals.

The work of Garton *et al.* (1961) and Wright (1961) suggested that the rate of hydrolysis of various triglycerides may be inversely proportional to the chain length and degree of

saturation of the constituent acids.

Hill, Saylor, Allen & Jacobson (1960) found that a greater degree of hydrolysis (10 ml. rumen liquor hydrolysed as much as 96% of 0.5 g. of soybean oil in 24 hr.) occurred under an atmosphere of CO_2 or air than under N_2 and that the activity was higher in samples of ingesta removed just prior to the morning or afternoon feeding than at any other time. Activity was also higher in rumen liquor from animals fed lucerne pasture than from animals on dry feed.

No intermediates (mono and diglycerides) in the fermentation of triglycerides have been reported.

Hobson and Mann (1961) succeeded in isolating bacteria from the rumen contents of sheep, which hydrolysed glycerides *in vitro*. The organisms were small, curved, Gram-negative rods which were strictly anaerobic.

(c) Fermentation of Glycerol

It is evident that a considerable proportion of the hydrolysis of glycerides occurs in the rumen, thereby releasing glycerol as a potential substrate. Johns (1953), in both *in vivo* and *in vitro* trials, found that glycerol was fermented by rumen micro-organisms but at a much slower rate than glucose. Propionic acid appeared to be the main end-product. Garton *et al.* (1961) reported that glycerol, arising from the hydrolysis of triglycerides *in vitro*, was almost completely metabolized. Propionic acid was the major end-product but did not account for more

than about half of the glycerol fermented. Bacteria responsible for glycerol fermentation in the sheep rumen have been isolated and identified as Selenomonas ruminantium var. lactilyticus (Hobson & Mann, 1961). These organisms produce mainly propionic acid and small amounts of lactic and succinic acids.

3. Effect of Supplementary Lipid on the Digestibility of Other Nutrients

The inclusion of fat as a source of energy in ruminant rations and its use as a bloat preventative raises the question of its effect on the digestibility of other nutrients. This effect appears to depend on the nature of the diet.

On feeding two levels of fat (approximately 3 and 7.5% of dry matter) to steers on a finishing ration, Willey et al.(1952) found that feed efficiency was improved but that there was no change in the rate of gain. The addition of 7% fat to a pelleted finishing ration for steers was reported by Erwin, Dyer and Ensminger (1956) to have significantly increased rate of gain. Hale, Mueller, Culbertson & Hammond (1956) found that 3% fat added to finishing rations for lambs produced a slight increase in both feed consumption and rate of gain. However, at a level of 10%, stabilized tallow had no significant effect on rate of gain, feed efficiency or feed consumption of sheep on a finishing ration (Jordan, Croom & Hanke, 1958). Therefore, the addition of fat ($<10\%$) to high quality rations for ruminants is of some benefit.

The effect of supplementary lipid on the digestibility of high fibre rations is apparently somewhat different. Brooks, Garner, Gehrke, Muhrer & Pfander (1954) reported that the addition of 32 or 64 g. corn oil daily to a ration composed mainly of cottonseed hulls significantly reduced cellulose digestibility. When corn oil (10 - 170 mg.) was added to 1 g. of dry matter containing 50% cellulose, in vitro cellulose digestion was reduced by 40 - 94%. In sheep on a similar ration, Pfander & Verma (1957) found that the addition of corn oil to the ration (46 g./day) depressed the digestibilities of organic matter, cellulose and nitrogen. However, if the oil was administered via a rumen fistula, there was no change in digestibility compared to the basal ration.

White, Grainger, Baker & Stroud (1958) supplemented high-fibre sheep rations with 5% corn oil and reported a progressive reduction in the digestibility of cellulose during the first 40 days of the trial. Brethour, Sirny & Tillman (1958) reported a reduction in weight gains and digestibility of dry and organic matter following addition of 10% maize oil or 15% animal fat to sheep rations containing cottonseed hulls. When beef fat or peanut oil was incorporated into a pelleted basal ration (lucerne and grain mix) at levels of from approximately 3 - 10%, Buysse (1962) found that at levels greater than 5%, the digestibility of crude fibre was reduced. There was no effect on digestibility of dry matter, organic matter, protein, or nitrogen-free extract, and digestibility of ether-extract increased.

The precise mode of action of lipid on cellulose digestibility

is thus uncertain. Several workers (Brooks et al., 1954; Pfander & Verma, 1957; Ward, Tefft, Sirn, Edwards & Tillman, 1957) have suggested that the depression of the digestibility of cellulose was due to the presence of a coating of fat on the fibrous part of the diet which acted as a physical barrier to the action of microbial enzymes on cellulose. However, White et al.(1958) found that cellulose digestibility was not completely restored until 17 days after the removal of oil from the ration and interpreted this to mean that bacterial activity must be affected by the fat. Brooks et al.(1954) and Ward et al.(1957) found that lucerne ash was effective in restoring cellulose digestibility. White et al.(1958) noted that calcium ions alone were as effective as lucerne ash in this respect and proposed that the cellulose depressing effect was due to the formation of a calcium-lipid complex which has been shown to occur in rats (Swell, Trout, Field & Treadwell, 1956).

Very little work has been reported on the effect of supplementary lipid on the concentration of fermentation end-products in the rumen. Chalmers (1960) found that the addition of oil to a ration which contained 3% or less nitrogen resulted in a depression in rumen ammonia levels, whereas, when added to rations with a nitrogen level of 4.5%, an increased concentration of ammonia resulted. Jayasinghe (1961) observed that 10 - 15% fat (groundnut oil) added to a protein supplement for sheep rations containing 3% nitrogen reduced the concentration of ammonia in the rumen.

The finding of Johns (1953), Garton et al.(1961) and Hobson &

Mann (1961) that glycerol was fermented in the rumen and that propionic acid was the main end-product, suggested that a change in the relative concentration of the volatile fatty acids (VFA) in the rumen following the addition of supplementary oils or fats might be expected. Shaw & Ensor (1959) noted that the inclusion of 300 ml/day of cod-liver oil, oleic acid or linoleic acid in a dairy cattle ration of lucerne hay and mixed concentrates decreased the molar proportion of acetic acid and increased the molar proportion of propionic acid. Linoleic acid had the greatest effect (acetic acid decreased from 78.9 - 52.2% and propionic acid increased from 9.9 - 29.9%). In addition, all three lipid supplements increased total VFA concentrations. Since linoleic acid apparently effected a greater change in the proportion of acetic to propionic acid than did cod-liver oil and since the total concentration of acids rose, propionate must have been formed from some source other than glycerol. Elam, Gutierrez & Davis (1960) could not detect any significant effect on either the total concentration or the proportions of VFA in the rumen, of the addition of 4 or 8% soybean oil to a basal finishing ration for steers. However, with the basal ration, the proportion of acetic acid was relatively low and of propionic acid relatively high representing 50 and 35% of the total acids, respectively. In later work, Elam and Davis (1962) found that the proportion of propionic acid increased if soybean oil, at a level of 8% of the weight of feed consumed, was given as a drench before each feed, whereas the same level, mixed with the feed, had no effect on acid proportions.

The difference was probably due to an effect on fermentation of the much higher concentration of oil present in the rumen after drenching.

B. Metabolism of Nitrogenous Compounds and Carboydrates by Ruminants

1. Metabolism of Dietary Nitrogen

The fate of dietary nitrogen in the rumen has recently been reviewed by several workers, including Lewis (1961), Chalmers (1961) and McDonald (1962) and only a general outline will be given here.

Chalmers & Synge (1954) and Johns (1955) found that of the total nitrogen in forage and in most other feeds commonly fed to ruminants, 80% or more is usually present as protein - the balance being distributed among various non-protein nitrogenous compounds such as free amino acids, peptides, nucleic acids, purine and pyrimidine bases, urea, nitrate and ammonia (NH_3). The level of nitrogen in these feeds can vary immensely. For example, oat straw generally contains less than 1% nitrogen, whereas immature ryegrass can contain more than 5% nitrogen. Protein supplements, such as oilseed or meat meals, usually have 5% or more nitrogen.

Sym (1938) and Pearson & Smith (1943a) were among the first to demonstrate that the microbial fraction of rumen contents possessed proteolytic activity. Pearson & Smith (loc. cit.) found that casein and gelatine were hydrolyzed *in vitro*, but when blood meal, a less water-soluble protein supplement, was used as a substrate, there was a net protein synthesis. Solubility was, therefore, considered an important factor in determining the degree of hydrolysis in the rumen. In more recent years, numerous other workers (McDonald, 1954; Chalmers,

Cuthbertson & Synge, 1954; Annison, 1956; McDonald & Hall, 1957; Blackburn & Hobson, 1960) have further substantiated this observation. However, some exceptions were noted by these workers. Hendericks & Martin (1963) have since demonstrated that solubility of proteins in synthetic rumen fluid was a more accurate index of the rate of hydrolysis in the rumen.

Since the concentration of free amino acids in the rumen is usually low (McDonald, 1952; Annison, 1956) and since NH_3 usually represents the major form of soluble nitrogen (McDonald, 1948; Annison, Chalmers, Marshall & Synge, 1954) it was concluded that either the amino acids were utilized as such, or were deaminated very rapidly by the rumen microorganisms. More recently, Lewis & McDonald (1962) demonstrated that almost all amino acids are degraded to some extent by washed cell suspensions and to a greater extent in the presence of rumen fluid. It is now recognized that NH_3 is the major nitrogenous end-product of proteolysis in the rumen.

Ammonia is also the major nitrogenous end-product of the degradation of non-protein nitrogen. Pearson & Smith (1943b) found that rumen contents had a high urease activity; Lewis (1951) demonstrated the conversion of nitrate to ammonia *in vitro*; Doetch and Jurtshuk (1957) reported that xanthine, guanine hydrochloride and uric acid could be completely degraded to ammonia, carbon dioxide and acetic acid.

The work of McDonald (1948) and Bouckaert & Oyaert (1952) showed conclusively that NH_3 was absorbed from the rumen. Lewis,

Hill & Annison (1957) found that NH_3 levels in the portal blood of sheep on different rations were directly related to rumen NH_3 levels. Except under conditions in which NH_3 levels in the rumen are exceptionally high (60 m-moles/l), the liver removes almost all the NH_3 from the portal blood converting it to urea. Some of the urea is returned to the rumen in the saliva (McDonald, 1948) and some enters across the rumen epithelium (Houpt, 1959; Ash & Dobson, 1963) but most of it is excreted. Therefore, since the amount of NH_3 absorbed and subsequently excreted is directly related to its concentration in the rumen, the latter has been used as a measure of the usefulness of a dietary protein to the animal (Preston, Whitelaw & MacLeod, 1963).

A considerable amount of work, particularly with non-protein nitrogen sources, has established that significant protein synthesis occurs in the rumen (Harris & Mitchell, 1941a; Mills, Booth, Bohstedt & Hart, 1942; Harris, Work & Henke, 1943; Pearson & Smith, 1943a).

In more recent years, attempts have been made to estimate the extent of conversion of food protein to microbial protein. Moir & Williams (1950) and Williams, Nottle, Moir & Underwood (1953) estimated the numbers of rumen organisms in sheep fed rations of varying protein content and found a relationship between bacterial count and the level of dietary nitrogen and starch in the food. Various methods of physical fractionation have also been used to determine changes in the level of protein in the rumen (Gray, Pilgrim & Weller, 1953; Kameoka, Morimoto, Takahashi & Kubota, 1953; Weller, Gray & Pilgrim, 1958). McDonald (1954) and McDonald & Hall (1957) used chemical

characteristics of zein and casein as a means of separating dietary protein from microbial protein.

The results of Bryant, Robinson & Chu (1959); Bryant & Robinson (1961) and Bryant, Doetsch & Robinson (1962) have shown that NH_3 and, to a lesser extent, amino acids are the main sources of nitrogen for bacterial growth. Bryant et al.(1962) found that, of the many strains studied, 25% required NH_3, 56% grew with either NH_3 or amino acids and only 6% required amino acids.

Since the microbial protein can form the major source of protein reaching the abomasum, its biological value is, therefore, of importance to the animal. Various workers (Reed, Moir & Underwood, 1949; McNaught, Owen, Henry & Kon, 1954) have found it to be a reasonably high quality protein. This is obvicusly an advantage to the animal when the dietary protein is of low biological value but a disadvantage when it is of high biological value. Methionine and iso-leucine were considered by Holmes, Moir & Underwood (1953) as possible limiting amino acids. Type of protein in the ration has little apparent effect on amino acid composition of bacterial protein.

2. Fermentation of Carbohydrates

Natural feeds contain a wide variety of carbohydrate constituents of varying chemical complexity. Grouped in order of decreasing solubility, these include monosaccharides, oligosaccharides, fructans, starch, galactolipids, pectins, hemicellulose and cellulose. Wylam (1953, 1954), Waite & Boyd (1953), Mackenzie & Wylam (1957), Waite

(1958), Bailey (1958), Routley & Sullivan (1958) and Sullivan, Phillips & Routley (1960) have done extensive work on the determination of the constituent monosaccharide units in the various carbohydrate fractions of grasses and clovers. Glucose, fructose, xylose, galactose, arabinose, galacturonic acid and glucuronic acid appear to be the major sugars present.

Tappeiner (1883) observed that the fermentation of cellulose in the rumen of the ox resulted in the production of large quantities of VFA, particularly acetic. Little nutritional significance was attributed to them at the time. Following on the discovery of McAnally and Phillipson (1942) that the high concentrations of VFA present in the rumen were not present in the abomasum, Barcroft, McAnally & Phillipson (1944) were able to demonstrate that the blood leaving the rumen, omasum and caecum contained significant amounts of VFA. In addition, they noted that peripheral blood concentrations were very low, suggesting that the acids were metabolized in the liver and perhaps in other tissues.

Elsden (1945, 1946) found that in sheep on natural diets, the average proportions of acetic, propionic and butyric acids were 64, 20 and 16%, respectively. In more recent work, it has been found that the rumen contents of animals fed diets high in soluble carbohydrate usually have a higher proportion of propionate (Balch & Rowland, 1957) and that in animals on diets high in protein, appreciable amounts of C_5 and C_6 acids appear (el-Shazly, 1952).

The importance of the VFA as an energy source can be gauged

from the estimates of Carroll & Hungate (1954) that at least 6000 - 12000 kcal. of energy are absorbed as VFA from the rumen of cattle every 24 hr . In sheep, Phillipson & Cuthbertson (1956) estimated that 600 - 1200 kcal./day were available to the animal from the VFA. Adult sheep and cattle require a minimum of about 1100 and 6500 kcal./day, respectively, for maintenance.

3. Effect of Nitrogenous Compounds on Cellulose Digestion

The effect of protein on the digestion of cellulose has not been fully established. Henneberg & Stohmann (1864) noted that the digestion of fibre by ruminants was depressed by a protein supplement. Hoflund, Quin & Clark (1948) found that the addition of 100 g. of casein to a poor quality grass diet for sheep reduced cellulose digestibility to almost zero but could detect no difference if only 50 g. of casein were added. Other workers, including Louw & van der Wath (1943), Burroughs, Gerlaugh, Edgington & Bethke (1949), Head (1953), Abou Akkada & El Shazly (1958), Minson & Pigden (1961), were unable to demonstrate any effect of supplementation of high fibre rations with protein.

However, Harris & Mitchell (1941b) showed that the addition of urea to a ration containing 0.126% nitrogen increased cellulose digestion. Similarly, Clark and Quin (1951), Swift, Thacker, Black, Bratzler & James (1947), Briggs, Gallup, Heller & Darlow (1948), Belasco (1954) found that cellulose digestibility increased when dietary nitrogen was increased by supplementation with urea. Head (1953) suggested that growth of the microorganisms responsible for

digesting cellulose was not affected by the inclusion of extra nitrogen if the substrate being digested contained about 1% nitrogen.

It would appear, therefore, that a minimum amount of nitrogen is required for efficient cellulose digestion in high fibre rations. If, however, the amount of available energy in the ration is increased by the addition of starch, the requirement for nitrogen as measured by cellulose digestion is increased (Burroughs et al., 1949; Burroughs, Gall, Gerlaugh & Bethke, 1950) probably because of greater microbial proliferation. Ellis & Pfander (1958) fed a semi-purified diet, containing large amounts of starch, to lambs to supply cellulose at one of three levels (21.4, 31.4 or 41.6%) and nitrogen at one of three levels (1.65, 2.05, or 2.45%). Increasing the nitrogen to 2.05% increased the digestibility of cellulose but a further increase to 2.45% had the opposite effect.

Several amino acids have also been found to improve cellulolytic activity. Bentley, Lehmkuhl, Johnson, Hershberger & Moxon (1954) found that valine and proline improved cellulose digestion and Macleod & Murray (1956) reported that the increased activity from a mixture of eighteen amino acids was due almost entirely to leucine, iso-leucine and valine. Dehority, Bentley, Johnson & Moxon (1957) confirmed these findings. These workers suggested that the increased activity might be due to a conversion of the amino acids to the corresponding VFA by Stickland (1934) reactions.

$$\text{eg. } 2RCH(NH_2)COOH + R'CH(NH_2)COOH + 2H_2O \longrightarrow$$

$$2RCH_2COOH + R'COOH + 3NH_3 + CO_2$$

Valeric, caproic, iso-butyric and iso-valeric acids had been shown by Bentley, Johnson, Hershberger, Cline & Moxon (1955) and in later work by Cline, Hershberger & Bentley (1958) to have cellulolytic factor activity. Bryant & Doetsch (1955) had also found that Bacteroides succinogenes requires either iso-butyric, iso-valeric or DL - methyl - n - butyric as well as valeric or caproic acids for growth on a purified medium. Similarly, Allison, Bryant & Doetsch (1962) found that a strain of Ruminococcus flavefaciens was unable to incorporate C^{14} - labeled leucine, yet synthesized leucine from C^{14} - labeled isovaleric acid. Stickland-type reactions offer a logical explanation for the improvement in cellulolytic activity observed following the addition of amino acids such as leucine to in vitro systems.

4. Effect of Carbohydrates on Protein Metabolism

As early as 1896, Wicke and Weiske claimed that protein utilization by ruminants was improved by the addition of carbohydrates to the ration. In vivo and in vitro experiments in recent years have shown that the type of carbohydrate added is of great importance. McDonald (1952) reported that the concentration of NH_3 in the rumen decreased when starch was added to the ration. Lofgreen, Loosli and Maynard (1951), working with dairy calves receiving protein at the maintenance level, found significantly greater retention of dietary nitrogen when the non-nitrogenous total digestible nutrients were increased by 15% above the requirements for maintenance. Head

(1953) reported that the addition of maize or potato starch to a hay ration for sheep reduced protein digestibility but increased nitrogen retention. Fontenot, Gallup & Nelson (1955) found that the effect on nitrogen retention of supplementing a wintering ration for steers with "cerelose" (glucose prepared by the hydrolysis of corn starch) depended on the protein content of the ration. Cerelose tended to increase the nitrogen loss in the faeces and to decrease the loss in the urine. The net effect of these changes was that at a protein level in the feed of 8%, cerelose depressed nitrogen retention; at a level of 10%, nitrogen retention was increased significantly and at 12% there was a small but not significant increase in nitrogen retention. The observation of Fontenot et al. (loc. cit.) that supplementary carbohydrate tended to reduce the loss of nitrogen in the urine is supported by the work of Preston, Breuer & Pfander (1961) who found that blood urea - N levels decreased in sheep following the addition of feeds high in carbohydrate to a basal ration.

Annison (1956) noted that the rate of disappearance of NH_3 and amino acids from the rumen after feeding casein was increased in the presence of carbohydrate. In more detailed work, Lewis & McDonald (1958) reported results of an experiment in which sheep were fed a basal diet of 700 g. of medium quality hay to which one of various carbohydrate materials had been added with or without 100g. of casein. The carbohydrates added included starch 250 g., cellulose 250 g., glucose 250 g., lactic acid 20 g., propionic acid 100 g., xylan 150 g. or grass levan 150 g. All carbohydrate supplements reduced the concentration of NH_3 in the rumen. Starch and levan had the greatest effect,

whereas cellulose had only a slight effect. These workers proposed that for maximum nitrogen retention in an animal fed a nitrogen source which gives rise to a rapid increase in the non-protein nitrogen in the rumen, an energy source that is attacked at a comparable rate should be included in the ration.

Various workers have also investigated the effect of carbohydrates on the utilization of non-protein nitrogen, particularly urea. Mills et al. (1942) found that starch was essential to protein synthesis from urea in animals fed a basal ration of Timothy hay. Bŏda, Tomas & Kona (1961) found sucrose, glucose or starch effective in improving nitrogen retention in sheep on diets containing varying amounts of urea.

Using in vitro techniques, McNaught (1951) tested a large range of carbohydrates for their activity in converting non-protein nitrogen, supplied as urea, to protein. Employing maltose as a standard, they found that maize and potato starch were much more effective boiled than raw. Raffinose, inulin, fructose, cellobiose and mannose were utilized but sorbose was not. L-arabinose and D-xylose were very effective but D-arabinose was not. The addition of one of three levels of dextrose, cane molasses, sucrose, starch, cellulose or ground corn cobs to an in vitro system was found by Arias, Burroughs, Gerlaugh & Bethke (1951) to increase urea utilization. Belasco (1956) reported that starch was more effective than xylan, pectin or cellulose in improving conversion of urea to protein in an artificial rumen.

In much of the work reviewed, workers have reported an increase in the amount of protein in the rumen or in the numbers of bacteria

present following the addition of carbohydrate. Bacterial growth requires energy and it is probably for this reason that supplementary carbohydrate has proved of value.

OBJECT OF THE PRESENT WORK

Little information is available concerning the effect of lipid on rumen metabolism in animals fed on pasture. Furthermore, the observed fluctuations in the lipid content of pasture species with stage of growth could result in considerable variation in lipid intake. Consequently, it was decided in the early phase of this work to investigate the effect of lipid on protein and carbohydrate metabolism in animals consuming freshly-cut pasture. Linseed oil was used as a supplementary source of lipid in several of the experiments because of the similarity in fatty acid composition to that of the lipid of grasses and clovers. However, linseed oil was found to reduce intake considerably and, therefore, whale oil, another relatively unsaturated lipid was substituted in some of the experiments.

Since almost all of the work reported on the hydrolysis and hydrogenation of lipids has been obtained from *in vitro* experiments, the rates of hydrolysis and hydrogenation of grass lipids and linseed oil in the rumen were followed in conjunction with the above *in vivo* experiments. The degree of hydrogenation of the free fatty acids, partial hydrolysis products of triglycerides and unhydrolysed triglycerides have been compared.

The effects of supplementary lipid on the concentrations of NH_3, total and individual volatile fatty acids in the rumen suggested that the lipid brought about a general decrease in the metabolic activity of the rumen microorganisms. This possibility was investigated by incubating rumen liquor - grass extract media with various amounts of linseed or whale oil and measuring gas production, ammonia and

volatile fatty acid concentrations.

The very high protein levels observed in pasture species, especially during periods of rapid growth, have led to speculation on the conditions required for efficient utilization of fodder high in protein. Since utilization of protein is likely to be considerably influenced by the availability of carbohydrates for microbial fermentation, investigations were carried out in order to determine the effect of supplementary starch on the concentrations of ammonia and total volatile fatty acids in the rumen of animals fed freshly-cut pasture. In addition, the effect on *in vitro* fermentation rates and concentrations of end-products, of each of a series of soluble sugars normally present in grasses and clovers was studied.

Part I. EFFECT OF TIME, DAY AND LOCATION OF SAMPLING ON CONCENTRATIONS OF AMMONIA AND VOLATILE FATTY ACIDS PRESENT IN THE BOVINE RUMEN

The contents of the rumen are generally considered to be heterogeneous in nature. Consequently, the method of obtaining samples in which concentrations of metabolites are to be determined is of great importance to the validity of any conclusions reached in work on rumen metabolism.

In cattle, there is a well-defined stratification of solids in the rumen (Pearson & Smith, 1943; Smith, Sweeney, Rooney, King & Moore, 1956; Lampila and Poijärvi, 1959). After a meal of hay, digesta in the dorsal sac of the rumen have been found to contain over 15% dry matter compared with levels of 5 - 7% in the reticulum and ventral regions (Balch, 1949).

The degree of stratification depends partly on the nature of the feed. Concentrates, succulents and finely ground roughages, which enter the dorsal region on ingestion, gravitate to the ventral region more rapidly than unground roughages (Balch, 1950; Nichols, 1954; Smith et al., 1956).

Evidence of stratification of the water soluble metabolites is less consistent. Smith et al. (1956) found that the concentration of VFA, sugars and total nitrogen was higher in the dorsal than in the ventral region of the rumen. Similarly, Lampila (1955) noted differences in VFA concentrations of up to 50% in favour of the dorsal region. Smith (1941) and Lampila (1955) were unable to demonstrate a definite anterior-posterior gradient in the concentrations of the metabolites measured but Bryant (1961) found that samples from the reticulum usually contained lower concentrations of total VFA

than samples from either the posterior dorsal blind sac or an area intermediate between the two. Reid, Bailey & Glenday (1967) observed that VFA concentrations were usually lower in the reticulum than in any of seven sites sampled in the rumen. Balch et al.(1955) and Balch & Rowland (1957) reported that differences were of no practical consequence since the maximum variation in total VFA concentrations which they observed was 1.1 m-mole/100 ml.

Time of sampling in relation to time of feeding is another major factor associated with the degree of stratification observed within the reticulorumen. As the time following feeding increases, the volume of the rumen contents decreases (Coop, 1949; Reid, 1965), the digesta becomes more fluid (Quin, Oyaert & Clarke, 1951; Reid, 1965) and the degree of stratification becomes progressively less well defined (Balch, 1961). Concentrations of NH_3 have been found to be higher in the dorsal rumen during the first half of the period between two feeds whereas no difference was found during the latter half (Lampila, 1960). Bryant (1964) found a dorsal-ventral gradient in total VFA concentrations and noted that the magnitude of the difference was partially dependent on the time which had elapsed since feeding. The relative proportions of individual VFA did not vary with site of sampling.

The total concentrations of NH_3 and VFA observed are also largely dependent on time of sampling since the levels of each generally increase significantly during the first few hours after feeding. The extent and duration of the increase is governed mainly by the nature of the diet (Annison et al., 1954; Balch & Rowland, 1957; Lewis, 1961).

For example, peak levels and the degree of diurnal variation in levels of NH_3 depend on the amount and solubility of the source(s) of nitrogen and carbohydrate ingested.

Evidence has been presented by several workers (Reid, Hogan & Briggs, 1957; Brethour, Sirny & Tillman, 1958) that the proportions of individual VFA also vary with time of sampling after feeding. Davey (1965) found that the proportions of acetic and propionic acid were inversely related and that the proportion of propionic acid was highest at the time when concentrations of total VFA were highest. However, Gray, Jones & Pilgrim (1960) and Shaw (1961) did not observe any consistent variations in proportions of VFA associated with time of sampling after feeding.

In an attempt to reduce potential errors due to sampling, various procedures have been employed. Mechanical mixing by means of a pumping device inserted through a fistula into the rumen, has been successfully used when the consistency of the rumen contents was relatively fluid (Sutherland, Ellis, Reid & Murray, 1962; Engelhardt, Hoeller & Hoernicke, 1963). These devices, by altering the environment, will have some effect on the rumen microorganisms. The magnitude of this effect is not known but is unlikely to be large. However, the usefulness of such an apparatus is limited because it is not able to circulate any appreciable quantity of coarse, fibrous material.

Agrawala, Duncan & Huffman (1953) obtained samples by first emptying and mixing the entire contents of the rumen. This

procedure possesses an apparent advantage over *in situ* techniques, particularly for obtaining representative samples for dry matter determinations. However, it has seldom been used in experiments in which samples are required several times daily. The lack of interest has been due to a belief that exposure of rumen contents to the atmosphere would alter the course of metabolism in the rumen since most rumen microorganisms are obligate anaerobes (Hungate, Bryant & Mah, 1964). This may not be a major problem. Recently Reid (1965) found that no gross depressions in either rate of breakdown or rate of passage occurred as a result of the use of this procedure. When compared with "grab" sampling, this method of sampling reduced standard errors associated with percentages of dry matter and, to a lesser extent, with concentrations of total VFA present in rumen contents (Reid *et al.*, 1967).

The sampling procedure most commonly used in studies of factors affecting changes in the levels of compounds present in solution has been to collect samples of rumen liquor *in situ*. Results are expressed as concentrations per unit of fluid. Techniques for obtaining such samples have included the collection of large samples (Hungate, Fletcher, Dougherty & Barrentine, 1955; Johns, 1955), sampling from a single fixed position within the rumen (Balch & Rowland, 1957; Bryant, 1959) and compositing samples obtained from the dorsal, mid and dorsal posterior blind sacs of the rumen (Bryant, 1961). Bryant (1961) concluded that representative samples of rumen liquor could be obtained by either the above composite sampling method or by removing a single large

sample from the mid region of the rumen.

There are obviously a number of sampling methods available which have been used with varying degrees of success by previous workers. The choice of a suitable sampling location and procedure for a specific experiment will depend upon many factors, including the type and quantity of ration fed, frequency and duration of feeding, species of animal and the type of information desired.

Results obtained by others (Hoflund et al., 1956; Balch & Rowland, 1957; Lewis & McDonald, 1958; Shaw & Ensor, 1959; Lewis, 1962) indicate that determination of concentrations of fermentation end-products can yield useful information concerning the metabolism of dietary constituents and could be a valuable technique in the study of dietary lipid metabolism in the rumen. Because of the degree of variation observed, particularly that associated with time of sampling, results of such a study would be meaningful only if feeding and sampling times and procedures were standardized. The methods adopted should be based on data obtained previously under a similar set of conditions.

With this object in mind, a preliminary experiment was conducted in order to determine the extent and major causes of variation in NH_3 and VFA concentrations when dairy cows were fed freshly-cut pasture herbage twice daily. However, some of the samples became contaminated with bacteria before the individual VFA were determined. As a result, the proportions and concentrations of these metabolites were found to be highly irregular and a second experiment was considered necessary. The results of both trials relating to concentrations of NH_3 and total

VFA were similar. Data are reported for the latter only.

Materials and Methods

Treatment of Animals

A Jersey steer weighing approximately 258 kg. and fitted with a rumen fistula was used in this experiment. The animal was fed indoors, for approximately 3 weeks before the experimental period, on freshly-cut bromegrass (Bromus inermis) of good quality. It was trained to consume its feed in two periods of approximately two hours beginning at 9:30 a.m. and 4:00 p.m. Water and iodized salt were available ad libitum. The same pasture herbage was fed during the experimental period.

Rumen Sampling

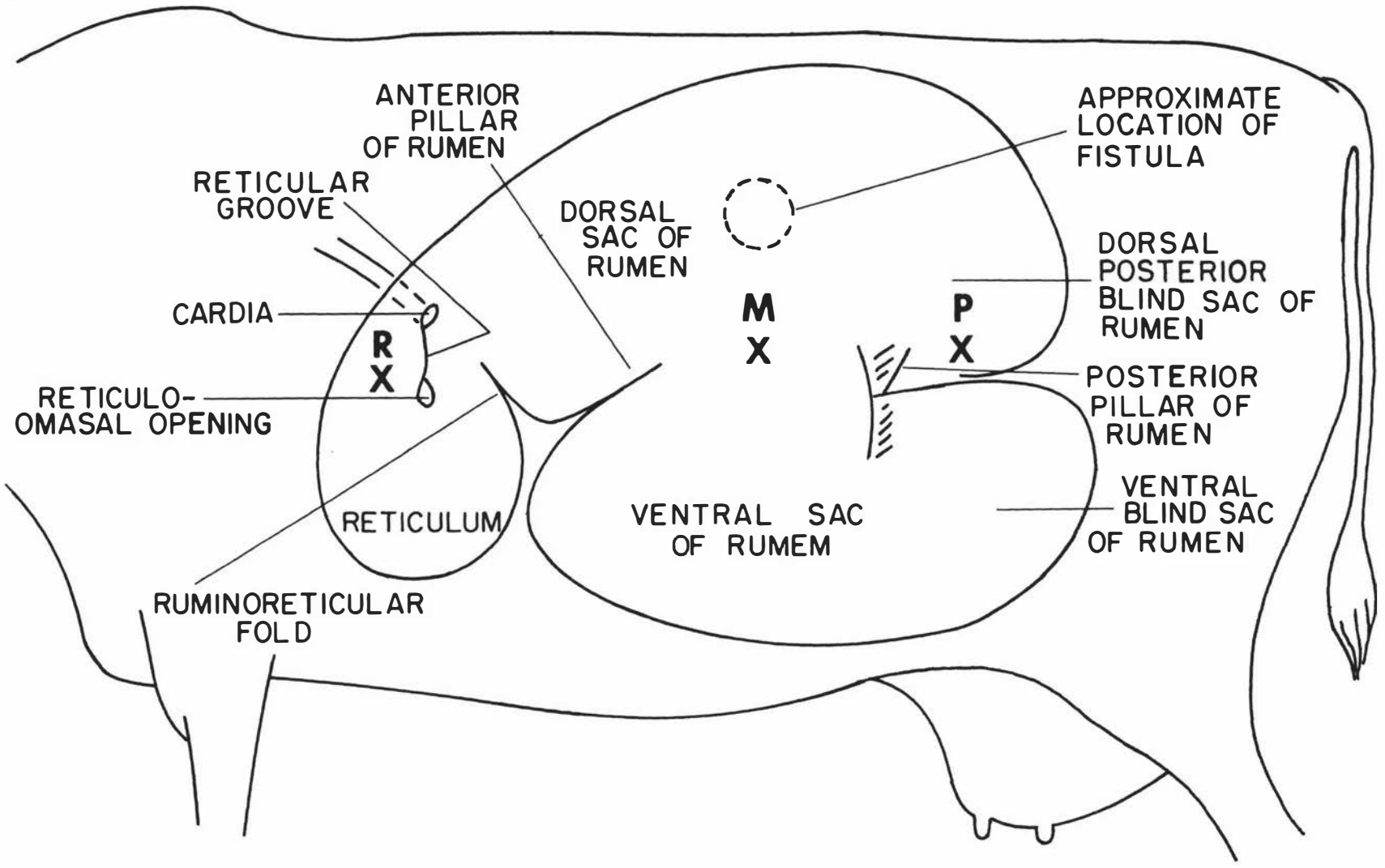
Duplicate samples, to give approximately 200 ml. rumen liquor after straining through two layers of muslin, were removed by hand from (1) the reticulum (R), (2) the posterior dorsal blind sac (P) and (3) an area intermediate between the dorsal and ventral positions (M) which was directly below the fistula (Fig. 1). Sampling was carried out just prior to the commencement of the morning feeding period and 2, 4.5 and 6.5 hr. later. This procedure was repeated on three consecutive days. The samples were preserved by the addition of 2% (v/v) of a saturated solution of mercuric chloride and stored at a temperature of 5°.

Analytical Methods

(a) Pasture Analysis

Dry matter was determined by drying samples at 100° for

Fig. 1. Diagramatic sketch of a mid sagittal section of a cow showing approximate location of sampling areas. R, reticulum; M, mid region; P, posterior dorsal blind sac.


ANTERIOR
PILLAR
OF RUMEN
RETICULAR
GROOVE
CARDIA
R
X
RETICULO-
OMASAL OPENING
RETICULUM
RUMINORETICULAR
FOLD
DORSAL
SAC OF
RUMEN
M
X
APPROXIMATE
LOCATION OF
FISTULA
DORSAL
POSTERIOR
BLIND SAC OF
RUMEN
P
X
POSTERIOR
PILLAR OF
RUMEN
VENTRAL SAC
OF RUMEM
VENTRAL
BLIND SAC
OF RUMEN

16 hr. in a forced-draught oven. Total nitrogen present in the dried samples was measured by the Kjeldahl method as modified for samples containing nitrate (A.O.A.C., 1960).

(b) Rumen Liquor Analysis

Ammonia was determined in 1 ml. of rumen liquor immediately after sampling by the boric acid-hydrochloric acid procedure of Conway and O'Malley (1942) using saturated potassium carbonate in the outer compartment.

Measurements of total and individual VFA were made with a gas chromatograph equipped with a hydrogen flame-ionization detector (F and M Scientific Corp., Avondale, Pa.).

The dual columns were of 1/4 in. o.d. stainless steel and were 6 ft. long. The column packing consisted of 5% by weight UCON 550 X on Haloport (F and M Scientific Corp., Avondale, Pa.). Flow rate of the carrier gas (helium) was 120 ml./min. Injector and detector temperatures were 230° and 270°, respectively. The columns were operated at an initial temperature of 100° with a programmed increase of 4°/min. to a maximum of 170°.

In preparation for gas chromatographic analyses, samples of rumen liquor were centrifuged at 1200 g. for 10 min. to remove suspended particulate material. 5 ml. aliquots were transferred to sample bottles containing 1 ml. 2 N sodium hydroxide and 0.2 m. eq. of an internal standard (caproic acid). The mixture was then evaporated to dryness on a water bath. Just prior to analysis, the samples were reacidified by the addition of 3 ml. of a solution of hydrochloric

acid (6N) and acetone (50:50, v/v). 3 µl. samples were introduced into the injection port of the gas chromatograph by means of a 10 µl. syringe. Fatty acid composition (moles %) was determined by dividing the area under the curve for each acid by the corresponding relative molar response factor.* The concentrations of both the individual and total VFA (m-mole/l.) were then calculated by comparison with the concentration of caproic acid.

* The relative molar response of a particular substance is the peak area obtained for one mole expressed relative to the peak area of one mole of a standard.

Results and Discussion

Feed Intake

The consumption of freshly-cut pasture herbage (expressed as kg. dry matter) was similar on all 3 days of sampling (Table 1). Except on the day prior to the first day of sampling, the steer consumed a higher proportion of its total daily intake during the afternoon feeding period.

Table 1. Intake of freshly-cut brome grass (kg. dry matter) by a stall-fed steer

Date	Morning	Afternoon	Total
29.7.64	3.0	2.0	5.0
30.7.64*	2.4	2.8	5.2
31.7.64*	2.4	2.8	5.2
1.8.64*	2.5	---	---

* Duplicate samples removed from the rumen before and at intervals after the morning feeding period.

Protein (N x 6.25) and dry matter content in the grass fed during the experimental period averaged 22.32 (% of dry weight) and 28.2%, respectively.

Ammonia

The accuracy of the method employed for estimating the concentration of NH_3 was determined by adding a known amount (20 mg./100 ml.), as ammonium sulphate, to each of eight samples of rumen liquor. Recovery of the added NH_3 averaged 100.1 $\pm$ 0.3%**.

Variations in the concentration of NH_3 between duplicate samples

** Standard error of means

of rumen liquor were not significant ($P < 0.05$), irrespective of day, location or time of sampling, indicating that the technique used to remove samples from the rumen was satisfactory.

Levels of NH_3 were lowest in samples obtained before feeding (8.3-13.5 mg. / 100 ml. rumen liquor) and significant differences occurred between each time of sampling (Table 2). Maximum concentrations, ranging from 30.2 to 40.3 mg. / 100 ml. rumen liquor, were present in the 4.5 hr. samples.

Increases in the concentration of NH_3 following feeding have been observed previously by many workers. Annison et al. (1954) found that the extent of the increase was largely dependent on the solubility of the protein and on the proportion of carbohydrate in the diet. Lewis (1961) demonstrated that the time required to reach maximum concentrations was, with one or two exceptions, directly related to the solubility of a particular protein in water. More recently, Henderickx & Martin (1963) found that solubility of a protein in an artificial rumen liquor was a more accurate index of the subsequent extent of degradation and, hence, of NH_3 concentrations in the rumen.

The concentrations of NH_3 tended to be lower on Day 1 than on Days 2 and 3 (Table 2). This was probably due to a lower consumption of dry matter on the afternoon prior to the first day of sampling than at any other time during the experimental period (Table 1).

Table 2. Mean concentrations of NH_3 (mg. / 100 ml.) in duplicate samples of rumen liquor

Day of sampling (D)	Location of sampling (L)	Time of sampling (T) after commencement of feeding (hr.)			
		0	2	4.5	6.5
1(30.7.64)	R*	8.3	27.6	30.2	22.3
	M*	9.1	29.1	36.8	21.3
	P*	9.2	28.1	33.5	21.5
2(31.7.64)	R	12.6	31.3	34.2	20.2
	M	13.5	30.9	36.6	21.3
	P	12.2	28.7	33.7	23.5
3(1.8.64)	R	9.2	27.8	33.6	23.9
	M	10.2	29.8	38.6	25.4
	P	10.5	29.1	40.3	22.8
		D_D** = 1.9			
		D_L = 1.9			
		D_T = 2.9			

* R = reticulum; M = mid region of rumen; P = posterior dorsal blind sac of rumen

** D = difference required for significance at $P \geq 0.05$ (Snedecor, 1956).

Location of sampling had no significant effect on NH_3 concentrations in samples obtained before feeding (Table 2). In general, this was also true of the 2 and 6.5 hr. samples and, although some variation did occur, no consistent differences between locations were noted. At only one sampling time (Day 1, 4.5 hr. samples) was there a significant

difference in concentration between each location.

It is important to note that the pattern of change following feeding was similar regardless of location and was of much greater magnitude than changes associated with either day or position of sampling.

Test of Method for Quantitative Determination of VFA

Mixtures containing known concentrations of free volatile fatty acids were analyzed by the gas chromatographic technique described in Materials and Methods. The relative molar response values obtained for each acid are presented in Table 3. For comparative purposes data obtained by Emery and Koerner (1961) and by Kabot and Ettre (1963) using a similar type of detector are also presented in Table 3.

The relative molar response values for acetic and propionic acids were somewhat lower than the theoretical values, whereas those for butyric and isovaleric deviated only slightly from the theoretical. This may indicate that in the case of the C_2-C_3 fatty acids, recovery is not completely quantitative. However, because of the general agreement between the factors obtained currently and those of Emery and Koerner (1961) and of Kabot and Ettre (1963), it is assumed that a lower relative response to acetic and propionic acids is a consistent characteristic of the hydrogen flame-ionization detector.

Table 3. Relative molar response factors for volatile fatty acids (C_2 - C_6)

Fatty acid	Moles % (actual)	Relative area under curve (av. of 3 determinations)	Relative molar response factor			
			Theoretical	Calculated 1*	2**	3***
Acetic	49.1	27.6 ± 0.2(2)	200	176	147	155
Propionic	14.6	13.2 ± 0.1	300	284	288	275
Isobutyric	4.6	6.1 ± 0.2	400	417	-	-
Butyric	9.3	11.8 ± 0.4	400	399	409	409
Isovaleric	4.7	7.5 ± 0.1	500	501	495	495
Caproic(1)	17.7	33.8 ± 0.3	600	600		

* Current results
** Emery and Koerner (1961)
*** Kabot and Ettre (1963)
(1) Internal standard
(2) Standard deviation

The results presented in Table 4 were obtained by gas-chromatographic analysis of the same mixture of acids following the addition of an excess of NaOH, evaporation to dryness and reacidification. It is apparent from a comparison of the corresponding molar percentages that this treatment of the samples prior to analysis had little, if any, effect on the proportion of each acid.

Table 4. Effect of treatment prior to gas chromatographic analysis on the proportion of volatile fatty acids

Fatty acid	Moles % (av. of 3 determinations)	
	Untreated	Treated
Acetic	49.0* ± 0.3**	49.6 ± 0.2
Propionic	14.7 ± 0.0	14.6 ± 0.1
Isobutyric	4.7 ± 0.3	4.7 ± 0.2
Butyric	9.3 ± 0.3	9.3 ± 0.4
Isovaleric	4.7 ± 0.0	4.8 ± 0.4
Caproic	17.6 ± 0.2	17.0 ± 0.5

* Proportions calculated using relative response factors as presented in Table 3.

** Standard deviation

A mixture of fatty acids containing proportions and concentrations of acetic, propionic and butyric acids similar to those which occur in the rumen was analyzed quantitatively (Table 5). The proportions of acetic and propionic acids were slightly higher than was expected. The converse was, of course, found for butyric acid. However, on a quantitative basis, no appreciable differences between the "actual" and the "calculated" concentrations of any of the individual VFA were found.

The method was then tested for accuracy in the determination of VFA concentrations in samples of rumen liquor. Sufficient acetic, propionic and butyric acid were added to each of four samples of rumen liquor to increase their respective concentrations in the samples by 50, 20 and 10 m-mole/l. The samples were then prepared for analysis as described under Materials and Methods. Recoveries of total added VFA averaged 79.9 m-mole/l. (standard error, $\pm$ 1.0) while the average recovery values for the individual acids were 50.5 $\pm$ 0.8, 19.9 $\pm$ 0.7 and 9.5 $\pm$ 0.5 m-mole/l. for acetic, propionic and butyric acid, respectively. In view of these results, the technique was considered suitable for the quantitative determination of VFA in the rumen liquor.

Table 5. Determination of the proportions and concentrations of volatile fatty acids by the use of caproic acid as an internal standard

Fatty acid	Moles %		Concentration (m-mole/l.)	
	Actual	Calculated (av. of 3 determinations)	Actual	Calculated
Acetic	70	70.3 $\pm$ 0.9*	112.0	113.2 $\pm$ 2.8*
Propionic	20	20.5 $\pm$ 1.0	32.0	33.0 $\pm$ 1.7
Butyric	10	9.2 $\pm$ 0.7	16.0	14.8 $\pm$ 0.7
Total	100	100.0	160.0	161.0 $\pm$ 3.1

* = Standard deviation.

Total and Individual Volatile Fatty Acids

Differences in the concentrations of total VFA between the duplicate samples of rumen liquor removed from each location of sampling were not significant ($P<0.05$).

Minimum concentrations of total VFA (56.8 - 92.7 m-mole/l. rumen liquor) were present in samples obtained just before the commencement of the morning feeding period (Table 6). Maximum levels of from 110.1 to 145.0 m-mole/l. were found in the samples obtained 4.5 hr. later. Differences between the minimum and maximum concentrations were significant on all 3 days (Table 6). On Days 1 and 3 differences in concentration between the 2 and 6.5 hr. samples from the reticulum and mid region of the rumen were not significant. On Day 2 no significant differences were found between concentrations in samples obtained before and 2 hr. after commencement of feeding. The pattern of change was, therefore, one of minimal values prior to feeding, maximal levels 4.5 hr. later followed by significant reductions in levels over the next two hours.

This pattern is typical of that observed previously, by others, for animals fed twice daily. For example, Balch & Rowland (1957) found that maximum levels of total VFA occurred between 2 and 6 hr. after feeding. They also reported that the difference between minimum and maximum levels varied depending on the type of ration fed. Hay alone gave values ranging from 8.1 to 12.1 m-mole/100 ml. Diets containing large proportions of concentrate, silage, pasture or finely ground hay produced a greater range in variation. The differences due to diet were related to at

least two factors: the higher level of readily fermentable carbohydrate present in concentrates and succulent feeds compared with hay and the larger surface area of concentrates and ground roughage, relative to hay fed in the long form, which would be exposed to microbial action immediately after ingestion.

Table 6. Mean concentrations (m-mole /l.) of total volatile fatty acids in duplicate samples of rumen liquor

Day of sampling	Location of sampling	Time of sampling after commencement of feeding (hr.)			
		0	2	4.5	6.5
1(30.7.64)	R	56.8	97.3	121.3	102.7
	M	64.1	100.4	145.0	107.1
	P	68.5	93.6	133.4	109.4
2(31.7.64)	R	83.9	90.2	110.1	88.2
	M	92.7	91.2	113.6	104.5
	P	85.7	93.8	126.2	112.0
3(1.8.64)	R	65.5	92.3	122.6	101.0
	M	71.2	99.9	137.9	106.1
	P	72.6	95.5	131.4	110.5
		D^*_D = 10.3			
		D_L = 10.3			
		D_T = 11.4			

* D = difference required for significance at $P \geq 0.05$ (Snedecor, 1956).

Concentrations of total VFA at corresponding times and locations of sampling on Days 1 and 3 were not significantly different (Table 6). Significantly higher concentrations were present before feeding on Day

2 than on either of the other two days. In contrast, concentrations after feeding tended to be lowest on Day 2. The cause of this variation is not known.

Although there was a tendency for concentrations to be lowest in samples from the reticulum, location of sampling generally had no significant effect on VFA levels before and at 2 and 6.5 hr. after feeding (Table 6). On only one occasion (Day 1, 4.5 hr. samples) was there a significant difference between the concentration at each location. This was also the only occasion on which the concentration of NH_3 at each location was significantly different (Table 2) and for this reason should be regarded as atypical.

The proportions and concentrations (m-mole/l.) of the individual VFA (acetic, propionic and butyric) are presented in Appendices I and II, respectively.

Proportions of acetic acid decreased following feeding reaching their lowest recorded levels 2.5 - 4.5 hr. after completion of feeding. They were inversely related to the proportions of propionic acid which were maximal 2.5 hr. after feeding. Butyric acid proportions were invariably lowest in samples obtained at the end of the feeding period. These results are in general agreement with those of Gray & Pilgrim (1951), Reid, Hogan & Briggs (1957) and Davey (1965). Bailey (1965) noted that of the total soluble sugars ingested in freshly-cut clover, 80% had disappeared from the rumen by the end of a 2 hr. feeding period. During the same period only 10% of the pectin consumed was digested and

almost no hemicellulose or cellulose was lost until 3 hr. after feeding. This variation in rate of degradation of carbohydrate fractions may explain the variation in individual VFA proportions since it has been found, *in vivo*, that rations high in soluble carbohydrates result in lower average acetate to propionate ratios than do those high in fibre (Phillipson, 1952; Balch *et al.*, 1955; Balch & Rowland, 1957).

Minimum and maximum concentrations of acetic acid were present in the pre-feeding and 4.5 hr. samples, respectively (Table 7). Concentrations in the 2 and 6.5 hr. samples were not significantly different.

Significantly higher concentrations of acetic acid were present in the pre-feeding samples on Day 2 than on Days 1 and 3, whereas significantly lower concentrations were present in the post-feeding samples on Day 2 than on Days 1 and 3 (Table 7). Differences between Days 1 and 3 were not significant.

Acetic acid levels in samples from Locations M and P did not differ significantly and tended to be higher than those present at Location R (Table 7).

These variations with time, day and location of sampling were similar to those found for total VFA and were to be expected since acetic acid constituted approximately 70% of the total concentration (Appendix I).

Table 7. Mean concentrations of acetic acid (m-mole /l.) in duplicate samples of rumen liquor

Day of sampling	Location of sampling	Time of sampling after commencement of feeding (hr.)			
		0	2	4.5	6.5
1(30.7.64)	R	42.9	73.8	87.1	72.8
	M	48.3	75.8	100.2	75.9
	P	49.2	69.6	93.6	77.7
2(31.7.64)	R	56.5	63.2	75.0	60.5
	M	61.0	63.9	78.5	73.1
	P	59.3	67.4	84.1	74.6
3(1.8.64)	R	46.7	68.8	86.4	70.7
	M	51.3	75.0	94.8	74.9
	P	51.4	70.2	91.1	77.5

$D_D = 7.4$

$D_L = 7.4$

$D_T = 8.2$

Concentrations of propionic acid increased after feeding with maximum levels present in the 4.5 hr. samples (Table 8).

Location of sampling had little effect on concentrations of propionic acid except in samples obtained 4.5 hr. after feeding on Day 1 and 6.5 hr. after feeding on Day 2 at which times significant differences occurred between each location. It will be recalled that significant differences in the concentrations of NH_3 and of total VFA between each location were also present in the 4.5 hr. sample on Day 1.

Concentrations of butyric acid were highest in the 4.5 hr. samples but in general were not significantly higher than the levels in the 6.5 hr. samples (Table 9). On Days 2 and 3, butyric acid concentrations tended to be lower in the 2 hr. samples than in those obtained before feeding.

It is apparent (Appendix I) that on all 3 days, the proportions of butyric acid were lower 2 hr. after feeding than before feeding.

Table 8. Mean concentrations of propionic acid (m-mole/l.) in duplicate samples of rumen liquor

Day of sampling	Location of sampling	Time of sampling after commencement of feeding (hr.)			
		0	2	4.5	6.5
1(30.7.64)	R	9.0	16.7	24.2	19.8
	M	10.4	17.6	30.2	20.5
	P	12.8	17.0	27.3	20.9
2(31.7.64)	R	16.4	18.1	23.1	17.3
	M	18.7	18.3	23.3	20.3
	P	16.1	17.6	27.1	24.5
3(1.8.64)	R	11.4	16.9	25.8	20.7
	M	12.4	18.2	28.6	20.4
	P	13.1	17.8	27.2	21.4

$D_D = 2.7$

$D_L = 2.7$

$D_T = 3.0$

Except in the pre-feeding samples there was no significant difference between concentrations of butyric acid on Days 1 and 3. Levels from samples obtained on Day 2 were generally significantly higher than on either of the other two days.

There was no definite trend in the concentrations of butyric acid between locations of sampling. In general, variations in concentrations between at least two of the three locations, of which location M was always one, were not significant.

Table 9. Mean concentrations of butyric acid (m-mole/l.) in duplicate samples of rumen liquor

Day of sampling	Location of sampling	Time of sampling after commencement of feeding (hr.)			
		0	2	4.5	6.5
1(30.7.64)	R	5.0	6.9	10.1	10.2
	M	5.5	7.1	14.6	10.8
	P	6.5	7.1	12.6	10.8
2(31.7.64)	R	11.0	8.9	12.6	10.4
	M	13.0	9.1	11.8	11.2
	P	10.3	8.9	15.1	14.9
3(1.8.64)	R	7.4	6.6	10.4	9.6
	M	7.6	6.7	14.6	10.9
	P	8.0	7.6	13.1	11.7

$D_D = 2.0$

$D_L = 2.0$

$D_T = 2.2$

In summary, variation associated with location of sampling was not of sufficient magnitude to be of practical importance. Almost without exception, samples from the mid region contained NH_3 and VFA levels which did not differ significantly from those in samples from at least one of the other two locations. Concentrations present in the reticulum were generally lowest. These findings are in general agreement with those of Bryant (1961) and Davey (1965) whose results were based on data obtained from cows grazing pasture. Of the two alternative sampling procedures recommended by Bryant (1961) the preparation of composite samples offers little improvement in the accuracy of sampling over the

removal of a single sample from the mid region of the rumen because the volume represented by each portion of the composite is not known.

Another alternative method of promise is that of total removal and mixing of contents prior to sampling which was employed to advantage recently by Reid et al. (1967). The potential of this technique for reducing variation, particularly in samples obtained for dry matter determinations, is recognized. However, under the conditions of the present experiment, location of sampling was a relatively minor source of variation and the additional time and labour required to obtain samples by this method appeared to outweigh the improvement in sampling accuracy likely to result.

Therefore, the removal of a large sample of digesta from the mid region of the rumen was selected as a suitable sampling technique for the subsequent in vivo experiments.

Of the sources of variation in chemical composition of the rumen contents determined in this experiment, that due to time of sampling was of the greatest magnitude. The concentration of a metabolite such as NH_3 or VFA, present in rumen fluid at any specific time, is dependent on its rate of production, rate of utilization and rate of absorption and/or passage from the rumen. In the present experiment, the similarity between the concentration curves for each of the three days of sampling indicated that these factors were in a similar state of equilibrium on all three days. This was undoubtedly due to several factors including the standardized feeding regimen and sampling times employed and to an adequate length of pre-experimental period. The effect of

any treatment imposed following the attainment of a similar condition in the animal would result in an alteration in the point of equilibrium and would be reflected by a change in concentration of one or more of the fermentation end-products. Although a concentration change will not indicate the relative effect of a treatment on each of the factors affecting the equilibrium, it will indicate the overall effect of a specific treatment.

In spite of this and a number of other limitations, the determination of changes in concentration of soluble metabolites in rumen fluid has added considerably to our present understanding of the fate of dietary constituents in the rumen and was adopted for general use in the following *in vivo* experiments. Additional information was obtained from several *in vitro* experiments.

Summary

1. A gas chromatographic technique for the quantitative determination of volatile fatty acids in samples of rumen liquor is described.

2. Concentrations of NH_3 and VFA were determined in samples of rumen liquor obtained from three fixed locations within the reticulo-rumen.

3. Differences in levels of NH_3 and VFA due to location, time and day of sampling are discussed in relation to the selection of a suitable sampling procedure for subsequent *in vivo* experiments.

Part 2. EFFECT OF LIPIDS ON THE CONCENTRATIONS OF AMMONIA, TOTAL AND INDIVIDUAL VOLATILE FATTY ACIDS AND ON THE DIGESTION OF CELLULOSE IN THE RUMEN

Materials and Methods

Treatment of Animals

Three pairs of identical twin, non-lactating Jersey cows with rumen fistulae were used in this series of experiments. The animals were fed indoors on either freshly-cut herbage, consisting of a mixture of perennial ryegrass (Lolium perenne) and white clover (Trifolium repens) (experiment i - iv), or perennial ryegrass hay (experiments v and vi). Except where indicated, they were fed twice daily at either 9:30 a.m. and 4:30 p.m. (experiments i, iii and iv) or 9:30 a.m. and 5:30 p.m. (experiments ii and v) for periods of approximately two hours. Water and iodized salt were available ad libitum.

Samples, estimated to contain approximately 200 ml. rumen liquor after being strained through muslin, were removed from the mid-region of the rumen (experiments i - v). In experiments i, ii, iii and v, oils were administered as oil/water emulsions, (50:50 w/v), using 1% Lissapol N X (Imperial Chemical Industries Ltd., London) as emulsifying agent.

Experiment (i)

The animals (pair b B) each weighed about 408 kg. Initially, a single dose of an emulsion containing 500 g. of linseed oil was infused into the rumen of cow b during one morning feeding period. A week later cow B received the same dosage on each of four consecutive mornings. Samples of rumen liquor were taken from each animal just before the morning feeding commenced and 2, 4 and 6.5 hr. later.

Experiment (ii)

The animals (pair a A) weighed approximately 272 kg. An emulsion containing 750 g. of linseed oil was given to each animal during the morning feeding on two consecutive days. The rumen of both animals was sampled on the day prior to dosage as well as on the two days of oil infusion immediately before and 2, 4, 6 and 8 hr. after the commencement of the morning feeding period.

Experiment (iii)

An emulsion containing 750 g. of whale oil (Perano Bros. Ltd., Picton, New Zealand) was infused into the rumen of Cow A during the morning feeding period on two consecutive days. Rumen liquor samples were taken from both animals before and at 2 hr. intervals to six hr. after the morning feeding commenced on the day prior to treatment and on each of the two days of dosage. One week later cow a received the same amount of emulsion on two consecutive days and both animals were sampled as before.

Experiment (iv)

In order to determine the maximum increases in concentration of propionic acid present in rumen liquor which could have arisen from glycerol fermentation in the preceeding experiments, it was first necessary to determine the approximate fluid volume of the rumen. Following a preliminary feeding period of one week, 100 g. of polyethylene glycol (P.E.G.) (average molecular weight, 4000) was sprayed over the surface of the rumen digesta of cows a and A just prior to the first feeding on two consecutive days. Samples of rumen liquor were removed

on both days immediately before the introduction of P.E.G. and again at 2 hr. intervals over the next 12 hr.

Experiment (v)

A third pair of animals (c, C) weighing approximately 272 kg. was used to determine the effect of lipid on cellulose digestion in the rumen. A method similar to that of Flatt, Warner & Loosli (1959) was adopted. Twelve weighed strands of white cotton thread (No. 40, Clarks) were suspended in the rumen of each cow, six in the dorsal region and six in the ventral region, after an overnight fast and prior to the morning feeding period. Two strands were removed from each region of the rumen after 10, 24 and 36 hr., washed in water and alcohol and the dry weight determined.

The experiment was in two parts, each of four days duration. During the first two days of each part, the digestion of cotton thread was measured in both cows without added oil. Another set of threads was then placed in the rumen of each cow. One of the animals (cow c) received 500 g. of linseed oil during the morning feeding period on each of the final two days of part (i) and the other animal (cow C) received the same dose on each of the final two days of part (ii).

Concentrations of NH_3 and VFA were determined in samples of rumen liquor obtained immediately before and 2, 4, and 6 hr. after the commencement of the morning feeding.

Experiment (vi)

Cow C was fed perennial ryegrass hay (Lolium perenne) twice daily for one week prior to the commencement of this experiment. Following

an overnight fast (18.5 hr.), 200 g. of linseed oil, as an oil/water emulsion (50:50, w/v) containing 1 g. of Lissapol NX, were infused directly into the omasum. This was accomplished by inserting one end of a ten foot length of pressure tubing through the reticulo-omasal orifice into the omasum and holding it in position during the period of infusion (10 min.). The opposite end of the tube was connected to a 1000 ml. separatory funnel to which the emulsion was added.

Three hr. prior to the infusion of oil, a pressure-sensitive transducer attached to a modified Sanborn, 4-channel recorder (Reid, Melville & Cornwall, 1960) had been placed in each of four locations - the reticulum, the dorsal, ventral and dorsal posterior blind sacs of the rumen. Pressure changes in these regions were recorded for 2.5 hr. before and 2 hr. after infusion. The animal was then fed for 2 hr., fasted overnight (18 hr.) and, on the following day, 500 g. of linseed oil emulsified with water, were again infused directly into the omasum. Pressure changes were recorded for 60 min. before and 130 min. after infusion.

Analytical Methods

Pasture Analysis

Dry matter and total nitrogen were determined by the methods outlined in Part 1 (p.37). Methods for the extraction of soluble sugars and the hydrolysis of the hemicellulose and cellulose fractions were as described by Jarrige (1960). Soluble sugars and products of hydrolysis were determined by the method of Bath (1958). All analyses were carried out on samples obtained at the beginning of each experiment.

Rumen Liquor Analysis

Ammonia was determined immediately after sampling by the method described in Part 1.

pH determinations were also made immediately after sampling during experiments (ii) and (iii). The samples were then frozen and held until such time as VFA determinations could be made.

The separation of total VFA from 5 ml. of rumen liquor was effected by steam-distillation in a Markham still using the method of Johns (1955 a). Sixty ml. of distillate were collected, followed by the collection of a further 60 ml. to serve as a blank. The distillates were then titrated with 0.05 N sodium hydroxide and phenolphthalein indicator. After addition of a small excess of alkali the salts of the fatty acids were concentrated in vacuo to a suitable volume (0.5 - 1.0 ml.) for gas-liquid chromatographic analysis.

The proportions of acetic, propionic and butyric acids in the volatile fraction were determined by gas-liquid chromatography (James & Martin, 1952) at 137° with the column packing described by Hawke (1957). The concentrated salts of the fatty acids were taken up in a syringe and approximately 0.05 ml. was acidified in a platinum boat containing a mixture of equal parts by weight of Celite 545 and dry potassium bisulphate. The boat was then pushed into the heated region of the column and the nitrogen supply connected.

Fluid Volume of the Rumen

The method of Hydén (1956, 1958) was used to determine fluid volume. The samples which could not be clarified by filtration after the addition of barium chloride, barium hydroxide and zinc sulphate were

centrifuged at 10,000 g. for 15 min. in an ultra-centrifuge (Ullyatt, pers. comm.).

Results

Effect of lipid on the NH_3, total and individual volatile fatty acid concentrations in the rumen of pasture-fed animals

Pasture Composition

Marked changes in the chemical composition of the pasture occurred between experiments i, ii and iii (Table 10). Higher concentrations of soluble sugars occurred in late winter (August) when growth was slower than during the periods of rapid growth in spring (November) and autumn (May). Conversely, protein was higher in the autumn sample than in the late winter and spring samples. Values for hemicellulose were similar to those for soluble sugars while cellulose remained relatively constant at about 18% of dry matter.

Table 10. Chemical composition (% dry wt.) of pasture for the period of the experiments in which linseed oil and whale oil were infused into the rumens of fistulated twin cows

Date of sampling	Dry matter (%)	Soluble sugars	Hemi-cellulose	Cellulose	Total carbo-hydrate	Protein (N x 6.25)	Protein/ soluble carbo-hydrate ratio
Expt.(i)							
(30.8.61)	14.1	18.4	19.9	17.7	56.0	21.0	1.14:1
Expt.(ii)							
(13.11.61)	20.7	16.1	16.2	18.0	50.3	18.2	1.13:1
Expt.(iii)							
(7.5.62)	10.6	9.0	9.8	18.9	37.7	28.7	3.2:1

Feed Intake

The infusion of 500 g. of emulsified linseed oil into the rumen of cow b in the first series of experiments appeared to depress intake slightly although intake levels each day were sufficiently variable to make this uncertain (Table 11). The treatment of cow B with the same weight of emulsified linseed oil, produced a reduction in intake to about 70% of the pretreatment level. The control animals maintained their intake close to the pretreatment levels with the exception of a low intake for cow b on one day.

Table 11. Intake of fresh pasture by stall-fed twin cows with and without added oil

Date	Oil added	Intake (kg. fresh pasture)					
		Morning		Afternoon		Total	
Expt.(i)		Cow b	Cow B	Cow b	Cow B	Cow b	Cow B
28.8.61	-	35.4	32.7	38.1	34.9	73.5	67.6
29.8.61	-	31.3	31.8	31.8	29.0	63.1	60.8
30.8.61	linseed, 500g.	24.9*	36.3	32.2	36.7	57.1	73.0
11.9.61	-	29.9	24.5	32.2	38.1	62.1	62.6
12.9.61	linseed, 500g.	24.5	28.6*	33.1	17.7	57.6	46.3
13.9.61	linseed, 500g.	22.2	24.5*	24.5	16.8	46.7	41.3
14.9.61	linseed, 500g.	31.8	22.7*	30.8	22.2	62.6	44.9
15.9.61	linseed, 500g.	34.5	25.9*	29.0	20.4	63.5	46.3
Expt.(ii)		Cow a	Cow A	Cow a	Cow A	Cow a	Cow A
13.11.61	-	20.0	16.8	29.0	29.0	49.0	45.8
14.11.61	linseed, 750g.	16.8	15.4*	24.0	4.1	40.8	19.5
15.11.61	linseed, 750 g.	14.1	0.9*	27.7	12.7	41.8	13.6
16.11.61	linseed, 750 g.	10.9*	0.0	4.1	4.1	15.0	4.1
17.11.61	linseed, 750g.	1.4*	1.4	3.6	5.9	5.0	7.3
Expt.(iii)							
7.5.62	-	19.1	25.4	25.9	27.2	45.0	52.6
8.5.62	whale, 750g.	24.9	25.4*	29.0	18.6	53.9	44.0
9.5.62	whale, 750g.	16.3	9.1*	24.5	12.7	40.8	21.8
16.5.62	-	25.4	20.0	23.1	20.0	48.5	40.0
17.5.62	whale, 750g.	29.0*	20.0	18.6	19.5	47.6	39.5
18.5.62	whale, 750g.	13.1*	20.0	15.4	22.2	28.5	42.2

* animal received oil

In experiment (ii) a marked drop in the intake of both animals (a and A) occurred at the feeding period which followed the infusion into the rumen of 750 g. of emulsified linseed oil (Table 11). Despite negligible intakes during some subsequent feeding periods considerable amounts of solid ingesta remained in the rumen.

A reduction in motility of the rumen was first observed when the rumen contents were sampled on the day following the first infusion (experiment ii). The delay in effect suggested that, if the oil was responsible, it was probably affecting a neural or hormonal control mechanism at some point further along the digestive tract. The period of quiesence continued for at least two days following the second infusion.

Diarrhea, characterized by yellowish, foul-smelling and very fluid faeces, appeared on the second day of infusion. The colour and consistency of the faeces gradually returned to normal over a period of approximately three days.

A decreased feed intake also resulted when animals a and A were given 750 g. of emulsified whale oil. The drop in intake (40-50% of the pre-experimental level) was not as great as that brought about by the infusion of the same weight of linseed oil (Table 11).

Ammonia Concentration in the Rumen

The addition of oil would appear to affect nitrogen metabolism in the rumen since levels of NH_3 were altered appreciably in all experiments. In cow b (experiment i) the NH_3-concentration rose from a normal maximum

of 20-25 mg./100 ml. rumen liquor (at 2 or 4 hr. after the commencement of feeding) to about 50 mg./100 ml. rumen liquor following the addition of oil (Fig. 2). At 6.5 hr. the NH_3-concentration had dropped considerably but was still in excess of that for the control animal. Following the rumenal infusion of oil into cow B the NH_3 level was similar to that for the control animal until the afternoon of the third day of treatment when it rose to 50 mg./100 ml. rumen liquor. The concentration in the sample taken prior to feeding on the fourth morning was 80 mg./100 ml. after which it dropped to a level below that found in samples from the control animal.

It was anticipated that increasing the amount of linseed oil from 500 g. to 750 g. would establish whether the irregular increases in NH_3 concentrations in experiment (i) were due to the addition of oil. However, although the NH_3 concentrations in the rumen of the experimental animals were generally higher than those in the controls (Fig. 3) the very large increases which were found occasionally in the first experiment were not obtained.

In experiment (ii) the concentrations of NH_3 in the rumen were lower in the control animals than in the previous experiment. This was probably due to the lower nitrogen levels in the pasture (Table 10). The levels of NH_3 for both animals on the day prior to the addition of oil were almost identical, rising to a peak of about 15 mg./100 ml. rumen liquor at 2 hr. and decreasing slowly over the next few hours. On the first day of treatment the initial rise at 2 hr. was

Fig. 2. Variations in rumen ammonia and volatile fatty acid concentrations in twin cows fed on freshly-cut mixed pasture with and without addition of 500 g. of linseed oil.

(a) 29/8/61 - cow b (without oil), o - - - o; cow B (without oil), o——o

30/8/61 - cow b (with oil), □ - - - □; cow B (without oil), □——□

(b) 11 - 15/9/61 - cow b (without oil), x - - - x (av. for 5 days)

11/9/61 - cow B (without oil), o——o

12/9/61 - cow B (with oil), □——□

13/9/9/61 - cow B (with oil), △——△

14/9/61 - cow B (with oil), ●——●

15/9/61 - cow B (with oil), ■——■

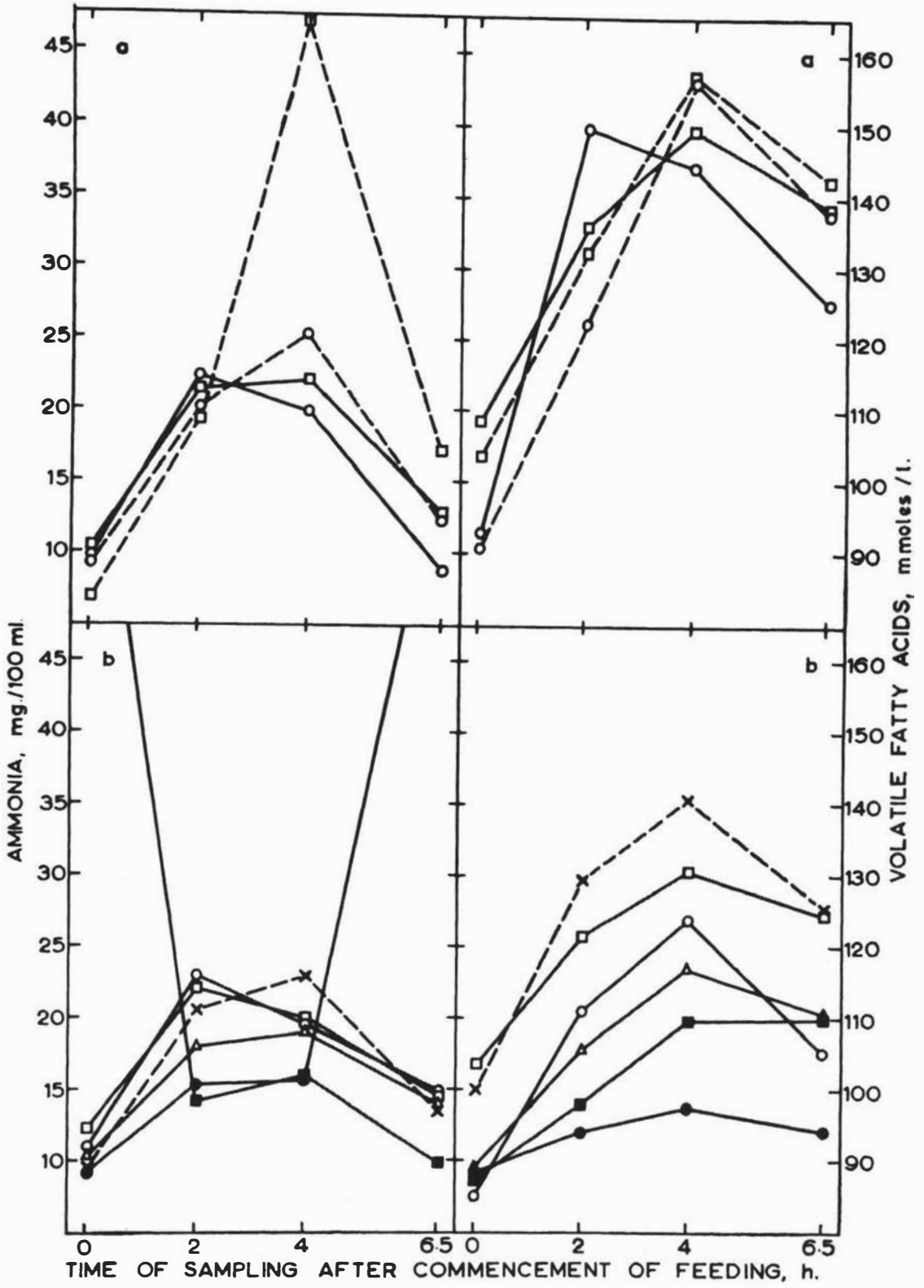

a
a
b
b
AMMONIA, mg./100 ml.
VOLATILE FATTY ACIDS, mmoles/l.
TIME OF SAMPLING AFTER COMMENCEMENT OF FEEDING, h.
0
2
4
6.5
10
15
20
25
30
35
40
45
90
100
110
120
130
140
150
160

Fig. 3. Variations in rumen ammonia and volatile fatty acid concentrations in twin cows fed on freshly-cut mixed pasture with and without additions of 750 g. linseed oil

(a) 13/11/61 - cow A (without oil), ○——○

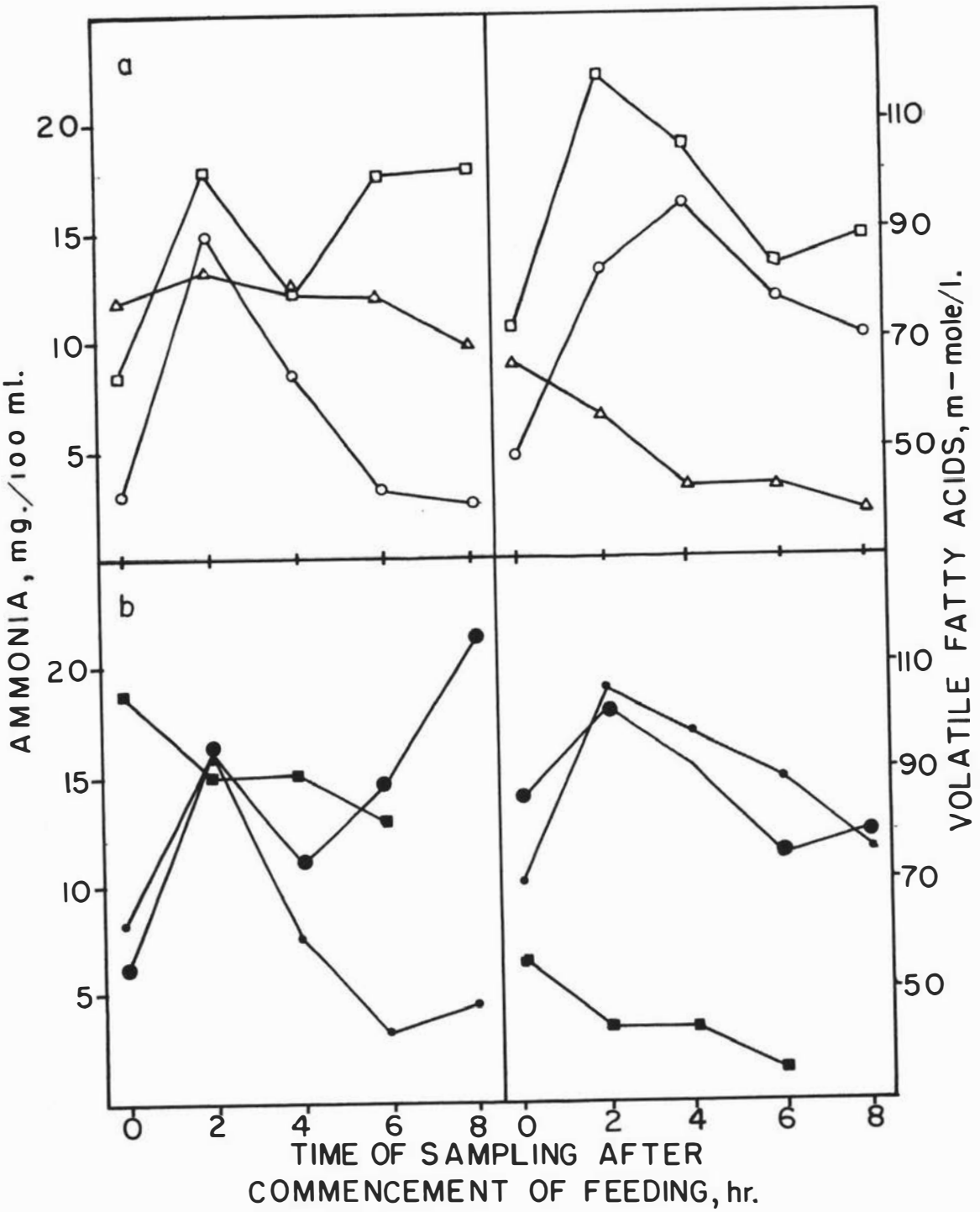
14/11/61 - cow A (with oil), □——□

15/11/61 - cow A (with oil), △——△

(b) 13 - 15/11/61- cow a (without oil), •——• (av. for 3 days)

16/11/61 - cow a (with oil), ●——●

17/11/61 - cow a (with oil), ■——■


a
b
20
15
10
5
110
90
70
50
0
2
4
6
8
AMMONIA, mg./100 ml.
VOLATILE FATTY ACIDS, m-mole/l.
TIME OF SAMPLING AFTER
COMMENCEMENT OF FEEDING, hr.

followed by further increases at 4 and 6 hr. reaching a maximum of about 20 mg./100 ml. rumen liquor at 6 hr. The level the following day remained almost constant at 10 - 15 mg./100 ml. despite a much lower feed intake.

It was thought that the severe effect of the larger dose of linseed oil on intake might have been due to a characteristic of the oil such as the presence of a cyanogenetic glucoside. Consequently, whale oil, another relatively unsaturated oil, was used in experiment (iii). The effect on NH_3 concentration following addition of 750 g. whale oil to cows a and A is shown in Fig. 4. Concentrations for both animals immediately prior to treatment were almost identical (max. 60 - 65 mg./100 ml. rumen liquor) and followed very similar patterns subsequent to treatment. The oil had no effect until the second day when concentrations remained almost constant at between 30 - 40 mg./100 ml. of rumen liquor.

pH Measurements of Rumen Liquor

In experiment (ii), the pH of the samples of rumen liquor obtained during the control period varied from maxima of approximately 7.0 to minima of 6.75 (Fig. 5). No appreciable variation from these values occurred following the first infusion of linseed oil. However, 2 hr. after feeding and infusion on the morning of the second day, the pH increased to approximately 7.6 and remained constant at this level for the duration of the day. Little variation was found between animals during either the control or experimental periods.

During the control period of experiment (iii), the changes following feeding were more pronounced with values ranging between 6.14

Fig. 4. Variation in rumen ammonia and volatile fatty acid concentrations in twin cows fed on freshly-cut mixed pasture with and without addition of 750 g. whale oil

(a) 7/5/62 - cow a (without oil), o- - -o ; cow A (without oil), o——o

8/5/62 - cow a (without oil), □- - -□ ; cow A (with oil), □——□

9/5/62 - cow a (without oil), △- - -△ ; cow A (with oil), △——△

(b) 16/5/62 - cow a (without oil), ●- - -● ; cow A (without oil), ●——●

17/5/62 - cow a (with oil), ■- - -■ ; cow A (without oil), ■——■

18/5/62 - cow a (with oil), ▲- - -▲ ; cow A (without oil), ▲——▲

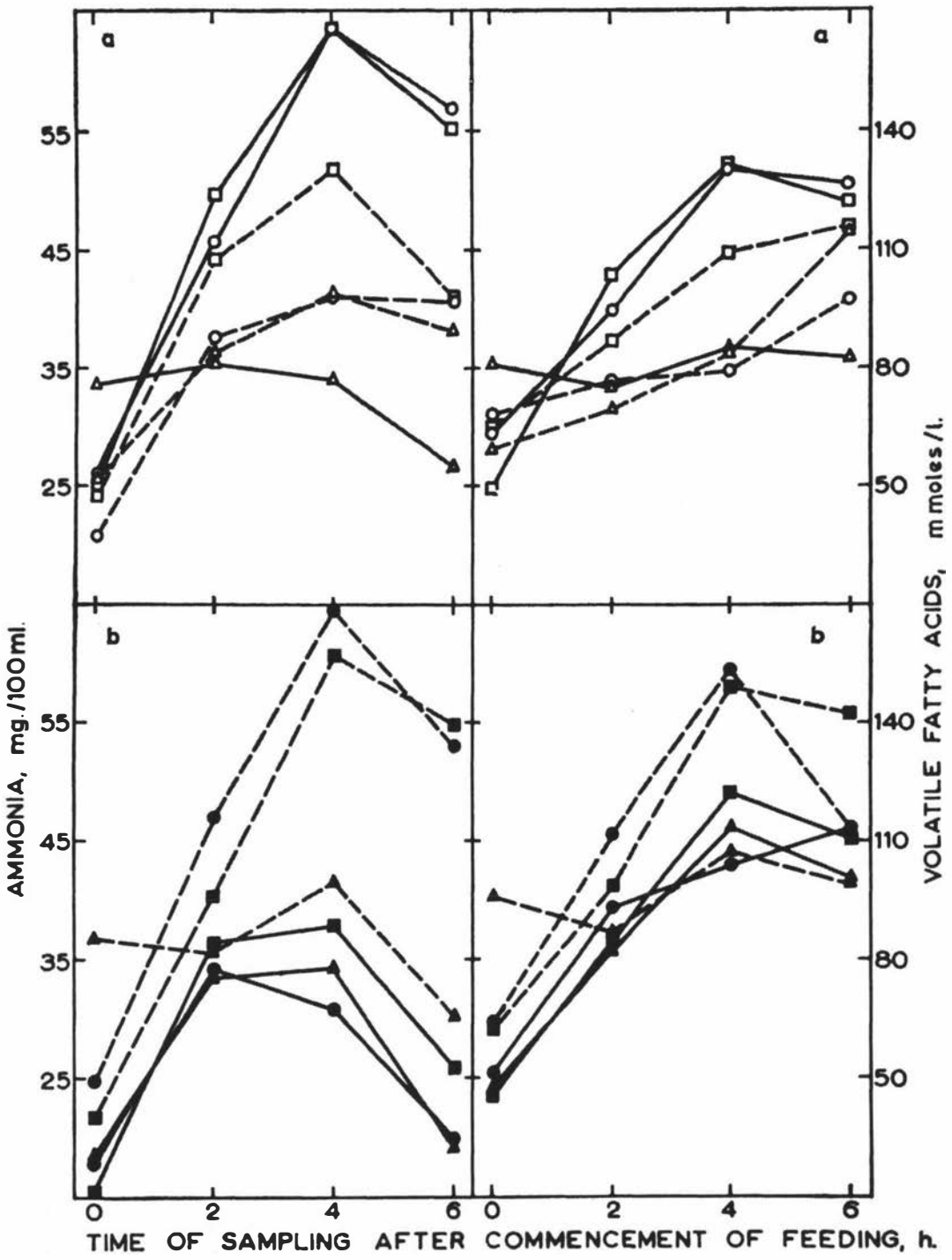

a
a
b
b
55
45
35
25
140
110
80
50
0
2
4
6
AMMONIA, mg./100ml.
VOLATILE FATTY ACIDS, m moles/l.
TIME OF SAMPLING AFTER COMMENCEMENT OF FEEDING, h.

Fig. 5 Variations in pH of rumen contents of twin cows fed on freshly - cut mixed pasture with and without additions of 750 g. of linseed oil

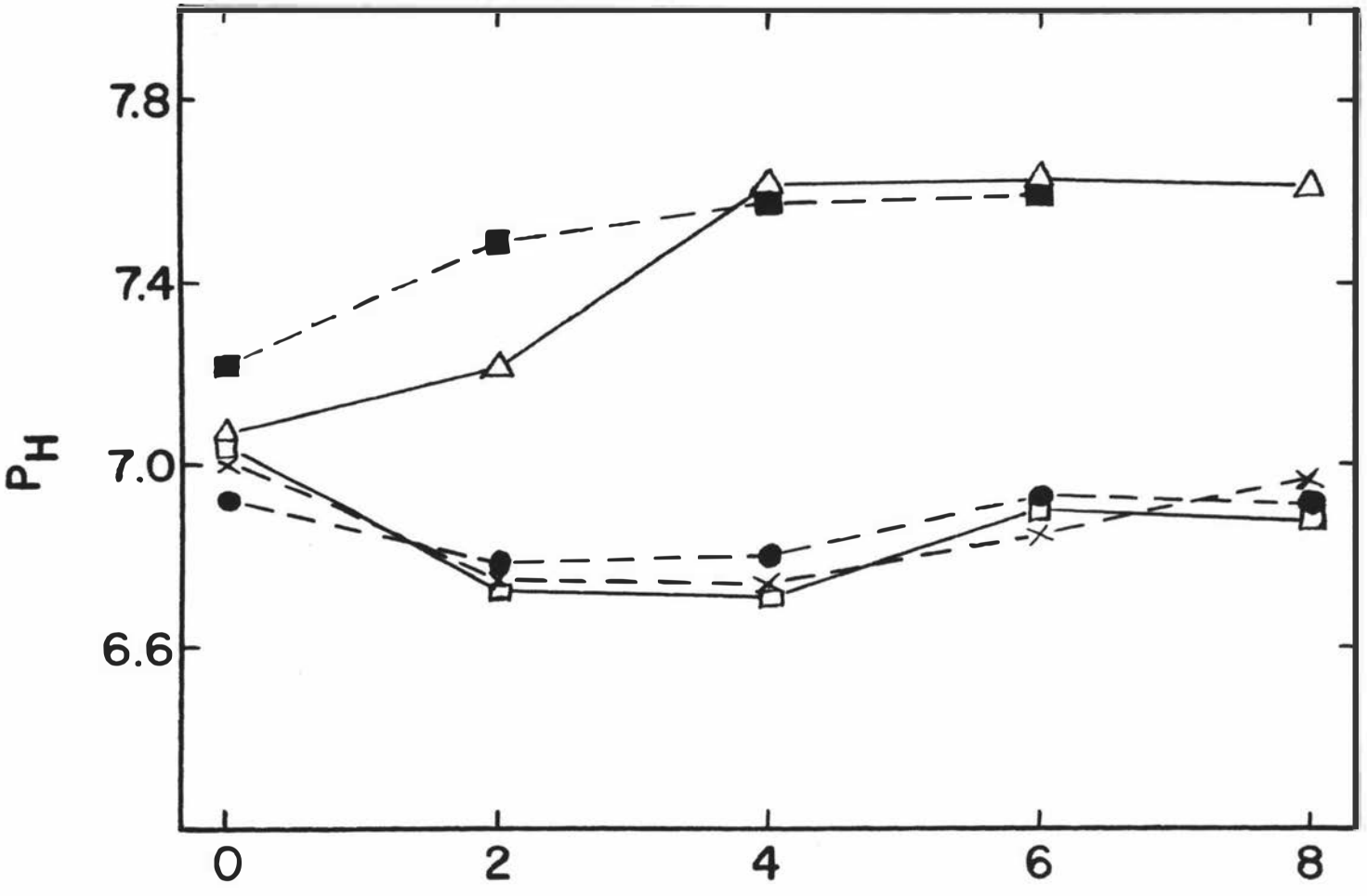
14 - 15/11/61 - cow a (without oil), x– – –x (av. for 2 days)

16/11/61 - cow a (with oil), ●– – –●

17/11/61 - cow a (with oil), ■– – –■

14/11/61 - cow A (with oil), □——□

15/11/61 - cow A (with oil), △——△


7.8
7.4
7.0
6.6
pH
0
2
4
6
8
TIME OF SAMPLING AFTER COMMENCEMENT
OF FEEDING, hr.

and 7.36 (Fig. 6). Infusion of 750 g. of whale oil had little effect until the second day of dosage when pH remained relatively constant throughout the day at between 6.6 and 6.8.

A highly significant correlation coefficient of - 0.911 between pH and total VFA concentrations was found following the pooling of the data from both experiments.

Total Volatile Fatty Acids

The reliability of the steam distillation procedure for determining total VFA concentration was checked by introducing a known concentration of a mixture of acetic, propionic and butyric acids to each of four samples of rumen liquor. The average recovery of the added constituents was 97.4% with a standard deviation of ± 0.2.

No apparent change occurred in the concentration of volatile acids in samples taken from cow b following infusion of 500 g. linseed oil (Fig. 2). Values for both animals varied from a minimum of approximately 90 m-mole/l. rumen liquor prior to feeding, to a maximum of 145 - 160 m-mole/l. rumen liquor 4 hr. after the commencement of feeding. The maximum concentrations in cow B decreased slightly on each successive day of treatment, except the last. This decrease was accompanied by a reduced intake at the afternoon following each infusion (Table 11).

VFA concentrations in cows a and A were similar prior to treatment in experiment (ii) (Fig. 3) and were well below those found during the pre-treatment period in experiment (i). The concentrations of VFA in samples from both animals on the first day of oil dosage, followed the

Fig. 6. Variations in pH of rumen contents of twin cows fed on freshly-cut mixed pasture with and without additions of 750 g. of whale oil.

(a) 7/5/62 - cow a (without oil), o— — —o ; cow A (without oil), o——o

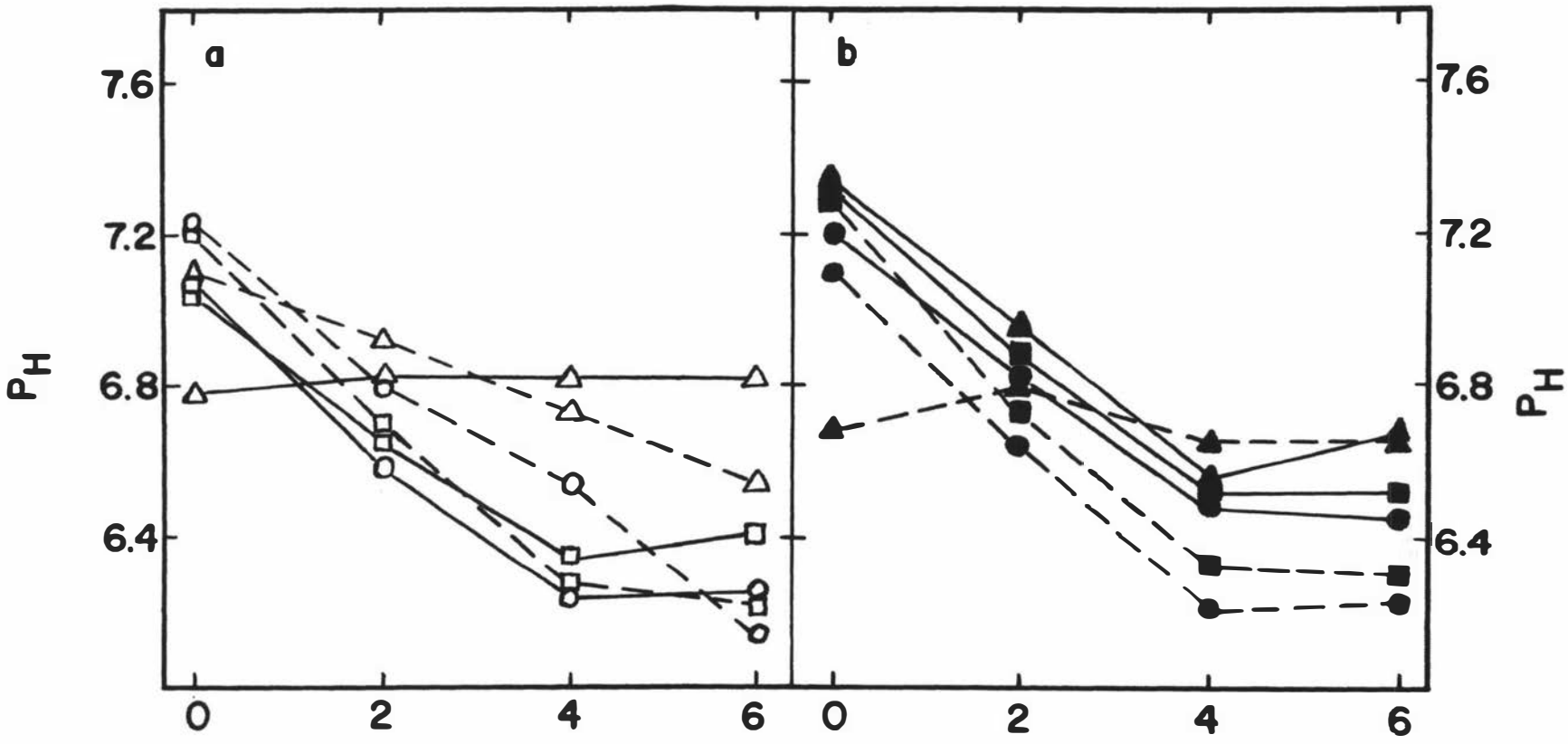
8/5/62 - cow a (without oil), □— — —□ ; cow A (with oil), □——□

9/5/62 - cow a (without oil), △— — —△ ; cow A (with oil), △——△

(b) 16/5/62 - cow a (without oil), ●— — —● ; cow A (without oil), ●——●

17/5/62 - cow a (with oil), ■— — —■ ; cow A (without oil), ■——■

18/5/62 - cow a (with oil), ▲— — —▲ ; cow A (without oil), ▲——▲


a
b
7.6
7.2
6.8
6.4
pH
0
2
4
6
TIME OF SAMPLING AFTER COMMENCEMENT
OF FEEDING, hr.

same pattern and were about the same level as during the control period but decreased in subsequent samples on the second day.

The administration of 750 g. of emulsified whale oil (experiment iii) had an effect on total VFA concentrations similar to that produced by the same weight of linseed oil (Fig. 4). On the first day of treatment the volatile fatty acid concentrations in samples from animals a and A followed those found on the pretreatment day. Concentrations obtained the following day showed much less overall variation and were lower than those found earlier in the experiment.

Individual Volatile Fatty Acids

The accuracy of the gas chromatographic technique for estimating the proportions of individual volatile fatty acids was determined by Bryant (1961) under conditions of analysis identical with those used in the present experiments. He found that the proportion of each acid did not differ by more than 2% from the calculated levels.

The concentrations of acetic, propionic and butyric acids (m-mole/l. of rumen liquor) were altered appreciably by the addition of 500 g. of linseed oil to the rumen (Table 12). In cow b, there was an almost immediate decrease in the concentration of acetic acid to about half that present in the pre-experimental period, and a considerable increase in the concentrations of propionic and butyric acids. The oil had no effect on the concentrations of the individual acids in cow B until the third day of infusion when the amount of propionic was well above previous levels despite a decrease in total concentration of acids. By the fourth day, the concentrations of acetic and propionic acids were almost equal whereas prior to oil infusion the acetic / propionic ratio

Table 12. Concentrations of the individual volatile fatty acids (m-mole/l.) in the rumen of twin cows stall-fed on freshly-cut mixed pasture with or without 500 g. linseed oil

Experiment (i)

Date	Cow b		Time of sampling after commencement of feeding (hr.) 0	2	4	6.5	Cow B		Time of sampling after commencement of feeding (hr.) 0	2	4	6.5
		A*	67.4	89.1	102.6	93.7		A	62.2	100.9	93.3	84.2
29.8.61	(no oil)	P	8.5	19.4	28.5	21.4	(no oil)	P	16.0	28.0	28.6	22.3
		B	14.8	13.7	25.3	22.5		B	13.8	20.8	22.5	18.9
		A	72.9	53.8	56.8	45.2		A	73.7	84.5	92.7	101.9
30.8.61	(linseed	P	13.8	45.2	67.5	59.2	(no oil)	P	17.7	31.0	27.7	21.8
	oil,500g.)	B	17.0	33.3	32.9	38.2		B	16.5	20.7	29.2	14.9
		A	69.9	75.7	100.0	95.5		A	-	83.9	89.1	63.5
11.9.61	(no oil)	P	15.1	24.9	22.6	20.0	(no oil)	P	-	16.2	17.9	24.0
		B	17.0	26.4	20.2	15.5		B	-	10.9	17.4	17.8
		A	-	86.0	98.3	87.7		A	78.1	72.3	84.1	79.6
12.9.61	(no oil)	P	-	29.1	27.2	24.0	(linseed	P	13.2	26.4	23.8	22.5
		B	-	21.1	20.0	17.2	oil,500g.)	B	12.3	22.8	22.7	22.5
		A	57.2	74.5	83.1	77.4		A	54.1	61.8	68.2	65.0
13.9.61	(no oil)	P	12.1	22.6	28.6	17.2	(linseed	P	21.7	28.5	32.0	29.6
		B	11.9	19.7	14.7	13.9	oil,500g.)	B	13.3	15.4	16.8	16.1
		A	76.6	90.5	92.8	83.6		A	46.3	55.4	51.2	45.9
14.9.61	(no oil)	P	16.7	22.8	27.6	25.3	(linseed	P	29.8	31.2	34.7	41.6
		B	11.3	20.8	22.5	18.5	oil,500g.)	B	11.9	7.5	11.6	6.6
		A	76.6	87.2	95.6	87.1		A	35.3	38.1	44.1	52.0
15.9.61	(no oil)	P	19.4	25.3	27.4	25.4	(linseed	P	42.8	42.6	48.8	49.2
		B	14.9	20.5	19.6	16.9	oil,500g.)	B	9.1	17.2	16.7	9.2

* A - acetic acid P - propionic acid B - butyric acid

had been approximately 4.5:1.

Changes in concentrations of the fatty acids following infusion of 750 g. of emulsified linseed oil were observed on the second day (Table 13). Although the total concentration of VFA decreased by approximately 50%, there was almost no change in the concentration of propionic acid. Acetic and butyric acids were both reduced to between one-third and one-half of the former levels.

When the same weight of whale oil was introduced into the rumen, no obvious effect on the concentration of individual acids occurred until the second day of infusion (Table 14). As in the previous experiment, the concentration of propionic acid remained at, or was higher than, the control level despite a decrease in the concentration of total fatty acids in the rumen.

Fluid Volume of the Rumen

The ingestion of freshly-cut pasture herbage caused a marked increase in the fluid volume of the rumen and a decrease in the concentration of P.E.G. Therefore, to improve the accuracy of the regressions, separate exponential curves were fitted to the concentrations of P.E.G. present in samples of rumen liquor obtained before and after each afternoon feeding period (Table 15). Except for Cow A, Day 1, extrapolation of both curves to zero-time gave reasonably similar estimates of the initial concentration of marker. The average rumen fluid volumes of Cows a and A immediately before the morning feeding were 57.6 and 52.9 l., respectively (volume for Cow A, Day 1, Period ii, omitted from calculation.).

Effect of Lipid on Cellulose Digestion

The intakes of the twin cows in the first part of experiment (v) were very similar both before and on the first day of the addition of lipid but it was apparent that 500 g. of emulsified linseed oil added to the rumen depressed the intake of hay on the second day of infusion (Table 16). The second part of the experiment, which commenced 4 days after the conclusion of the first, was less satisfactory because of a carry-over effect of lipid. As a result, the intake of the control animal (cow c) was well below that of cow C when the second trial began. However, the effect of lipid may be assessed in part (ii) from the reduction in the dry matter intake of cow C from 5.5 kg. on the first day of lipid addition to 4.2 kg. on the second day.

Added lipid depressed the digestion of cotton threads suspended in the rumen (Table 17). Part (i) showed this to better effect because of the carry-over effect of lipid in Part (ii). Judged by the loss of weight of the cotton thread, cellulolytic activity was greater in the ventral than in the dorsal rumen. The residual weights of cotton threads were greater at 24 hr. in the presence than in the absence of lipid. At 36 hr. the threads had remained unbroken in 3 of the 4 determinations when lipid was added but had disintegrated in the absence of lipid.

The pre-feeding concentration of NH_3 in the rumen varied between 2.6 and 5.1 mg./100 ml.; peak levels recorded 2 hr. after feeding began ranged between 4.9 and 14.9 mg./100 ml. with the majority between 9 and 12 mg./100 ml. (Appendix III). Four hours after the commencement of feeding, the NH_3 levels had returned to approximately the pre-feeding concentrations.

Table 13. Concentrations of the individual volatile fatty acids (m-mole/l.) in the rumen of twin cows stall-fed on freshly-cut mixed pasture with or without 750 g. linseed oil

Experiment (ii)

Date	Cow a		Time of sampling after commencement of feeding (hr.) 0	2	4	6	8	Cow A		Time of sampling after commencement of feeding (hr.) 0	2	4	6	8
13.11.61	(no oil)	A*	45.5	73.5	69.0	61.6	58.7	(no oil)	A	37.2	63.9	71.9	55.3	53.2
		P	8.3	18.9	16.8	14.3	9.2		P	5.7	10.9	13.2	14.1	10.6
		B	5.8	6.5	12.4	11.8	6.9		B	4.6	8.2	9.3	7.9	6.6
14.11.61	(no oil)	A	59.9	76.2	75.7	63.1	59.1	(linseed oil,750g.)	A	52.9	80.6	74.8	52.5	52.7
		P	12.3	23.1	19.6	13.3	12.5		P	11.7	22.8	17.3	18.6	23.3
		B	9.8	13.1	15.1	16.6	9.3		B	6.8	15.0	13.7	12.0	12.0
15.11.61	(no oil)	A	45.7	69.9	58.3	62.4	49.5	(linseed oil,750g.)	A	35.6	31.6	21.3	24.3	21.3
		P	11.8	23.2	14.0	13.4	12.4		P	25.8	18.9	18.4	14.8	12.9
		B	11.0	11.6	9.5	10.5	9.2		B	4.1	5.4	2.9	4.0	3.2
16.11.61	(linseed oil,750g.)	A	64.7	74.6	75.0	52.1	51.6							
		P	12.4	16.9	12.1	11.6	15.8							
		B	7.2	10.4	3.4	10.7	11.5							
17.11.61	(linseed oil,750g.)	A	--	27.5	26.8	24.0	--							
		P	--	11.2	12.4	8.7	--							
		B	--	4.2	4.1	2.5	--							

* A - acetic acid P - propionic acid B - butyric acid

Table 14. Concentrations of the individual volatile fatty acids (m-mole/l.) in the rumen of twin cows stall-fed on freshly-cut mixed pasture with or without 750 g. whale oil

Experiment (iii)

Date	Cow a		Time of sampling after commencement of feeding (hr.) 0	2	4	6	Cow A		Time of sampling after commencement of feeding (hr.) 0	2	4	6
		A*	46.4	51.6	49.8	61.3		A	44.3	66.3	85.7	85.3
7.5.62	(no oil)	P	11.1	15.9	19.7	19.4	(no oil)	P	10.5	16.8	23.1	18.1
		B	10.7	9.0	9.8	16.7		B	8.8	11.7	21.5	23.1
		A	45.0	61.2	73.3	73.6		A	37.6	71.2	81.6	70.4
8.5.62	(no oil)	P	11.0	17.6	21.8	22.7	(whale oil,	P	10.2	18.8	26.7	26.6
		B	9.1	8.2	14.0	19.3	750 g.)	B	11.6	13.4	23.4	25.0
		A	41.1	45.9	57.3	76.1		A	43.0	36.2	40.1	42.2
9.5.62	(no oil)	P	11.0	12.4	16.0	23.4	(whale oil,	P	26.6	27.7	33.6	29.4
		B	7.8	11.5	10.9	15.8	750 g.)	B	11.5	11.9	11.5	11.4
		A	44.2	80.3	104.7	72.9		A	36.6	60.9	71.0	76.1
16.5.62	(no oil)	P	9.9	20.6	29.6	19.7	(no oil)	P	8.6	19.1	19.3	21.8
		B	9.9	10.3	19.5	20.3		B	5.9	12.9	12.9	14.4
		A	45.1	64.4	99.3	93.1		A	31.6	60.0	84.0	77.3
17.5.62	(whale oil, 750 g.)	P	7.2	20.5	27.7	26.2	(no oil)	P	7.6	17.7	24.5	21.4
		B	9.9	13.7	21.9	22.9		B	6.3	7.6	13.9	12.0
		A	49.2	46.8	57.3	55.2		A	33.2	57.0	73.3	70.0
18.5.62	(whale oil, 750 g.)	P	26.9	26.7	34.5	32.7	(no oil)	P	7.8	18.2	23.6	18.6
		B	19.8	12.7	15.4	12.9		B	6.6	7.7	17.1	11.7

* A - acetic P - propionic acid B - butyric acid

Table 15.

Concentration of P.E.G. in rumen liquor (g./l.) and the fluid volume of the rumen (l.) of twin cows as determined following the introduction into the rumen of 100 g. of P.E.G.

Experiment (iv)

	Period	Mean concentration (P.E.G.)	Standard errors of estimate	Concentration - time curve ($y=y_0e^{-kx}$)	Fluid volume of rumen
Cow a					
Day 1	(i)*	0.900	0.183	$1.74e.^{-.1723x}$	55.5
	(ii)**	0.183	0.010	$1.98e.^{-.2311x}$	48.5
Day 2	(i)	0.987	0.010	$1.63e.^{-.1291x}$	59.3
	(ii)	0.161	0.005	$1.45e.^{-2140x}$	67.0
Cow A					
Day 1	(i)	0.910	0.061	$1.92e.^{-.1994x}$	50.1
	(ii)	0.137	0.004	$1.24e.^{-.2148x}$	78.6
Day 2	(i)	0.883	0.090	$1.75e.^{-.1784x}$	55.1
	(ii)	0.113	0.016	$1.80e.^{-.2703x}$	53.5

* (i) - period between morning and afternoon feedings (3 samplings at 2 hr. intervals)

** (ii) - period following afternoon feeding (3 samplings at 2 hr. intervals)

Table 16.

Intake of ryegrass hay (kg. dry matter) by stall-fed twin cows with or without added linseed oil (500 g.)

Experiment (v)

Date	Oil Added	Morning		Afternoon		Total	
		Cow c	Cow C	Cow c	Cow C	Cow c	Cow C
Part (i)							
17.9.63	--	1.9	2.0	2.3	2.2	4.2	4.2
18.9.63	--	2.4	2.5	2.2	2.3	4.6	4.8
19.9.63	Linseed,500g.	2.2*	2.3	2.4	2.2	4.6	4.5
20.9.63	Linseed,500g.	1.8*	2.1	0.5	1.0	2.3	3.1
Part (ii)							
24.9.63	--	1.6	3.3	1.0	2.8	2.6	6.1
25.9.63	--	2.5	3.3	1.9	2.4	4.4	5.7
26.9.63	Linseed,500g.	2.2	2.6*	2.6	2.9	4.8	5.5
27.9.63	Linseed,500g.	2.8	2.1*	2.0	2.1	4.8	4.2

* Animal received oil

No consistent differences in the concentrations of ammonia were apparent following the addition of linseed oil to the rumen (Appendix III).

The pre-feeding levels of total VFA were in the range 51 - 86 m-mole /l., increasing to peak levels of from 81 to 109 m-mole/l. at 2 hr. after feeding began (Appendix IV). Concentrations of total VFA over the period of sampling (2 - 6 hr.) appeared to be slightly depressed by the addition of lipid, although the higher initial values obtained on the days subsequent to the addition of lipid may indicate that the fermentation was more prolonged.

The only noticeable difference in the concentrations of the individual volatile fatty acids following the infusion of lipid into the rumen was an increase in propionic acid, particularly on the second day of infusion (Appendix V). Before the addition of lipid, the concentration

Table 17. The digestion of cotton thread (% of original wt. which remained undigested) in the rumen of cows fed on ryegrass hay with or without the addition of 500 g. linseed oil

Experiment (v)

Date	Cow c	Location of thread in rumen	Time in rumen (hr.) 10	24	36	Cow C	Location of threads in rumen	Time in rumen (hr.) 10	24	36
Part (i)										
17.9.63	(no oil)	Dorsal	97.4	78.4	--	(no oil)	Dorsal	96.5	69.4	--
to			96.8	78.6	--			96.4	69.9	--
18.9.63										
		Ventral	97.1	76.3	--		Ventral	97.7	57.8	--
			97.3	74.3	--			98.8	59.2	--
19.9.63	(linseed oil	Dorsal	98.4	87.3	62.8	(no oil)	Dorsal	97.7	73.2	--
to	500 g.)		98.1	85.5	59.1			97.3	73.1	--
20.9.63										
		Ventral	100.0	71.2	47.8		Ventral	97.3	56.0	--
			99.1	73.3	44.8			98.3	51.7	--
Part (ii)										
24.9.63	(no oil)	Dorsal	97.7	90.3	79.6	(no oil)	Dorsal	101.4	76.3	--
to			98.1	90.6	76.1			99.9	69.8	--
25.9.63										
		Ventral	101.7	85.3	55.7		Ventral	103.2	54.2	--
			98.0	79.7	46.6			95.2	53.8	--
26.9.63	(no oil)	Dorsal	99.6	78.6	56.4	(linseed oil	Dorsal	97.5	84.5	53.7
to			98.0	80.1	55.3	500 g.)		96.6	85.1	55.1
27.9.63										
		Ventral	96.5	64.5	--		Ventral	97.1	71.9	--
			97.3	65.7	--			97.0	72.3	--

of propionic acid prior to feeding and 6 hr. after feeding was generally between 10 and 13 m-mole/l. rumen liquor. The levels at these times increased to 17 and 22 and to 19 and 16 m-mole/l. on the second day of infusion for cows c and C, respectively.

Effect of Lipid on Rumen Motility

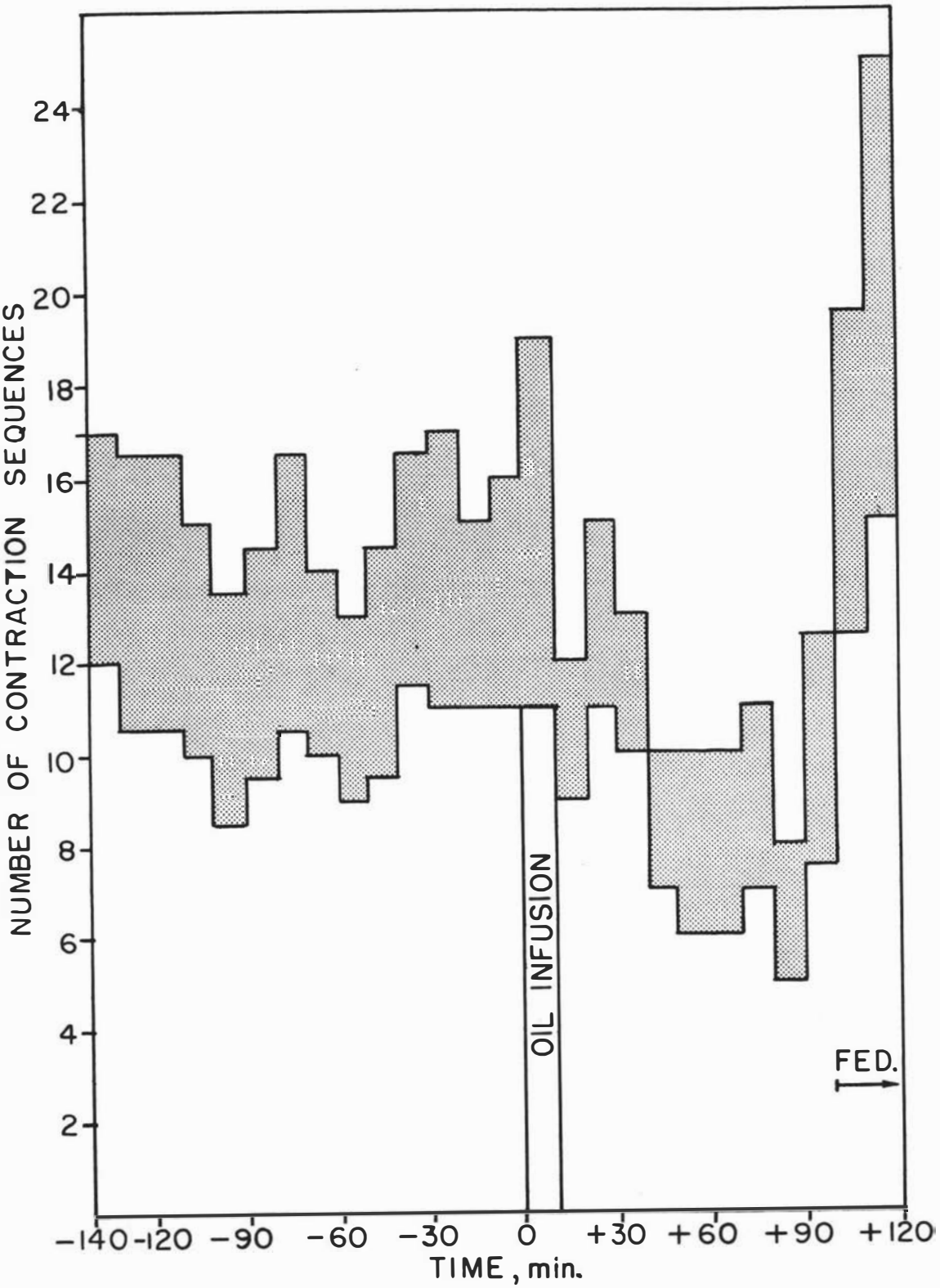
Two basic cycles of coordinated contractions occur in the reticulorumen. These will be referred to as "A" and "B" sequences following the nomenclature adopted by Reid (1963). The "A" sequence commences with a double contraction of the reticulum followed closely by a contraction of the rumen which begins in the anterior dorsal region and spreads to the posterior and ventral regions. "B" sequences begin with a contraction of the posterior ventral blind sac, spread anteriorly through the dorsal regions of the rumen and terminate with a contraction of the main ventral sac. Contractions of the reticulum are not associated with "B" sequences.

The effect of 200 g. of linseed oil, infused into the omasum, on the frequency of the two types of sequences is shown in Fig. 7. During the period before infusion (140 min.), the number of "A" and "B" sequences averaged 10.9 and 5.1/10 min., respectively, with standard deviations of $\pm$0.9 and $\pm$0.7. For the period following infusion until feeding commenced (90 min.), the average number of "A" and "B" cycles was 7.6 $\pm$ 2.0 and 3.7 $\pm$ 0.7/10 min. Little change in the frequency of "A" sequences occurred until 25 min. after infusion; hence, the large standard deviation for the post-infusion period. The number of "B" sequences decreased immediately after infusion.

Fig. 7. Variations in the number of coordinated contraction sequences of the reticulorumen before and after the infusion, over a 10 min. period, of 200 g. of linseed oil into the omasum

"A" sequences/10 min. 

"B" sequences/10 min. 


NUMBER OF CONTRACTION SEQUENCES
24
22
20
18
16
14
12
10
8
6
4
2
OIL INFUSION
FED.
-140 -120 -90 -60 -30 0 +30 +60 +90 +120
TIME, min.

Feeding resulted in a rapid increase in both types of contraction cycles but during the period recorded (20 min.), had little effect on the duration of each sequence. The time required to complete both types of sequences was similar, ranging from 22 to 28 sec.

"B" sequences were almost invariably associated with eructations both before and after infusion. Eructations also occurred either in conjunction with "A" sequences or independently of both. A sharp transitory increase in pressure, which reached a maximum almost simultaneously in all regions of the reticulorumen, characterized each eructation.

The following day, during the 60 min. in which pressure changes were recorded before the infusion of 500 g. of linseed oil, the number of "A" sequences averaged 10.7 $\pm$ 0.7*/10 min. (Fig. 8). Little apparent decrease in frequency occurred until almost 60 min. after infusion. During the next 70 min. the average frequency of contraction sequences was 6.75 $\pm$ 1.23*/10 min.

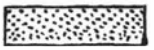
Due to mechanical failure of the transducers located in the main ventral sac and the dorsal posterior blind sac it was not possible to obtain recordings of pressure changes in these regions. Consequently, the frequency of "B" sequences could not be determined.

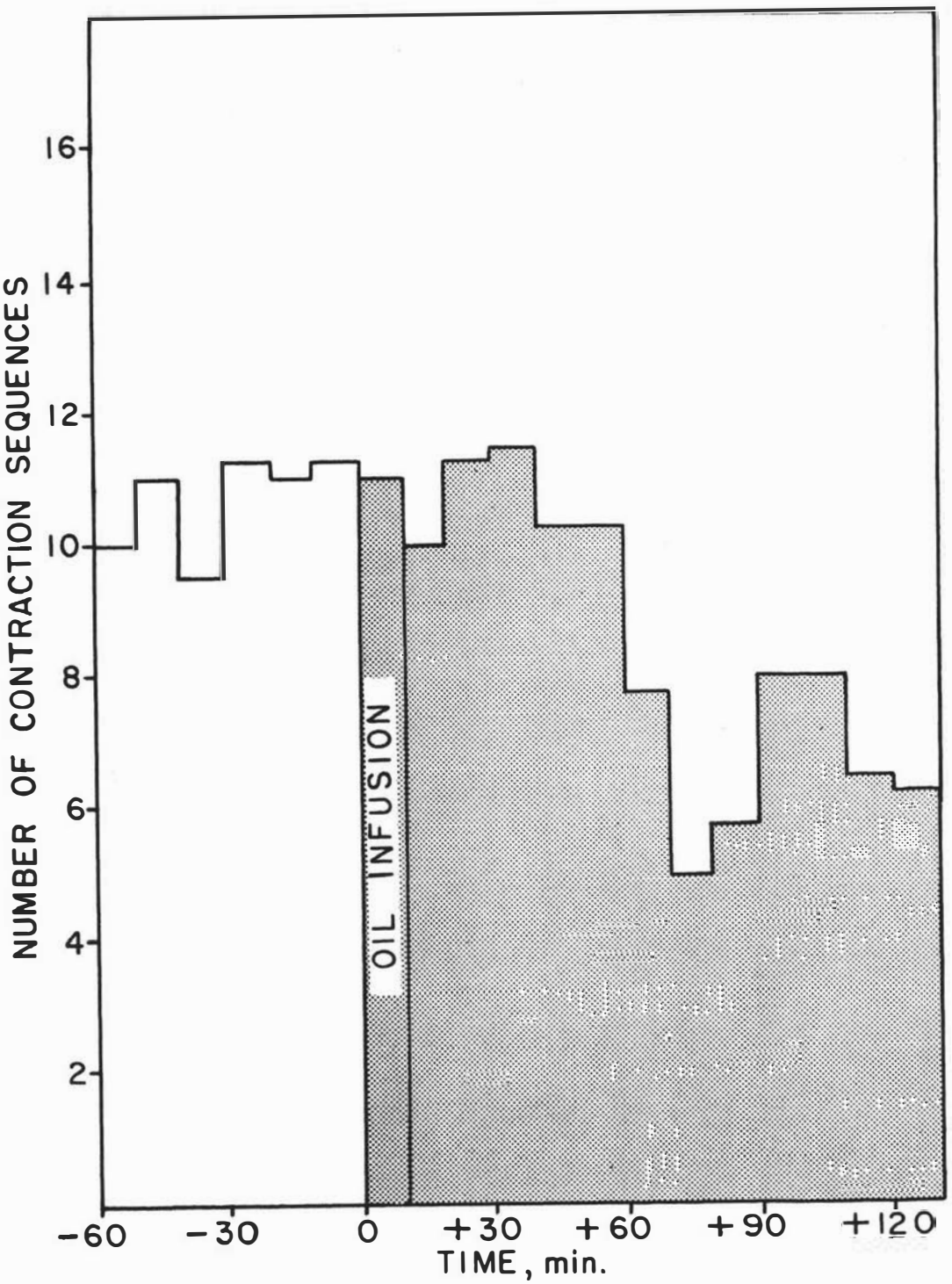
Rumination occurred intermittently prior to infusion on the second day only. It was characterized by the occurrence of an extra reticulum contraction just prior to the first of the diphasic contractions of the reticulum which normally initiate an "A" sequence. The commencement and

*standard deviation

Fig. 8. Variations in the number of coordinated contraction sequences of the reticulum before and after the infusion, over a 10 min. period, of 500 g. of linseed oil into the omasum

"A" sequences/10 min. before infusion 

"A" sequences/10 min. after infusion 


NUMBER OF CONTRACTION SEQUENCES
16
14
12
10
8
6
4
2
OIL INFUSION
-60
-30
0
+30
+60
+90
+120
TIME, min.

conclusion of each period of rumination was marked by an interval of 1 - 2½ minutes during which no major contractions of the reticulum or rumen occurred.

On both days of infusion, a short period of rumination commenced at the time when the rubber tubing was being inserted through the reticulo-omasal orifice and continued for up to 4.5 min. after the infusion was completed and the tube withdrawn. No further periods of rumination occurred during the post-infusion period on either of the two days.

Discussion

The addition of linseed oil generally resulted in increased concentrations of NH_3 in the rumen of pasture-fed animals. Increased levels of NH_3 were also found in the rumen by Arth, Kaufmann & Rohr (1966) following the addition of 0.5 or 1.0 kg. of tallow, coconut oil, palm oil or oleic acid to rations for dairy cattle. Unless the oil acted as a physical barrier to absorption it is unlikely that these increases were due to decreased absorption from the rumen since the higher pH values in the animals given oil compared with those for the controls, especially in experiment (ii), would have favoured an increase in the absorption rate (Coombe, Tribe & Morrison, 1960). Therefore, the increases in the concentration of NH_3 appear to have been due to an effect of oil on protein metabolism and would have occurred as a result of either increased deamination of the amino acids in the rumen or reduced utilization of NH_3 by the rumen microorganisms. Davison & Woods (1960) found that the addition of corn oil to rations high in roughage resulted in a decreased retention of nitrogen and from the present work, it appears that increased NH_3 levels in the rumen might have caused higher losses of urinary and faecal nitrogen. On the other hand, a decrease in NH_3 concentration occurred after the addition of whale oil. This was possibly due to the decrease in feed intake and, hence, protein intake, although decreases in intake did not have this depressing effect on NH_3 concentration when linseed oil was used. The relationship between NH_3 production and the amount of oil and protein ingested appears to be rather complex since Chalmers (1960) has found that when nitrogen levels in the feed were 3% of the intake of dry matter, the addition of oil to the ration depressed

rumen NH_3 concentrations but when nitrogen levels were 4.5% of the dry matter intake, increased NH_3 concentrations occurred. In the present experiments (i-iii) the nitrogen levels in the pasture were approximately 3 and 4.5% of the dry matter for the linseed and whale oil experiments, respectively. In experiment (v) in which linseed oil had no effect on NH_3 concentrations, the nitrogen level in the hay was 1.2% of the dry matter.

The high protein levels found in New Zealand pastures, particularly in late autumn and early spring (Johns, 1955 a) might be expected to lead to conditions of NH_3 toxicity. In the present experiments maximum NH_3 concentration in the rumen (60-65 mg./100 ml.) occurred at the time when pasture levels of protein were highest and of carbohydrate lowest. Although no toxic symptoms were observed, these levels were of the magnitude found by other workers (Head & Rook, 1955; Lewis et al., 1957) to cause an increase in the level of NH_3 in peripheral blood with eventual toxic effects on the animal.

The highly significant negative correlation coefficient between rumen pH and total VFA concentrations was slightly lower than that obtained by Bryant (1961). However, both values indicate that a close inverse relationship exists between these two variables.

In considering the effect of the metabolism of glycerides on the amounts of VFA produced, it can be calculated that complete hydrolysis of 500 and 750 g. of linseed oil and the quantitative fermentation of glycerol to propionic acid could account for increases in the concentration of propionic acid in the rumen of approximately 10 and 15 m-mole/l.,

respectively, (fluid volume as determined by the P.E.G. method was approximately 55 l.). In experiments (i) and (iii) increases in propionic acid concentration equalled or exceeded the calculated value. However, other workers (Johns, 1953; Garton et al., 1958) have shown that glycerol is fermented relatively slowly in the rumen and that the volatile acids, predominantly propionic, did not account for more than 50% of the glycerol metabolised (Garton et al., 1961). Therefore, very little of this increase can be attributed to the oil itself and appears likely to have been brought about by the effects of oil on the digestion of carbohydrates.

The most pronounced effect of added oil on the metabolism of carbohydrates was a decrease in acetic acid concentration in the rumen. Some of this decrease was undoubtedly due to the reduction in intake which occurred in varying degrees following oil infusion. However, in experiment (i) in particular, large reductions in the concentration of acetic acid occurred and were accompanied by only small reductions in feed intake. It was, therefore, probable that the oil had a direct effect on the metabolism of the carbohydrate constituents in the pasture. Previous work by Brooks et al. (1954), White et al. (1958) and Davison & Woods (1960) showed that cellulose digestion was reduced when corn oil was added to low quality rations. That the reduction in cellulose digestion would reduce concentrations of acetic acid in the rumen is supported by the results of many workers (Phillipson, 1952; el Shuzly, 1952; Gray et al, 1952; Annison, 1954; Balch & Rowland, 1957; Shaw, Ensor, Tellechea & Lee, 1960). Blaxter (1962) summarized these results by stating:

> "The fermentation of hexoses and the simpler carbohydrates results in some production of lactic acid; propionic acids

tends to be the most common of the fatty acids formed; and acids higher than butyric acid are produced. The less easily hydrolyzed carbohydrates yield no lactic acid, few acids higher than C_4, and the major end-products seem to be acetic and propionic acids, acetic acid predominating in most but not all instances."

Pure culture studies of the nutritional requirements and major fermentation end-products of rumen microbial species have added to our understanding of the reasons for these results. Bryant (1963) has compiled a list of nutritional features useful for identifying many of the culturable, anaerobic rumen bacteria. The bacteria which use cellulose as substrate, which include Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, and R. albus, produce acetic acid. Propionic acid is not a major fermentation end-product of any of these species.

The average fluid volumes of the rumen of cows a and A (57.6 and 52.9 l., respectively) were similar to that found by Hydén for a cow being fed hay (mean, 50 l.; range, 45-55 l.) but are considerably higher than the volume (approximately 32 l.) obtained by Reid (1965) for a cow fed freshly-cut red clover (Trifolium pratense). However, in the latter case, the animal was only fed once a day for a period of 2 hr. As a result, the intake of water in the feed was considerably lower than in the present experiment (20.0 and 37.3 kg. of water/day, respectively). When fed freshly-cut pasture (experiments i-iv) the cows seldom drank any additional water. Therefore, much of this difference in fluid volume was probably due to the difference in the volume of water ingested.

The coordinated contraction sequences of the reticulorumen followed patterns similar to those reported previously for cattle (Reid & Cornwall, 1959) and sheep (Reid, 1963).

Infusion of linseed oil into the omasum was followed by a reduction in motility of the reticulorumen. Titchen & Reid (1965) have indicated that a gradual depression in motility is normally associated with the change from a fed to a fasted condition. However, in the present experiment, the length of the fasting period and the similar frequency of contraction sequences observed before infusion indicate that the subsequent reduction was due to an effect of the oil. Similar results have recently been reported by Titchen, Reid & Vleig (1966) following rapid infusion (23 ml./30 min.) of linseed oil, a linseed oil hydrolysate, olive oil or oleic acid into the duodenum of sheep. However, the change in relative frequency of "A" and "B" sequences after infusion, observed by these authors, did not occur in this experiment. They found that the intensity of the depressing effect varied depending on the substance infused. Differences between animals were also observed. In the only specific example cited, these workers noted that a reduction in frequency of "A" sequences became apparent 37 minutes after the start of an infusion of linseed oil and continued for the next 150 minutes. This dose would be similar, per unit of body weight, to the 200 ml. of linseed oil administered to cow C during the first part of the present experiment.

In monogastric animals, motility of the stomach is known to be inhibited by the presence of lipid in the small intestine (Best & Taylor, 1961). Long chain fatty acids have been found to be more effective

inhibitors than triglycerides (Long & Brooks, 1965). This inhibition has been shown by several workers (Farrell & Ivy, 1926; Ivy & Gray, 1937) to be caused by a hormone, enterogastrone, produced in the mucous membrane of the duodenum and carried to the stomach by the blood.

No experimental data on the effects of enterogastrone preparations administered to ruminants have been reported in the literature. However, Hill (1965) has stated:

> "As in non-ruminant animals, the liberation of enterogastrone by the presence of fat in the duodenum is followed by complete or partial cessation of gastric secretion and inhibition of abomasal and forestomach motility."

If this statement is correct, the production of enterogastrone appears to be the most probable cause of the depression in rumen motility.

Summary

1. The infusion of linseed or whale oil into the rumen of pasture-fed twin cows resulted in marked reductions in intake, and in the concentrations of acetic and butyric acids. Concentrations of propionic acid, however, remained at or above the levels found during the pretreatment periods. The effect on NH_3 concentrations was variable.

2. The digestion of cellulose, as measured by the loss of weight of cotton threads suspended in the rumen, was reduced following the addition of linseed oil.

3. Added lipid also depressed rumen motility.

4. The fluid volume of the rumen was determined using polyethylene glycol as a reference substance.

Part of the material presented in this section has been published in the Journal of the Science of Food and Agriculture, 1964, 15, 274, 890.

Part 3. *In vivo* HYDROLYSIS AND HYDROGENATION OF LIPID

Materials and Methods

Treatment of Animals and Collection of Samples

The samples of rumen liquor on which the analyses described below were carried out were obtained from rumen-fistulated twin cows (pair b, B) during experiment (i) (Part 2). Consequently, the treatment of the animals and the method of obtaining the samples were as described on page 52.

Extraction of Lipid from Rumen Fluids

The lipid concentrations and compositional changes in the lipids were followed in total rumen fluids as obtained above and in the clear supernatant which was obtained by centrifugation of a further portion of the total rumen fluids for 30 min. at 14,600 g. Both the rumen fluids and the supernatant fraction were diluted with an equal volume of ethanol and evaporated to dryness at approximately 60° *in vacuo* in a rotary evaporator. The residue was extracted with diethyl ether and the extract washed several times with water to remove non-lipid impurities. The ether extract was then evaporated to dryness, the final stages being carried out *in vacuo*. The extraction procedure described by Hilditch (1956) was used to separate fatty acids from the neutral lipid.

Separation of Lipids by Thin Layer and Column Chromatography using Silicic Acid

Qualitative separations of rumen lipids into fatty acids, di-, and tri-glycerides were made on thin layers of Silica Gel G (Merck, Germany). Slurries of silicic acid were prepared by the usual technique (Mangold, 1961) and applied to glass plates by the method of Lees and De Muria

(1962). Petroleum ether-diethyl ether-acetic acid (90:10: 1, by vol.) light petroleum-diethyl ether-acetic acid (60:40:1, by vol.) and toluene-ethyl acetate-ethanol-acetic acid (10:5:5:0.1, by vol) solvent mixtures were used to develop the chromatograms and the lipids were detected under UV light after spraying with 0.2% 2', 7' - dichlorofluorescein in ethanol (Malins & Mangold, 1960).

Rumen lipids were also chromatographed on columns of silicic acid (Mallinckrodt) by the procedure described by Hirsch & Ahrens (1958) except that smaller columns were used. These columns (12 cm. x 1 cm.) of silicic acid (7 g.) did not separate triglycerides and fatty acids but enabled monoglycerides to be eluted in less than 500 ml. solvent. Triglycerides and fatty acids were separated after column chromatography by the usual solvent extraction procedures (Hilditch, 1956).

Separation and Determination of Fatty Acids

The lipid fractions were saponified by refluxing with ethanolic potassium hydroxide for 3 hr. and the non-saponified material was removed by ether extraction. The fatty acids were then converted to methyl esters by refluxing with 1% H_2SO_4 in methanol (Hilditch, 1956). These were analyzed by gas-liquid chromatography in a Pye Argon Chromatograph. To identify the methyl esters, R_F values were determined on 4 ft. x 4 mm. i.d. columns of two types, (a) 20% Apiezon M on Celite 545 (48 - 85 mesh) held at 200° and (b) 20% succinic acid-diethylene glycol on Celite 545 48 - 85 mesh) held at 150° (Hawke, Hansen & Shorland, 1959). The proportions of each component were determined by measurement of peak areas.

Results

Variations with Feeding in the Amounts of Lipid in Rumen Liquor

Experiments (i-iii) (Part 2) showed a regular pattern of changes in the levels of ammonia and volatile fatty acids in the rumen following stall-feeding on fresh pasture. Increases in the lipid content also occurred soon after feeding commenced (Table 18) in both the total rumen liquor and in the supernatant fraction. The lipid of the total rumen liquor would have included lipid from bacteria, protozoa, partly digested plant material and the rumen fluid. The rumen fluid did make some contribution to this increase but soon after the commencement of feeding, it is assumed that partly digested plant material would have been a major contributor. The lipid levels returned to approximately the pre-feeding levels in 6.5 hr. even when linseed oil was infused into the rumen. A large proportion of the linseed oil probably adhered to the solid ingesta, since, in later *in vitro* experiments (Part 4) in which 4 g. of linseed oil were added to 100 ml. of rumen liquor, it was found that approximately 80% of the oil became adhered to the solid material.

The proportions of free fatty acids in the total lipid of the supernatant varied considerably. High values (>35%) were obtained on two days and on one day very much lower values (8-28%) were obtained. In general, the free fatty acid levels increased in the period of feeding and a high level of lipase activity in the rumen was evident.

Fatty Acid Composition of Rumen Liquor

Palmitic, stearic, oleic, linoleic and linolenic acids were usually

Table 18. Lipid levels in rumen liquors (mg./100 ml.) obtained from twin cows fed on fresh pasture with and without the addition of linseed oil

Cow	Sampling time after commencement of feeding (hr.)	31.7.61		29.8.61		30.8.61*		12.9.61**	
		Total liquor	Supernatant[1]	Total liquor	Supernatant[1]	Total liquor	Supernatant[1]	Total liquor	Supernatant[1]
b	0	72	11 (61)	71	11	50	20 (8)	173	38 (74)
	2	108	19 (75)	132	23	464	148 (22)	216	42 (69)
	4	83	5 (40)	132	31	151	14 (27)	261	42 (71)
	6.5	55	7 (69)	100	18	-	10 (10)	105	34 (71)
B	0	68	7 (45)	66	14	34	12 (14)	165	30 (41)
	2	126	14 (57)	122	55	192	16 (28)	684	138 (69)
	4	53	16 (35)	96	14	135	19 (14)	104	48 (75)
	6.5	47	4 (50)	52	10	-	13 (10)	104	42 (86)

* 500 g. linseed oil added to the rumen of cow b after taking the first sample (0 hr.)

** 500 g. linseed oil added to the rumen of cow B after taking the first sample (0 hr.)

[1] % free fatty acids in brackets

the major fatty acid constituents of rumen liquor obtained from cows feeding on fresh pasture. Appreciable proportions of myristic acid were found on one occasion (Table 19) but otherwise 1.0 - 3.0% was the more usual proportion of this acid. In addition to myristic, palmitic and stearic acids, the remaining saturated fatty acids from C_{12} to C_{20} were present as minor constituents. Small proportions (<3.5%) of C_{15} and C_{17} branched-chain acids were also present in rumen liquor. In order to simplify the comparisons of major constituents, values for the minor constituents have not been presented in the tables. Fatty acids above C_{20} were not determined.

Table 19. Changes in the fatty acid composition of total rumen liquor after feeding fresh pasture (24.5 kg. to Cow B - 31.7.61)

Sampling time after commencement of feeding (hr.)	Fatty acids (moles %)					
	C_{14}	C_{16}	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
0	9.2	27.4	41.6	21.6	1.5	-
2	23.1	16.3	24.7	20.7	7.8	7.3
4	6.0	16.7	49.2	21.0	6.4	0.8
6.5	-	39.1	30.5	15.6	10.9	3.9

The proportions of the major fatty acid components, after an overnight fast, varied considerably (Tables 19 and 20). Stearic acid was quantitatively the most important fatty acid, followed by palmitic and oleic acids. The unsaturated fatty acids did not make a large contribution to the total fatty acids present and again appreciable fluctuations in the proportions of these acids occurred.

Table 20. Comparison of changes in the fatty acid composition of total rumen liquor from twin cows

(Cow b fed on fresh pasture (24.9 kg.) and linseed oil (500 g.) added to the rumen. Cow B fed on fresh pasture (36.3 kg.) - 30.8.61)

Sampling time after commencement of feeding (hr.)	Fatty acids (moles %)				
	C_{16}	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
Composition of linseed oil	6.1	2.9	16.7	17.6	56.7
Cow b (linseed oil added)					
0	23.8	32.9	26.4	12.2	4.7
2	6.7	3.4	17.0	18.7	54.2
4	8.0	9.6	33.9	29.2	19.3
6.5	10.6	18.3	37.9	28.9	3.3
Cow B					
0	23.0	54.5	6.1	12.3	4.1
2	17.0	57.2	13.8	4.5	7.5
4	18.1	44.7	19.1	8.6	9.5
6.5	31.2	45.2	17.2	6.2	0.2

Despite the considerable variability in the relative proportions of the major components, a distinct pattern of changes in composition was apparent with increased time after the feeding of fresh grass and clover. These changes were consistent with the known hydrogenation reactions in the rumen and, following a rise in the proportions of the unsaturated fatty acids after feeding, there was a progressive shift from linoleic to oleic. The data obtained from Cow B (Table 20), showed that there may be some delay in release of plant lipid as there were considerable increases in the proportions of palmitic acid 6.5 hr. after the commencement of feeding.

Four and 6.5 hr. after the infusion of linseed oil into the rumen there was a very rapid decrease in the proportions of linolenic acid, accompanied by increases in the proportions of linoleic, oleic and stearic acids as a result of *in vivo* hydrogenation. A decrease in the amount of lipid present in the rumen accompanied these changes (Table 18).

Fatty Acid Composition of Rumen Supernatant

Only small amounts of lipid were released into the supernatant after feeding on fresh forage alone (Table 18). At the conclusion of the feeding period, i.e. at 2 hr., increased proportions of oleic acid were present and at 4 hr. some linoleic acid also appeared (Table 21, cow B). In addition, appreciable proportions of myristic acid and some unidentified fatty acids of lower molecular weight (C_{12} - C_{14}) were present.

Even a short time after the infusion of linseed oil, only about one-third of the total lipid in the rumen liquor was in the supernatant fraction and the degree of unsaturation of the lipid in this fraction decreased rapidly, very little unsaturated fatty acid remaining at 6.5 hr. (Table 21, cow b).

Further examination of the lipids in the rumen supernatant 4 hr. after infusion of linseed oil into the rumen was carried out after fractionation into free fatty acid, triglyceride, diglyceride and monoglyceride on silicic acid columns (Table 22). Only 41% of the lipid eluted from these columns was triglyceride and hydrolysis products of triglyceride. The remainder was green and yellow pigment which was eluted with light petroleum and phosphorus - containing lipid, mixed with green pigment, which was eluted with methanol. Compared with the results of a similar experiment given in Table 21, the lipids showed a greater overall hydrogenation at 4 hr. The free fatty acid fraction was more saturated

Table 21. Comparison of changes in the fatty acid composition of the major constituents of the supernatant obtained from rumen liquor
(Samples corresponding to those in Table 20 for total rumen liquor)

	Sampling time after commencement of feeding (hr.)	Fatty acids (moles %)										
		C_{14}	C_{15iso}	C_{15}	C_{16}	$C_{16}^{1=}$	C_{17iso}	C_{17}	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
Cow b (linseed oil added)	0	2.2	2.2	1.6	35.5	-	0.5	1.6	51.6	3.2	1.6	tr.
	2	tr.*	-	-	14.0	-	-	-	12.0	22.0	16.0	36.0
	4	2.9	-	1.9	23.3	-	-	-	16.9	12.3	39.8	2.9
	6.5	6.4	3.8	5.1	35.9	-	-	-	46.2	tr.	tr.	tr.
Cow B	0	7.3	5.2	3.1	46.9	-	-	-	37.5	-	-	-
	2	14.8	2.9	3.4	41.2	1.0	1.0	1.5	31.3	2.9	-	-
	4	5.3	4.4	2.2	38.7	2.2	0.9	1.8	26.7	13.8	4.0	-
	6.5	9.4	4.7	4.0	43.7	tr.	-	1.3	27.5	9.4	tr.	-

* tr. = trace

Table 22. Fatty acid composition of triglycerides and the hydrolysis products of triglycerides in the supernatant of rumen contents four hours after infusion of linseed oil
(total lipid = 106 mg./100 ml. supernatant rumen fluid)

Lipid Fraction	Amount (wt.-%)	Fatty acids (moles %)					
		C_{16}	$C_{16}^{1=}$	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
Triglyceride	24.2	37.3	2.6	20.5	18.2	15.0	6.4
Diglyceride	13.3	29.1	2.5	30.4	24.0	14.0	tr.*
Monoglyceride	27.3	28.7	3.4	31.6	16.1	19.1	1.0
Fatty acid	35.2	29.1	2.1	49.0	14.6	4.9	2.3

* tr. = trace

than the triglyceride fraction, while the partially hydrolysed glycerides were of intermediate saturation. Despite being the least saturated of the four lipid components examined, the composition of the triglycerides was very different from that of the linseed oil infused into the rumen (Table 20), the most abundant fatty acids being palmitic and stearic acids with oleic, linoleic and linolenic acids in decreasing order of abundance.

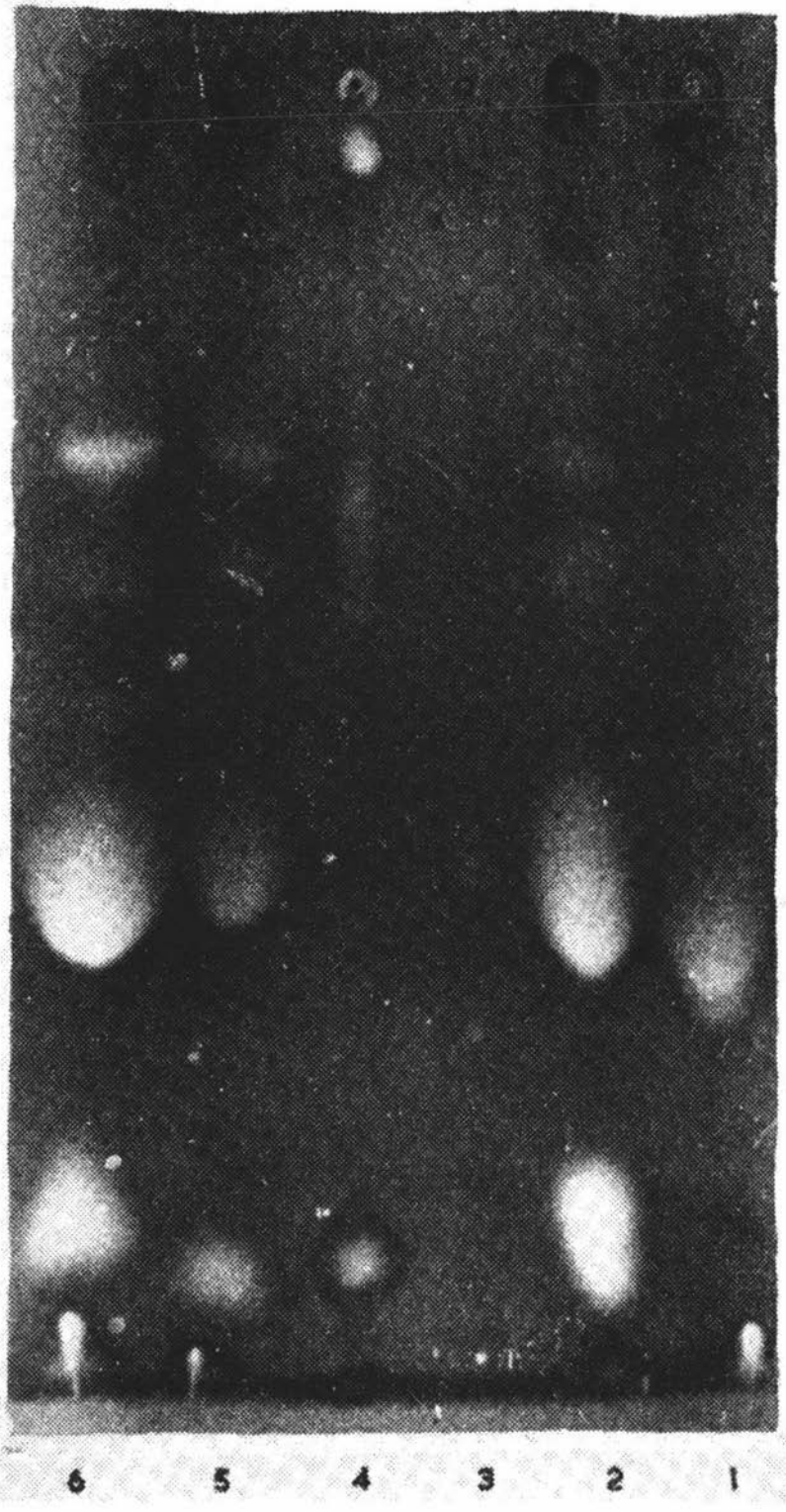
Qualitative Separation of Lipid by Thin Layer Chromatography

The presence of various lipids and hydrolysis products in the rumen supernatant obtained from cows on a grass and clover diet, with and without added linseed oil, was demonstrated by chromatographing lipid extracts on thin layers of silicic acid and spraying with 2', 7', -dichlorofluorescein. Fatty acids were found to be present before and after feeding grass and clover but diglycerides only after feeding. Triglycerides could not be detected, but unidentified fluorescent spots ran ahead of triglyceride at or near the solvent front in light petroleum-diethyl ether-acetic acid (60:40:1, by vol.). Fluorescent material which remained at the origin in this solvent was shown, by developing chromatograms with toluene-ethyl acetate-ethanol-acetic acid (10:5:5:0.1, by vol.),

to include mono- and di-galactolipid. Slower moving spots also present were probably partial hydrolysis products of galactolipids.

Fluorescent compounds corresponding to triglycerides and diglycerides were found in the rumen supernatant, in addition to fatty acids, after linseed oil had been added (Fig. 9). The intensity of the diglyceride spots increased with time over the 6.5 hr. sampling period. Monoglycerides could not be detected but green pigmented material which ran at the same rate as the monoglyceride standard could have masked the monoglycerides if they were present.

Fig. 9. Separation of neutral lipids from rumen liquor by TLC on silicic acid. From right to left: 1. pre-feeding sample; 2. sample 2 hr. after commencement of stall-feeding on mixed pasture and addition of 500 g. linseed oil; 3. oleic acid; 4. (in descending order) galactolipid, monopalmitin, α, α'-distearin, trimyristin; 5. 4 hr. sample; 6. 6.5 hr. sample. Developing solvent: light petroleum-diethyl ether-acetic acid (60:60:1 v/v). Detection with 0.2% 2', 7' - dichlorofluorescein in ethanol and photographed under UV illumination.


Gal. L. (origin)
M.G.
D.G.
F.A.
T.G.
6
5
4
3
2
1

Discussion

The wide variations in lipolytic activity of rumen fluids reported by other workers (Garton *et al.*, 1961; Wright, 1961; Allen *et al.*, 1959) have been found to occur in the present *in vivo* experiments and may be similarly attributed to variations in the microbial population and in the ability of the micro-organisms to metabolize lipid. Reasonable agreement was obtained between pairs of animals at the one time, but, despite a similar feeding regime, appreciable differences in the lipid levels and in the proportions of non-esterified fatty acids occurred between experiments. This may indicate that compositional changes in pasture plants influence lipid metabolism as well as affecting protein and carbohydrate metabolism (Part 2).

The rapid return of the lipid content of rumen liquor and rumen supernatant to pre-feeding levels, even when emulsified linseed oil was introduced, was also observed by Hoflund *et al.* (1956). It is unlikely that passage of the emulsion from the rumen would occur so rapidly and *in vitro* experiments (Part 4) showed that the rapid decrease in lipid levels in rumen liquor is probably due to the occlusion of lipid on particulate material in the rumen.

The presence of mono- and di-glycerides in the rumen after the infusion of linseed oil was of particular interest: Garton *et al.*, (1961) could not detect these intermediates of triglyceride hydrolysis after incubation of linseed oil with rumen liquor obtained from sheep being fed on hay and concentrates. Furthermore, diglycerides were also detected in rumen liquor obtained from a fistulated cow fed on fresh pasture alone; this may indicate a difference in metabolism in the rumen of

animals fed on fresh pasture and on hay and concentrates. Since mono- and di-galactolipids are the most abundant lipids in grasses and clovers (Weenink, 1959) and α and β -galactosidases have been found in rumen microorganisms (Conchie & Levvy, 1957) these lipids would seem to be the most likely precursors of diglycerides. However, in more recent *in vitro* experiments (Hawke & Weenink, pers. comm.) diglycerides could not be found among the hydrolysis products when galactolipids were incubated with rumen liquor.

In vitro experiments (Shorland *et al*, 1955; Garton *et al*, 1961) have shown that linolenic acid, the most abundant fatty acid constituent in pasture grasses and clovers (Weenink, 1959) in very rapidly hydrogenated by rumen micro-organisms. Because changes are being followed in a dynamic system, *in vivo* experiments are less satisfactory for following quantitative transformations between fatty acids. Nevertheless, the information obtained here does show that, in cows on a diet of fresh grass and clover, there is rapid hydrogenation in the rumen. Six and a half hours after the addition of 500 g. of emulsified linseed oil, above-normal proportions of linoleic acid persisted in the rumen but the proportions of linolenic acid had returned to about the pre-feeding level. Thus, it can be readily appreciated why variations in the nature and the amount of lipid in the normal diet of ruminants are not likely to affect greatly the composition of the depot and milk fat. However if, as suggested by Shaw *et al*, (1960), dietary changes can markedly alter hydrogenation activity, variations in the composition of pastures at different stages of growth could still be one of the factors responsible for the regular seasonal changes in the proportions of unsaturated fatty acids, principally

oleic acid, in the milk fat of grazing cows (Hawke, 1963). Furthermore, in view of this rapid hydrogenation within the rumen, the presence of 11.5% of linolenic acid in plasma lipids of pasture-fed heifers (Duncan & Garton, 1962) is unexplained.

The present work has confirmed the finding of Garton et al (1961) that fatty acids released from triglycerides by lipolysis undergo more extensive hydrogenation than triglyceride fatty acids. Compared with the composition of linseed oil, however, the triglycerides extracted from rumen liquor 4 hr. after the addition of linseed oil, were much more saturated; the proportions of the unsaturated fatty acids in partially hydrolysed lipid were not greatly different to those of the triglycerides. The presence of large proportions of palmitic acid lends support to the suggestion (Garton et al, 1961) that this fatty acid might be derived from the C_{18} fatty acids of linseed oil. Increases in the proportions of palmitic acid during the post-feeding period were also observed when cows were receiving a diet of grass and clover only. Gray, Pilgrim, Rodda & Weller (1952) could find no evidence for the synthesis of palmitic acid from short chain fatty acids, although this is a well-established route to palmitic acid in other biological systems (Wakil, 1961).

Summary

1. The hydrolysis and hydrogenation of grass and clover lipids and linseed oil have been followed in fistulated twin cows.

2. Mono- and di-glycerides, in addition to fatty acids, were found in the rumen liquor after infusion of linseed oil into the rumen.

3. The proportions of linoleic acid found in rumen liquor 6.5 hr. after infusion of linseed oil were above normal but the proportion of linolenic acid had returned to about the pre-feeding level in this time.

4. The free fatty acids were more saturated than the unhydrolysed triglycerides, but the proportions of unsaturated fatty acids in the partially hydrolysed glycerides were not greatly different to those of the triglycerides.

The material presented in this section has been published by the Journal of the Science of Food and Agriculture, 1964, 15, 283.

Part 4. EFFECT OF LIPID ON MICROBIAL ACTIVITY *in vitro*

Materials and Methods

Preparation of Rumen Liquor and Substrates

Non-lactating fistulated cows, which were fed indoors and fasted overnight before samples were taken, provided the source of rumen liquor. Rumen samples were strained through muslin and the liquor held in a vacuum flask (previously warmed to about 40°) during the setting up of the manometric apparatus.

Rumen liquors were obtained from cows fed on fresh grass (Lolium perenne) when soluble ryegrass constituents were used in the incubation experiments, whereas the cows were on a hay diet in the experiments with ryegrass fibre. These feeding regimes were chosen following the suggestion of Boda & Johns (1962) that selection of diet is necessary in order to decrease adaptation effects when investigating separately the fermentation of soluble and fibrous dietary constituents.

Plant juices and fibre were prepared by passing freshly-cut perennial ryegrass (Lolium perenne) through a Bentall 'Protess' extraction plant. The juices, which contained 9.8% dry matter, were frozen in 600 ml. batches and the fibre was washed in running tap-water for 3 hr. before it was freeze-dried. Both fractions were held at -25° until required.

Linseed oil and whale oil emulsions in water (50:50, w/v) were used as substrates along with plant juices or plant fibre.

Measurement of Fermentation Rates

Incubations of rumen fluids were carried out at 39 $\pm$ 0.5° in bottles of one pint capacity fitted with rubber stoppers; one lead through the stoppers was connected to a manometer (260 ml.) and the other was fitted with a valve to permit the addition of oil emulsion and plant juices and

the acidification of the contents (Boda & Johns, 1962). To ensure release of dissolved CO_2, gas volumes were measured after acidification with 4 ml. 10 N-sulphuric acid at the conclusion of the incubation period.

Incubation of Rumen Fluids with Lipid and Grass Juice

Eight bottles were used in each experiment and were placed on shaker racks in the water bath at 39°. Rumen fluid (100 ml.) obtained from a cow feeding on freshly-cut grass was added to each bottle and the bottles were gassed for one minute with a mixture of 95% N_2/5% CO_2. Ryegrass juice (20 ml.) was added to six bottles and water (20 ml.) to the remaining two bottles which acted as blanks. The appropriate volume of oil/water emulsion (2, 4 or 8 ml.) was added to three of the six bottles containing ryegrass juice and an equal volume of water added to each of the two blanks and the remaining three bottles which contained ryegrass juice. The bottles were then shaken momentarily by hand to ensure adequate mixing and the manometers adjusted to zero.

One of the blanks was acidified at zero time and the other at 3 hr. The difference in manometer readings between the two blanks measured the gas produced by fermentation of material present in the rumen liquor. Of the remaining bottles, one with added oil and one without added oil were incubated for 1, 2 and 3 hr. respectively. Any difference in these pairs measured the effect of added oil on the metabolic acitivity of the rumen micro-organisms.

Incubation of Rumen Fluids with Lipid and Grass Fibre

The effect of lipid on the fermentation of plant fibre by rumen fluids was determined in a similar manner to that described above.

Because the amounts of volatile fatty acids formed were likely to depress the pH of rumen liquor over a considerable portion of the 48 hour experimental period, a bicarbonate/phosphate buffer (pH 7.0) which was similar in composition to saliva (McDougall, 1948), was used. One g. of washed ryegrass fibre, 100 ml. of rumen fluid, obtained from a cow on a hay diet, and 30 ml. of buffer were incubated with or without 4 ml. linseed oil emulsion for 12, 24 and 48 hr.

Analytical Methods

Ammonia, total volatile fatty acids, proportions of individual volatile and non-volatile fatty acids in rumen liquor, and soluble sugars in the grass extracts were determined as described previously (Parts, 1, 2 and 3). A measure of the effect of lipid on fibre digestion in 48 hr. under *in vitro* conditions was obtained by determining the residual crude fibre (A.O.A.C., 1960) before and after fermentation. Total nitrogen was determined by the Kjeldahl method (A.O.A.C., 1960).

Results and Discussion

Effect of Lipid on the Fermentation of Soluble Ryegrass Constituents

A series of preliminary experiments were conducted in each of which eight bottles (two containing 100 ml. of rumen liquor and 20 ml. of water and six containing 100 ml. of rumen liquor and 20 ml. of ryegrass juice) were used. Two of the units containing ryegrass juice were acidified after incubation for periods of 1, 2 and 3 hr. Excellent agreement was obtained between duplicates in terms of ammonia concentrations and of gas and total volatile acid production (Table 23). Therefore, although direct comparisons between experiments are not valid due to the variation in the initial values for rumen liquor, differences between the values obtained for the control and experimental units (i.e. no lipid vs. lipid) within each of the later experiments should indicate an effect of the addition of lipid.

The addition of ryegrass juice to rumen liquor considerably increased the amount of fermentation products (Table 24). It is likely that the soluble sugars present in the ryegrass juice (34 mg./ml. extract) were primarily responsible for the increase in gas and VFA over the 3 hr. incubation period. Similarly, the ryegrass juice which was high in crude protein (17.7 mg./ml.) produced approximately twofold increases in NH_3 concentration.

Despite standardization of the feeding regime and of the sampling time, the rumen liquors before incubation contained between 4.0 and 6.7 mg. of NH_3/100 ml. of rumen liquor and 38.9 - 68.6 m-moles of volatile acids/ l. of rumen liquor (Table 24). Similarly, differences in the concentrations of NH_3 and of total VFA were found on several occasions after incubation of rumen liquor with ryegrass extract. This would appear to be due to day-to-

day variations in either the total number of organisms or in the relative proportions of different microbial species (Wilson & Briggs, 1955).

Table 23. The formation of gas, NH_3 and VFA from rumen liquor incubated with ryegrass juice

	Incubation time (hr.)	9 / 4 / 62			11 / 4 / 62			18 / 4 / 62		
		B*	C**	C**	B	C	C	B	C	C
Gas production (ml.)	0	160	160	160	97	97	97	147	147	147
	1		185	186		140	137		190	194
	2		201	205		158	159		215	213
	3	176	211	213	124	174	172		230	230
NH_3 (mg./100 ml.)	0	10.13	10.13	10.13	14.61	14.61	14.61	17.17	17.17	17.17
	1		21.93	21.86		14.61	24.37		29.91	29.95
	2		24.47	24.51		30.34	30.42		33.46	33.71
	3	15.5	28.19	28.30	19.1	34.57	34.60	22.5	37.23	36.97
VFA (m-mole/l)	0	53.3	53.3	53.3	71.2	71.2	71.2	64.2	64.2	64.2
	1		67.9	68.3		85.7	85.2		83.8	83.2
	2		74.0	74.1		93.9	94.2		92.0	92.1
	3	56.6	78.8	78.2	70.4	101.1	101.5	64.0	99.4	99.0

* = blank, rumen liquor only

** = controls, rumen liquor and ryegrass juice

The incubation of 1, 2 and 4 g. of linseed or whale oil with rumen liquor had no appreciable or consistent effect on the rate of fermentation as measured by gas production (Table 24). Thus, the rate of fermentation of soluble ryegrass constituents by rumen organisms *in vitro* appeared to be little affected by lipid.

Table 24. The formation of gas, NH_3, and total VFA from rumen liquors incubated with linseed oil or whale oil in the presence of ryegrass juice

	Incubation time (hr.)	Linseed oil added									Whale oil added								
		-	-	1g.	-	-	2g.	-	-	4g.	-	-	1g.	-	-	2g.	-	-	4g.
		B*	C**	E***	B	C	E	B	C	E	B	C	E	B	C	E	B	C	E
Gas production (ml.)	0	75	75	75	165	165	165	156	156	156	103	103	103	135	135	135	168	168	168
	1		128	130		229	224		220	-		157	161		201	200		210	212
	2		164	159		261	256		253	254		183	182		224	223		223	228
	3	90	194	192	184	277	286	167	272	278	116	215	215	153	248	246	178	252	-
NH_3 (mg./ 100 ml.)	0	4.01	4.01	4.01	6.65	6.65	6.65	6.45	6.45	6.45	5.80	5.80	5.80	6.03	6.03	6.03	5.89	5.89	5.89
	1		10.27	10.48		13.66	13.35		12.68	-		12.71	13.20		13.12	14.90		10.02	10.63
	2		13.99	14.90		17.83	18.07		16.61	16.08		15.69	15.85		16.50	16.22		11.68	11.71
	3	7.56	15.83	16.17	9.76	20.14	19.75	8.19	17.28	18.70	8.67	17.72	18.62	8.61	17.97	18.68	7.23	13.68	13.80
VFA (m-mole/l)	0	68.6	68.6	68.6	41.3	41.3	41.3	41.7	41.7	41.7	50.4	50.4	50.4	49.8	49.8	49.8	38.9	38.9	38.9
	1		90.5	93.0		63.1	63.6		63.0	-		70.0	71.4		71.7	72.9		46.9	51.7
	2		105.5	104.4		76.8	78.3		76.9	77.0		81.9	80.4		82.2	81.5		63.1	65.1
	3	74.5	115.0	114.1	55.1	84.1	83.2	46.3	81.8	84.6	53.4	89.9	92.8	55.3	92.0	90.9	42.7	72.5	73.0

* = blank, rumen liquor only.

** = control, rumen liquor and ryegrass juice.

*** = experimental, rumen liquor, ryegrass juice and oil.

Concentrations of NH_3 in the rumen liquors incubated with lipid were generally higher than those for the controls. Increasing the amount of lipid from 1 to 4 g. did not appear to accentuate this effect. The increases in NH_3 concentration, although small, lend support to the findings of earlier *in vivo* work (Part 2) in which linseed oil produced irregular increases.

After 1 hr. the concentrations of total VFA in the presence of lipid were from 0.8 to 10.2% higher than those for the controls although after 3 hr. no differences were apparent. The effect of either linseed or whale oil on acetic acid and butyric acid concentrations was small and variable (Table 25), but concentrations of propionic acid were from 2.2 to 9.4% higher when lipid was added. In the short incubation times used it is unlikely that glycerol, produced on hydrolyses of lipid, would have made a significant contribution to the increase in propionic acid (Garton *et al*, 1961).

The addition of 2 g. of lipid to 100 ml. rumen liquor and 20 ml. ryegrass juice gave a lipid concentration comparable to the addition of about 900 g. lipid to a rumen with a fluid volume of 55 l. (Part 2). Consequently, the absence of any marked differences in gas production, NH_3 or total VFA concentrations in these short-term incubations suggests that the variations found previously when lipids were added to the rumen were not due to a decreased activity of the rumen micro-organisms in the fermentation of soluble constituents which are present in ryegrass juices.

Hydrogenation of Linseed Oil by Rumen Liquor

During the short-term incubation periods used it was found that the activity of the rumen micro-organisms changed the proportions of the fatty

Table 25. Concentrations of acetic, propionic and butyric acids (m-mole/l.) formed on incubation of rumen liquor with linseed oil or whale oil

	B*	C**	E***	B	C	E	B	C	E
	0 hr.	3 hr.	3 hr.	0 hr.	3 hr.	3 hr.	0 hr.	3 hr.	3 hr.
Linseed oil	-	-	1g.	-	-	2g.	-	-	4g.
Acetic	51.4	79.6	77.1	30.6	61.7	61.7	31.1	60.0	61.2
Propionic	11.2	23.5	25.1	5.9	14.8	15.2	6.0	14.9	16.3
Butyric	6.0	10.1	11.9	4.8	7.6	6.3	4.6	6.9	7.1
Whale oil	-	-	1g.	-	-	2g.	-	-	4g.
Acetic	36.3	62.5	66.4	37.1	64.1	62.1			
Propionic	7.3	18.2	19.1	6.8	18.0	18.4			
Butyric	6.8	7.7	7.3	5.9	9.9	10.4			

* = Blank, rumen liquor.

** = Control, rumen liquor and ryegrass juice.

*** = Experimental, rumen liquor and ryegrass juice and oil.

acids originally present in linseed oil (Table 26). Hydrogenation of each of the tri-, di-, and mono-ethenoid C_{18} acids had occurred but the greatest changes involved the more abundant linolenic acid. Reductions of 11 and 5% in linolenic acid occurred in the supernatant and solid fractions of the rumen fluids, respectively, between 1 and 3 hr. after the addition of oil. The increases in the proportions of the dienoic and monoenoic acids indicate that the more unsaturated acids were preferentially hydrogenated. Incubations for 24 hr. of linseed oil and rumen liquor carried out by Garton *et al.* (1961) resulted in a much greater degree of hydrogenation, the proportion of C_{18}-trienoic acid decreasing from 54.5 to 5.1% of the total fatty acids. Hydrogenation appears to be more rapid *in vivo*, because very little C_{18}-trienoic acid remained 6.5 hr. after infusion of linseed oil (Part 3).

Separation of the rumen liquor into a supernatant liquid and a residue showed that about four-fifths of the added lipid became occluded on the small particles present in the strained rumen liquor (Table 26). It seems that a similar occlusion of oil to particulate material occurs in the rumen (Part 3).

Before the addition of lipid, rather more palmitic acid and less stearic and linolenic acids were found in the residue than in the supernatant liquid. However, after the addition of linseed oil the differences in the proportions of the major fatty acid components in these two fractions were small and largely within analytical error. The residual fractions, probably representative of the composition of the micro-organisms present, contained higher proportions of the branched-chain and odd-carbon fatty acids. In order to facilitate comparisons of the major fatty acid components, the amounts of these acids which were present only in traces in the samples

Table 26. The amounts (g.) and composition (moles %) of lipid in fractions of rumen liquor before and after incubation of strained rumen contents with linseed oil

Rumen liquor (100 ml.) with and without 4 ml. linseed oil*

	Supernatant				Residue			
	Rumen liquor alone	Rumen liquor and lipid Time of incubation (hr.) 1	2	3	Rumen liquor alone	Rumen liquor and lipid Time of incubation (hr.) 1	2	3
Wt. of lipid (g.):-	0.072	0.706	0.655	0.711	0.076	2.718	2.719	2.343
Fatty acid								
C_{12}	2.1				1.3			
C_{13}^{br}	0.7				1.0			
C_{13}	1.3				0.4			
$C_{14}^{1=or\ br}$	0.4				1.7			
C_{14}	2.9	tr.	tr.	0.8	4.8	0.4	tr.	0.7
C_{15}^{br}	0.8				7.8			
C_{15}	0.6				5.4			
$C_{16}^{1=}$	0.2			0.8	2.5			
C_{16}	24.8	9.8	9.2	9.2	30.3	8.7	8.6	8.9
C_{17}^{br}	0.6				2.7			
C_{17}	1.0				2.7			
$C_{18}^{3=}$	13.0	47.4	45.8	42.3	5.6	49.0	48.8	46.6
$C_{18}^{2=}$	5.9	14.4	13.9	16.1	5.8	14.8	14.6	16.1
$C_{18}^{1=}$	11.8	22.6	24.8	24.6	11.3	21.5	23.3	22.2
C_{18}	33.7	5.8	6.3	6.2	16.7	5.6	5.7	6.5

* = Fatty acid composition of linseed oil (moles %): C_{16}, 6.1; $C_{18}^{3=}$, 56.7; $C_{18}^{2=}$, 17.6; $C_{18}^{1=}$, 16.7; C_{18}, 2.9.
br. = branched; tr. = trace.

incubated with linseed oil are not recorded in Table 26.

Effect of Lipid on the Fermentation of Grass Fibre

Over a 48 hr. period, gas production from 100 ml. rumen liquor in the presence of 1 g. ryegrass fibre was 2 to 3 times greater than endogenous fermentation. Ryegrass fibre produced very much slower rates of fermentation than ryegrass juice when incubated with rumen liquor (Tables 23 and 27).

Gas production was slightly higher in the presence of 2 g. of linseed oil at 12 and 24 hr. but was lower at 48 hr. In two experiments, the respective weights of residual crude fibre after 48 hr. incubation with lipid were 21.3 and 7.2% of the initial weight of crude fibre added, compared with 16.9 and 1.6% without lipid. Linseed oil depressed ammonia production at 48 hr. (Table 27). This effect is opposite to that found in the presence of soluble constituents (Table 23).

Consistent with the differences in residual crude fibre, added lipid slightly depressed the levels of total volatile fatty acids produced over 48 hr. (Table 27) although the formation of VFA was faster and produced slightly higher levels at 12 and 24 hr. However, the concentrations of acetic and propionic acids at 48 hr. were markedly affected (Table 28) with decreases in acetic acid (10 - 12 m-mole/l.) being approximately balanced by increases in propionic acid (8 - 11 m-mole/l.). The concentrations of butyric acid were little affected.

The longer incubation periods used in these experiments probably allowed sufficient time for a substantial amount of hydrolysis of the linseed oil. Complete hydrolysis of 2 g. lipid and a quantitative conversion of the glycerol moiety to propionic acid would account for an increase in the concentration of propionic acid of slightly less than 0.30 m-mole/l. The observed increases (8.5 and 11.3 m-mole/l.) far

Table 27. The formation of gas, NH_3 and total VFA from rumen liquor incubated with linseed oil in the presence of ryegrass fibre

	Incubation Time (hr.)	Experiment (i)			Experiment (ii)		
		B*	C**	E***	B	C	E
Gas Production	0	124	124	124	104	104	104
	12	-	191	205	-	166	179
	24	-	252	261	-	228	236
	48	186	294	272	165	287	252
NH_3 (mg./100 ml.)	0	5.73	5.73	5.73	4.57	4.57	4.57
	12	-	15.41	14.94	-	11.53	13.71
	24	-	20.46	20.05	-	21.96	24.01
	48	23.73	34.03	27.01	28.23	37.58	28.92
Total VFA (m-mole/l.)	0	56.0	56.0	56.0	66.2	66.2	66.2
	12	-	80.6	86.5	-	92.1	94.9
	24	-	101.5	104.8	-	111.6	114.4
	48	74.5	115.8	115.4	87.3	128.3	125.0

* = blank, rumen liquor.

** = control, rumen liquor and fibre.

*** = experimental, rumen liquor, fibre and 2 g. lipid.

Table 28. Concentrations of acetic, propionic and butyric acids (m-mole/l.) formed on incubation of rumen liquor with 2g. linseed oil in the presence of ryegrass fibre

		B*	B*	C**	E***
Time of incubation (hr.): -		0	48	48	48
Experiment (i)	Acetic	40.5	51.8	80.9	70.7
	Propionic	9.2	13.0	20.0	31.3
	Butyric	6.3	9.8	14.8	13.4
Experiment (ii)	Acetic	49.2	64.2	92.9	80.4
	Propionic	9.7	12.6	20.0	28.5
	Butyric	7.3	10.6	15.4	16.1

* = Blank, rumen liquor.

** = Control, rumen liquor and ryegrass fibre.

*** = Experimental, rumen liquor, ryegrass fibre and linseed oil.

exceeded the theoretical value and must therefore have been due to an effect of lipid on carbohydrate digestion.

The results of the present in vitro experiments with linseed oil and ryegrass juice extracts high in protein (20 ml. ≡ 350 mg. crude protein) are consistent with the results of earlier work (experiments (i and ii), Part 2) which showed that the addition of linseed oil to cows feeding on pastures high in protein - N increased NH_3 concentrations in the rumen. However, linseed oil produced the opposite effect when juice extracts were replaced by ryegrass fibre (1 g. ≡ 200 mg. crude protein). This relationship found in vitro between the level of substrate protein and the effect of lipid on NH_3 concentration is similar to that reported by Chalmers (1960) and Jayasinghe (1961) who found that when nitrogen levels in the feed were 3% of the dry matter, the addition of lipid resulted in

a depression in the concentration of NH_3 in the rumen whereas at nitrogen levels of 4.5%, the added lipid resulted in an increased concentration of NH_3. Despite the agreement between our *in vitro* experiments and the *in vivo* work of others, the present *in vivo* experiments with ryegrass hay containing 1.2% N (with and without lipid), (experiment v, Part 2) did not show any consistent differences in the concentrations of NH_3.

That the utilization of soluble plant constituents is little affected by lipid is confirmed by the *in vitro* experiments with plant juice extracts in which only slight differences in the concentration of volatile fatty acids and in the volume of fermentation gases were observed in the presence and absence of lipid. On the other hand, the present *in vitro* and *in vivo* investigations with ryegrass fibre and cotton thread respectively, showed that lipid (16.7 g./l. *in vitro* and 10.9 g./l. *in vivo*) depressed digestion of fibrous carbohydrates. *In vitro* this effect was accompanied by a decrease in the level of acetic acid which is normally the predominant acid formed by the fermentation in the rumen of feeds high in cellulose (Balch, 1960). From this and earlier work it appears that reductions in the digestion of cellulose by rumen micro-organisms can be brought about by lipid under a variety of dietary conditions. Increased formation of propionate in the presence of lipid also appears to be related to the digestion of the fibrous rather than the soluble constituents of pasture species.

Summary

1. In the anaerobic incubation of strained rumen liquors at 39°, lipids increased the formation of ammonia from ryegrass juice high in protein - N, but had the opposite effect when the juice extract was replaced by ryegrass fibre low in protein - N.

2. Lipid decreased the digestion of fibrous ryegrass constituents.

3. Increased formation of propionic acid also occurred in the presence of lipid. Formation of acetic acid was reduced by lipid when ryegrass fibre was fermented.

4. The high rates of fermentation of the constituents of ryegrass juice were not affected by lipid and were accompanied by rapid hydrogenation of the constituent unsaturated fatty acids of the lipid itself.

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Part 5. EFFECT OF ADDED CARBOHYDRATE ON METABOLIC ACITIVITY *in vivo* AND *in vitro*

Because the type and relative amounts of dietary protein and carbohydrate regulate the ammonia concentration and presumably the extent of microbial synthesis in the rumen (Lewis & McDonald, 1958), the high concentrations of ammonia frequently found in the rumen of animals grazing on pasture has led to speculation on the efficiency of utilization of nitrogenous constituents of pasture species (Barnett & Reid, 1961). The concentrations of sugars in grasses and clovers are influenced by factors such as stage of growth and climate (Mackenzie & Wylam, 1957; Bailey, 1958; Hirst, Mackenzie & Wylam, 1959) and accompanying fluctuations in the levels of protein and other nitrogenous constituents result in considerable variation in the soluble carbohydrate/protein ratio throughout a growing season, with low values in early spring and in late autumn when growth is rapid (Johns, 1955).

The significance of the variation in the protein level during a growing season is difficult to ascertain in a seasonal study because of the introduction of uncontrollable factors. Furthermore, the influence of the physical and chemical properties of a protein on its rate of utilization makes supplementation of pasture protein with protein from another source an unreliable guide to the effects of variations in the level of protein in pasture on rumen digestion. Paired feeding experiments in which the protein intake was varied by growing fresh fodder with different nitrogen levels, provided a method for simulating natural variations in pasture protein and examining the effects of carbohydrate supplementation. Starch was infused into the rumen to examine carbohydrate supplementation because it had been found to increase the rate of disappearance of ammonia in the rumen of cows on hay and concentrate diets and to improve the conversion of urea to protein (Belasco, 1956).

The role of soluble sugars in the conversion of ryegrass protein to bacterial protein was followed *in vitro*. Ryegrass extract served as an appropriate protein substrate.

Materials and Methods

Treatment of Animals for in vivo Experiments

Lactating twin Jersey cows (pair a A) with rumen fistulas, which were being stall-fed twice daily (9:30 a.m. and 4:30 p.m.) on freshly-cut white clover (Trifolium repens)- perennial ryegrass (Lolium perenne) pasture were used to investigate the influence of readily fermentable carbohydrate on the utilization of dietary protein as measured by ammonia levels in the rumen. One-half of the available pasture had previously been top-dressed with ammonium sulphate at the rate of 224 kg./hectare. After a preliminary period of 9 days, during which Cow a was fed top-dressed pasture and cow A normal pasture, 500 g. of commercial cornflour (wheat starch) was infused, as a 50:50 aqueous suspension, into the rumens of both animals during the morning (9:30 - 11:30 a.m.) and afternoon (4:30 - 6:30 p.m.) feeding periods for two consecutive days (experiment i). Rumen samples were collected immediately before the morning feeding period and at regular intervals after both feeding periods on the day before the infusion of starch and on the two days of infusion.

After the second day of infusion, cow a was fed normal pasture and cow A fed top-dressed pasture for a period of 14 days. Both animals then received 900 g. starch twice daily for two consecutive days (experiment ii), with infusion and sampling times as described above.

Treatment of Animals for the in vitro Measurement of Fermentation Rates

A non-lactating Jersey cow with a rumen fistula, which was being stall-fed on freshly-cut red clover (Trifolium pratense L.) and fasted

overnight before sampling, provided the source of rumen liquor. The collection of rumen fluids, preparation of ryegrass juice extract, and the measurement of fermentation rates at 39° were as described previously (Part 4).

Incubation of Rumen Liquor with Carbohydrates

In the first series of experiments, the eight bottles of one pint capacity used as experimental vessels were arranged as follows: two blanks containing rumen liquor (100 ml.) and water (20 ml.); two or three controls containing rumen liquor (100 ml.) perennial ryegrass juice (10 ml.) and water (10 ml.); three or four bottles containing rumen liquor (100 ml.), perennial ryegrass juice (10 ml.) and carbohydrate solution (10 ml.) which contained either 0.1 or 0.2 g. of carbohydrate. In order to determine gas formation, one of the blanks was acidified at zero time and the remaining bottles after incubation for 3 hr.

In the second series of experiments, four bottles were used as controls (rumen liquor, 100 ml.; ryegrass juice, 10 ml.; water, 10 ml.) and three with 10 ml. water replaced by 10 ml. of a 10% solution of sugar. As the supply of α-D-galacturonic acid was limited, 10 ml. of a 5% solution were used. One of the controls was acidified at zero time and one control and one containing sugar after incubation for 0.5, 1.5 and 3 hr.

Ammonia and VFA concentrations were measured after acidification in all experiments.

Analytical Methods

Moisture: 100 g. freshly-cut herbage was chopped into 1-2" lengths and dried in a forced-draught oven on aluminium trays at 100° for 16 hr.

Crude protein: Crude protein was determined as described previously (Part 2).

Non-protein nitrogen: 5 g. of dried herbage were boiled with 250 ml. 80% ethanol for 3 min. and filtered. The filtrate was evaporated to dryness on a water-bath and re-dissolved in 10 ml. of water (Bathurst & Allison, 1949). Total nitrogen was determined on a 5 ml. aliquot by the Kjeldahl method (A.O.A.C., 1960).

Ammonia and total VFA in the rumen liquors and total nitrogen and soluble sugars in the grass juice extracts were determined as described previously (Parts 1 and 3).

Separation and identification of soluble sugars in grass juice extract: An aliquot (approximately 100 ml.) of the grass juice extract was centrifuged at 40,000 g. for 30 min. to remove suspended particulate material (Bailey, 1958). Soluble sugars in the supernatant were then separated by chromatography on either Whatman No. 1 or No. 3 MM paper. The most useful eluting solvent for the separation of glucose, galactose, mannose, arabinose, ribose and fructose was ethyl acetate - acetic acid - water (3:1 :3, by vol.). p-Anisidine hydrochloride was used as the spray reagent for pentoses and hexoses (Hough, Jones & Wadman, 1950) and urea phosphate for ketoses (Wise, Dimler, Davis & Rist, 1955).

Quantitative estimation of sugars: The concentrations of the various sugars in the ryegrass juice extract were estimated after purification by chromatography (Pridham, 1956) on Whatman No. 3 MM paper. A volume of 0.003 - 0.015 ml. of supernatant prepared from ryegrass juice was applied to the paper as spots by an 'Agla' micrometer syringe, and the papers (46 in. x 18 in.) eluted for 32 - 70 hr. with ethyl acetate - acetic acid - water (3:1:3, by vol.). The papers were dried and, except for fructose determinations, dipped in aniline hydrogen phosphate (Bailey, pers. comm.) and heated at 105° for 10 min., 50-100/µg. of the sugars

being estimated were used as standards throughout the procedure. Elution and the determination of the sugars was by the method described by Richards (1963).

For the determination of fructose, an additional marker strip containing the standard was chromatographed. This strip was cut off, sprayed with urea phosphate solution and heated. The areas on the main chromatogram corresponding to the position of fructose on the marker strip were cut out and eluted in 6 ml. of water. Fructose was estimated in 2 ml. of the eluted solution by the method of Bell (1955), and total soluble sugar by the method of Bath (1958).

Results

A. In vivo Experiments

Nitrogen Levels in the Feed and the Level of Intake

Total nitrogen in pasture top-dressed with ammonium sulphate at the rate of 224 kg./hectare ranged from 3.18 to 3.72% of the dry matter and was 7.4 to 14.8% higher than the levels in untreated pasture obtained on corresponding days (Table 29). Levels of non-protein N (NPN) were also higher in top-dressed pasture, and consequently the relative proportions of the NPN and the protein components in the two pastures were similar.

With the availability of pastures of different nitrogen levels, the two cows in paired feeding experiments were maintained on appreciably different nitrogen intakes except on the day before the first infusion of starch (experiment ii) when cow a, feeding on pasture of lower nitrogen content, had an unusually high intake (Table 29).

Ammonia Concentrations in the Rumen and the Effect of Addition of Starch

Changes in the ammonia concentration in the rumen followed much the same pattern throughout the six days of the experiment, with peak concentrations occurring 2-4 hr. after the commencement of each feeding period (Fig. 10). Concentrations of NH_3 were appreciably higher in the rumen when the cows were fed pasture with the higher nitrogen content. Without starch, the average ammonia concentration 6 hr. after the start of feeding on high N-pasture was more than twice that for normal pasture (15.8 mg./100 ml. rumen liquor compared with 6.9 mg./100 ml.).

The addition of 500 g. of starch twice daily had a variable effect on NH_3 levels. On the second day of addition, together with a diet of high-N pasture, the recorded maxima and minima were similar to those

Table 29. Nitrogen intake of twin cows fed on pasture containing different levels of nitrogenous constituents

	Pasture top-dressed with 224 kg. $(NH_4)_2SO_4$/hectare						Pasture without $(NH_4)_2SO_4$					
		N content of pasture (% of D.M.)		Total N intake (g.)				N content of pasture (% of D.M.)		Total N intake (g.)		
Date	Animal	Total-N	NPN	Morning	Afternoon	Total	Animal	Total-N	NPN	Morning	Afternoon	Total
Experiment (i)												
31.10.62	Cow a	3.56	0.76	157	149	306	Cow A	3.28	0.71	121	131	252
1.11.62*	" "	3.41	0.74	159	167	326	" "	3.16	0.69	131	136	267
2.11.62*	" "	3.72	0.84	172	169	341	" "	3.16	0.72	108	137	245
Experiment (ii)												
15.11.62	Cow A	3.18	0.74	140	157	297	Cow a	2.96	0.64	161	161	322
16.11.62**	" "	3.25	0.79	164	180	344	" "	2.83	0.70	130	143	273
17.11.62**	" "	3.33	0.75	148	132	280	" "	2.94	0.66	137	129	266

* = 500 g. of an aqueous suspension of starch infused into rumens of both animals during morning and afternoon feeding periods.

** = 900 g. of an aqueous suspension of starch infused into rumens of both animals during morning and afternoon feeding periods.

Fig. 10. Variations in rumen ammonia concentrations in twin cows fed on freshly-cut mixed pasture with and without the addition of starch.

(a) Experiment (i)

——— , cow a - fed top-dressed pasture.

- - , cow A - fed untreated pasture.

o , 31/10/62 - no starch.

□ , 1/11/62 - 500 g. starch added to rumen during each feeding period.

△ , 2/11/62 - " " " " " " " " "

(b) Experiment (ii)

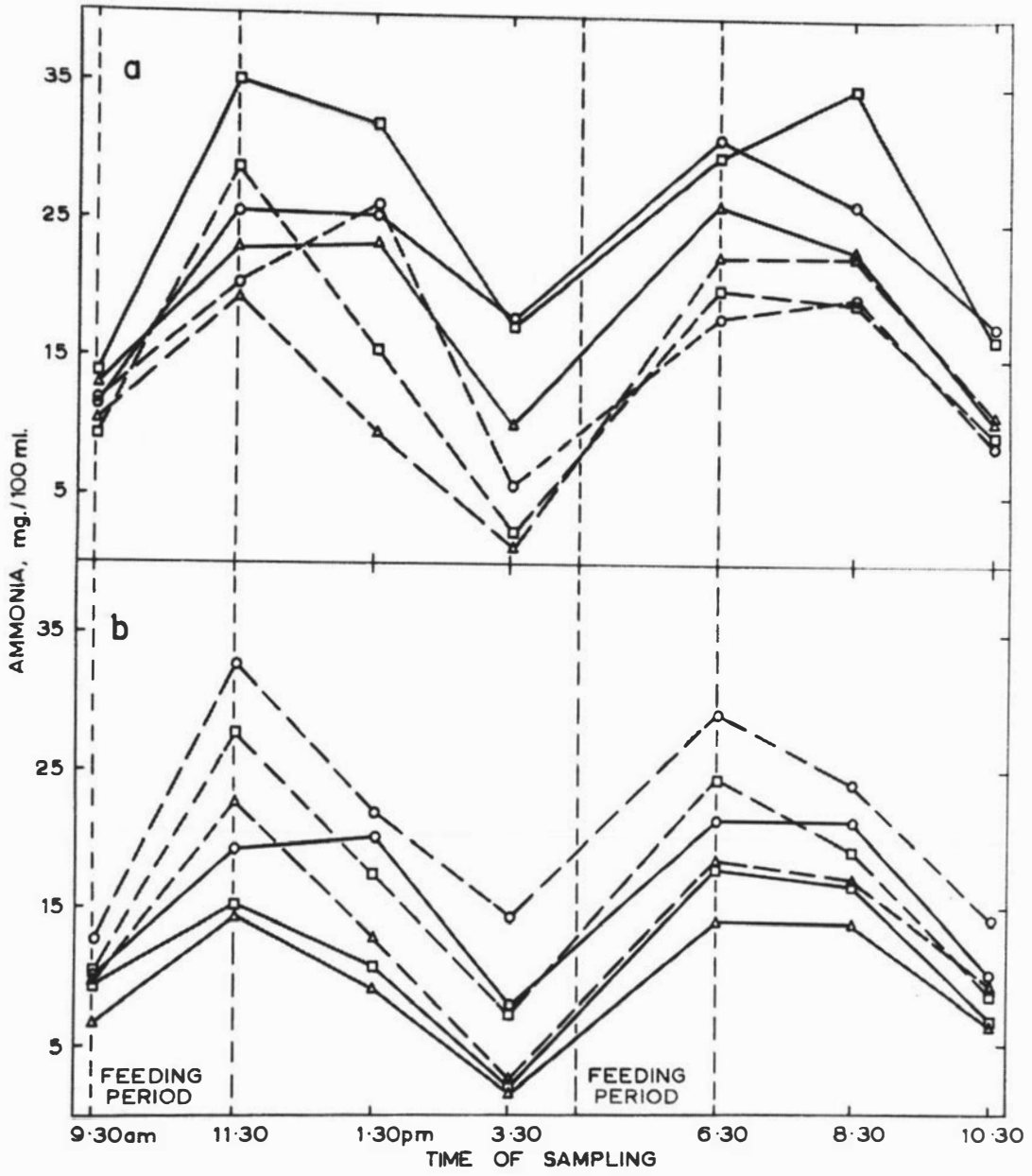
——— , cow a - fed untreated pasture.

- - , cow A - fed top-dressed pasture.

o , 14/11/62 - no starch.

□ , 15/11/62 - 900 g. starch added to rumen during each feeding period.

△ , 16/11/62 - " " " " " " " " "


a
b
35
25
15
5
AMMONIA, mg./100 ml.
FEEDING PERIOD
FEEDING PERIOD
9·30am
11·30
1·30pm
3·30
6·30
8·30
10·30
TIME OF SAMPLING

for untreated pasture without starch. However, on the first day, when approximately 30 g. of additional N were present in the high-N pasture ingested at each feeding period, this level of supplementation was insufficient to bring about a lowering of NH_3 concentrations. Increasing the dose to 900 g. starch twice daily brought about appreciable and consistent reductions in ammonia levels; in general, concentrations in the rumen of cows feeding on high-N pasture were of the same order as those fed normal pasture without starch. The very low minima recorded 6 hr. after feeding (<5 mg./100 ml. on several occasions) was a further feature when starch was added.

VFA Concentrations in the Rumen

The concentrations of VFA generally rose steadily throughout the day, reaching maximum levels after the afternoon feeding period (Fig. 11). Values ranged from minima of 50-94 m-mole/l. to maxima of 124-162 m-mole/l. and, in general, the cow with the higher intake had slightly higher VFA levels. There was no apparent effect of either the nitrogen level in the feed or of added starch.

B. *In vitro* Experiments

Soluble Sugars and Crude Protein in Ryegrass Juice Extract

Seven sugars were identified in the grass juice extract used as a substrate in the *in vitro* incubations (Table 30).

The concentrations (mg./ml.) of six sugars found in the grass juice extract as measured by quantitative paper chromatography were: galactose, 1.38; glucose, 4.00; mannose, 1.55; fructose, 7.88; arabinose, 0.85; ribose, 0.29. Galacturonic acid was also identified. The total concentration of the individual sugars determined by this method (15.95 mg./ml.

Fig. 11. Variations in rumen volatile fatty acid concentrations in twin cows fed on freshly-cut mixed pasture with and without the addition of starch.

(a) Experiment (i)

——— , cow a, fed top-dressed pasture.

- - , cow A, fed untreated pasture.

O , 31/10/62 - no starch.

□ , 1/11/62 - 500 g. starch added to rumen during each feeding period.

△ , 2/11/62 - " " " " " " " " "

(b) Experiment (ii)

——— , cow a, fed untreated pasture.

- - , cow A, fed top-dressed pasture.

O , 14/11/62 - no starch.

□ , 15/11/62 - 900 g. starch added to rumen during each feeding period.

△ , 16/11/62 - " " " " " " " " "

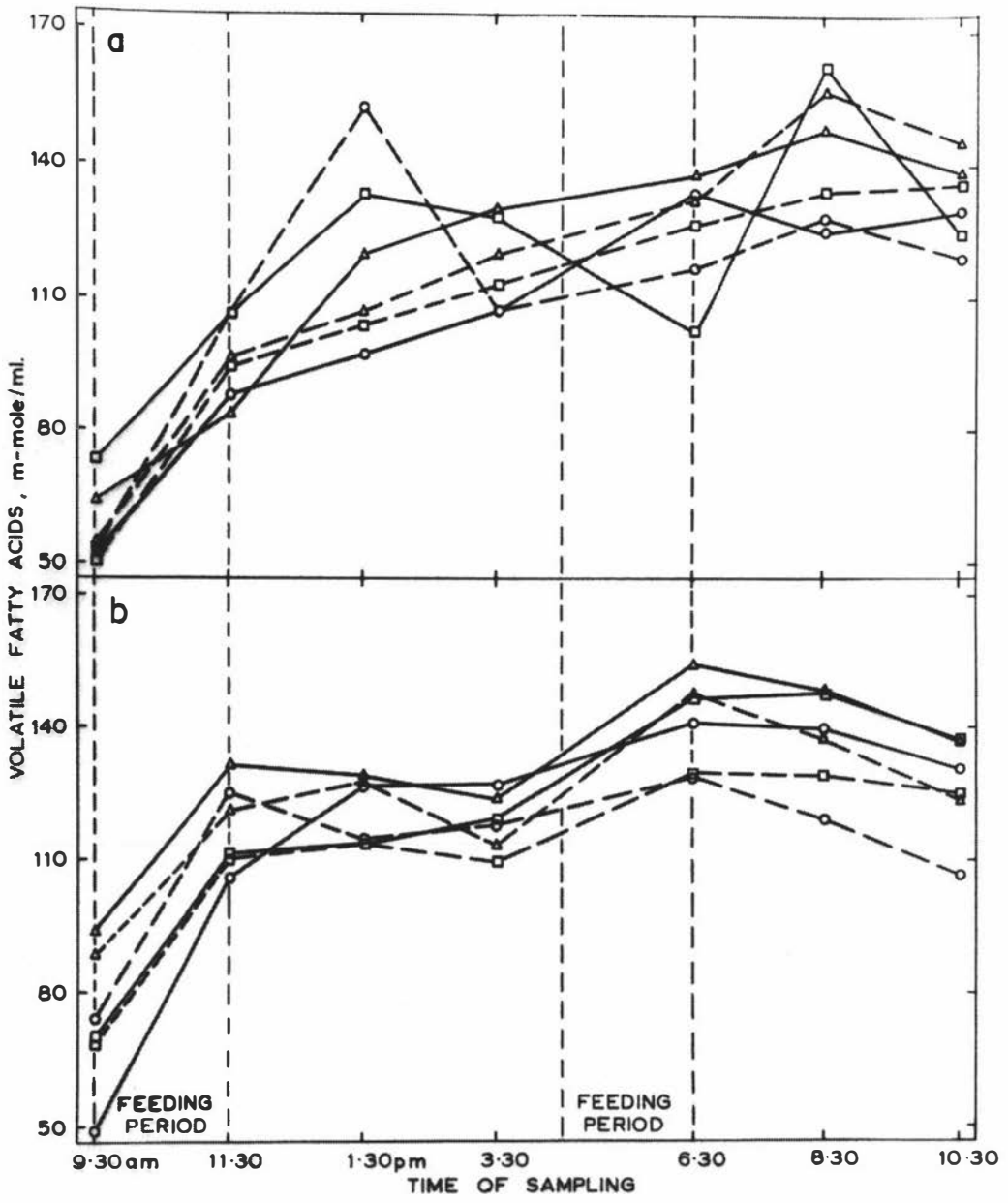

a
b
170
140
110
80
50
VOLATILE FATTY ACIDS, m-mole/ml.
FEEDING PERIOD
FEEDING PERIOD
9·30 am
11·30
1·30 pm
3·30
6·30
8·30
10·30
TIME OF SAMPLING

Table 30. Identification of sugars in ryegrass juice extract by paper chromatography

Sugar	Spray Reagent	Colour	Solvents 1* Standard $R_{glucose}$	1* Extract $R_{glucose}$	2** Standard $R_{glucose}$	2** Extract $R_{glucose}$	3*** Standard $R_{glucose}$	3*** Extract $R_{glucose}$
Galacturonic acid	p-anisidine HCL	red	0.68	0.68				
Galactose	" "	yellow-brown	0.88	0.86	0.92		0.89	0.88
Glucose	" "	" "	1.00	1.00	1.00	0.97	1.00	0.98
Mannose	" "	" "	1.30	1.29			1.19	1.18
Fructose	urea phosphate	blue	1.39	1.38	1.21	1.23	1.18	1.17
Arabinose	p-anisidine HCL	pink	1.52	1.51	1.31	1.32	1.19	1.18
Ribose	" "	"	2.58	2.58	1.63	1.66	1.58	1.59

* = ethyl acetate:acetic acid:water (3:1:3, by vol.).

** = ethyl acetate:acetic acid:formic acid:water (18:3:1:4, by vol.)

*** = n-butanol:ethanol:water:ammonia (40:10:49:1, by vol.)

extract) was 0.7 mg./ml. higher than the value obtained for total soluble sugars by the method of Bath (1958). The ryegrass extract contained 4.4 mg. crude protein/ml.

Effect of Addition of 0.1 and 0.2 g. of Soluble Carbohydrates on Metabolic Activity in vitro

In the absence of added substrate, small changes occurred in gas production, NH_3 and VFA concentration after incubation of rumen liquors, obtained from a cow fasted overnight, for 3 hr. (Table 31). The considerable variations (55 - 155 ml.) in the gas released on acidification of collected rumen liquor was related to the initial level of VFA, and therefore to the pH-high VFA concentrations giving low pH values and hence low bicarbonate concentrations in rumen liquor. Compared with values for the incubation of rumen liquor alone, the increases brought about by the addition of 10 ml. of ryegrass juice extract to 100 ml. of rumen liquor were as follows: gas formation, 33-76 ml.; ammonia concentration, 12.1 - 15.7 mg./100 ml.; VFA concentration, 18.1 - 32.9 m-mole/l.

Comparison of Rates of Fermentation of Soluble Carbohydrates

Preliminary experiments showed that the effects of glucose on gas production, ammonia and VFA concentrations increased as the levels of glucose were raised from 0.1 g. to 1.0 g. (Table 32). With the exception of NH_3, these changes were approximately proportional to the increases in the amount of glucose added. Consequently, it was decided to test a series of carbohydrates at the 1.0 g. level since differences could then be expected to be more apparent than at the lower levels used above. Practical difficulties permitted rate measurements to be made using only one carbohydrate at a time: therefore direct comparisons of gas formation

.ble 31. Gas, ammonia and total volatile fatty acid formation in rumen liquor (100 ml.) incubated with ryegrass juice alone (10 ml.) or with ryegrass juice (10 ml.) and soluble carbohydrates for 3 hr. at 39°.

	Exp. No.	Blank 0 hr.	Blank 3 hr.	Ryegrass juice alone	Ryegrass Juice and Glucose 0.1g.	Glucose 0.2g.	Sucrose 0.1g.	Sucrose 0.2g.	L-arabinose 0.1g.	L-arabinose 0.2g.	Fructose 0.2g.	Galactose 0.2g.	Xylose 0.2g.
Gas production (ml.)	1	155	176	209	222	-	220	-	-	-	-	-	-
	2	122	138	180	192	-	193	-	188	-	-	-	-
	3	67	82	151	-	170	-	170	-	158	-	-	-
	4	126	142	205	-	226	-	225	-	-	225		
	5	55	77	153	-	175	-	168	-	-	-	173	167
NH_3 concentration (mg./100 ml.)	1	15.2	22.8	34.9	33.7	-	34.0	-	-	-	-	-	-
	2	21.0	27.5	40.1	38.0	-	38.4	-	40.3	-	-	-	-
	3	18.6	22.5	38.2	-	34.6	-	34.6	-	37.8	-	-	-
	4	16.3	21.7	36.4	-	31.7	-	32.9	-	-	32.2	-	-
	5	18.7	26.2	41.4	-	37.7	-	37.3	-	-	-	36.1	35.8
Total VFA (m-mole/l.)	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	56.5	53.5	76.4	85.6	-	84.3	-	84.4	-	-	-	-
	3	76.1	76.0	94.1	-	110.2	-	110.7	-	109.5	-	-	-
	4	52.7	53.4	79.5	-	85.8	-	85.7	-	-	85.6	-	-
	5	81.1	79.2	112.1	-	117.5	-	117.8	-	-	-	118.5	117.8

Table 32. Gas, ammonia and total volatile fatty acid formation in rumen liquor (100 ml.) incubated with ryegrass juice alone (10 ml.) or with ryegrass juice (10 ml.) and glucose (0.1 - 1.0 g.) for 3 hr. at 39°

	Blank		Ryegrass juice alone	Ryegrass juice and glucose			
	0 hr.	3 hr.		0.1g.	0.2g.	0.5g.	1.0g.
Gas production (ml.)	91	124	196	208	211	237	278
Ammonia (mg./100 ml.)	28.1	33.9	50.6	48.6	46.9	44.7	43.6
Total volatile fatty acid (m-mole/l.)	66.0	73.8	85.0	87.5	92.7	103.6	111.5

and VFA and NH_3 concentrations are not valid because of the variations in the initial values for the different samples of rumen liquor.

A consistent feature of the incubation experiments was the high rate of gas, ammonia and VFA formation in the first half-hour of incubation (Table 33). The increase in ammonia levels in the controls during this period was usually between one-half and three-fourths of the total increase over the 3 hr. period, whereas increases in gas and VFA formation represented between one-third and one-half and between one-quarter and one-half of the 3 hr. increases respectively. Although gas and VFA formation in the controls increased at a slower rate between 30 min. and 3 hr., the presence of certain sugars had the effect of sustaining the initial high rates. These same sugars had an even more marked effect on the levels of ammonia during the later stages of fermentation as shown by the subsequent decrease in ammonia concentration, whereas the levels continued to increase in the controls.

Comparison of the effect of sugars on gas production and ammonia formation shows that there is a close relationship between the fermentation of readily available substrate and the utilization of nitrogen for protein synthesis (Fig. 12). Galactose, glucose, sucrose and lactose, which were fermented rapidly, brought about the greatest reduction in ammonia concentration. Galacturonic acid appears to behave in a similar way allowing for the lower concentration used. On the other had, when D-arabinose was present, a negligible effect on fermentation rate was accompanied by almost no change in ammonia concentration.

Similarly, galactose, sucrose, lactose, glucose and galacturonic acid caused the greatest increases in VFA formation (16.8 - 30.7 m-mole/l.) above the controls after incubation for 3 hr. Xylose was less effective (11.6 m-mole/l.) and D-arabinose had a very small effect (4.4 m-mole/l).

Table 33. Gas, ammonia and total volatile fatty acid formation *in vitro* with and without 1 g. of additional carbohydrate

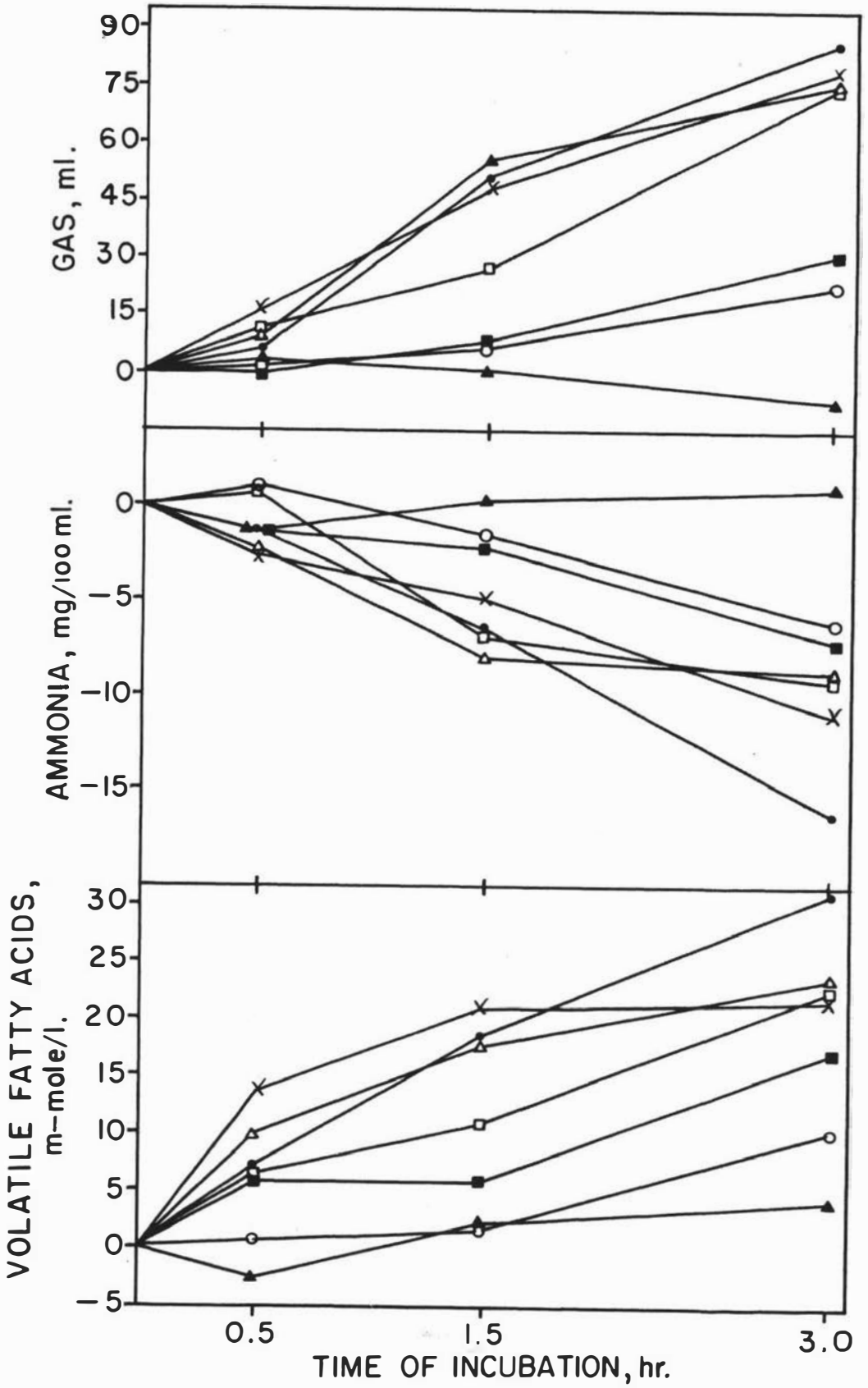
		Gas production (ml.)				Ammonia (mg./100 ml.)				Total volatile fatty acid (m-mole/l.)			
Incubation time (hr.):		0	0.5	1.5	3.0	0	0.5	1.5	3.0	0	0.5	1.5	3.0
Carbohydrate:													
Glucose	C*	150	183	201	218	23.8	34.8	36.7	37.9	54.2	61.3	70.0	81.0
	E**	150	200	249	296	23.8	32.1	31.6	26.9	54.2	75.0	92.5	103.0
Galactose	C	127	168	186	199	27.3	38.5	38.8	42.2	59.9	66.1	78.0	83.1
	E	127	174	238	285	27.3	37.1	32.4	26.1	59.9	73.4	96.6	113.8
Lactose	C	107	132	152	164	22.5	29.8	32.9	33.2	71.8	80.4	86.1	91.6
	E	107	144	179	239	22.5	30.5	26.1	24.2	71.8	87.0	96.9	113.8
Sucrose	C	129	164	185	195	21.0	30.0	32.4	33.2	62.0	72.4	80.3	85.7
	E	129	174	240	270	21.0	27.9	24.4	24.5	62.0	82.4	98.4	109.0
Xylose	C	175	200	218	233	17.9	24.6	28.8	30.9	45.3	52.9	61.9	63.8
	E	175	202	224	256	17.9	25.4	27.1	24.9	45.3	53.8	63.5	75.4
D-arabinose	C	137	173	186	215	26.2	35.6	37.3	39.7	69.0	79.0	85.4	94.8
	E	137	176	187	208	26.2	34.1	37.7	40.5	69.0	76.5	87.2	99.2
α-D-galacturonic acid (0.5 g.)	C	170	194	219	240	18.6	28.3	31.9	33.7	49.7	56.4	67.7	69.9
	E	170	195	226	270	18.6	27.1	28.9	27.5	49.7	62.1	73.4	86.7

* = control, rumen liquor (100 ml.) and ryegrass juice extract (10 ml.).

** = experimental, rumen liquor (100 ml.), ryegrass juice extract (10 ml.) and carbohydrate solution (10 ml.)

Fig. 12. Differences in gas production and ammonia and volatile fatty acid concentrations brought about by the incubation of one of a series of soluble carbohydrates with rumen liquor (100 ml.) and ryegrass juice extract (10 ml.). ● , galactose; ■ , galacturonic acid; ▲ , D-arabinose; ○ , xylose; □ , lactose; Δ , sucrose; x , glucose.

1 g. of each carbohydrate added except for galacturonic acid of which 0.5 g. added.


GAS, ml.
90
75
60
45
30
15
0
AMMONIA, mg/100 ml.
0
−5
−10
−15
VOLATILE FATTY ACIDS, m-mole/l.
30
25
20
15
10
5
0
−5
0.5
1.5
3.0
TIME OF INCUBATION, hr.

Discussion

The higher ammonia levels present in rumen samples from animals fed top-dressed pasture illustrate a relationship between the nitrogen content of the pasture, nitrogen intake, and the concentration of ammonia in the rumen. Other workers (McDonald, 1952; Annison *et al*, 1954; Annison, 1956) have shown that the time required for ammonia levels to reach a maximum after feeding, and the magnitude of the increases, depend largely on the solubility of the dietary protein. For example, a dose of 100 g. of casein gave a maximum ammonia concentration of 63.5 m-mole/l. 3 hr. after administration, whereas zein, which is much less soluble, gave a peak of 11.0 m-mole/l. 10 hr. after administration. In the present work, maximum concentrations occurred 2 - 4 hr. after feeding, which indicates that the plant protein was readily metabolised.

Levels of ammonia were frequently lower 6 hr. after the morning feeding than before feeding. McDonald (1952) reported a similar observation and suggested that, as a result of large amounts of energy becoming available for protein synthesis a few hours after feeding, ammonia utilization would exceed formation. Later, when the rate of bacterial growth dropped, production could again exceed utilization.

Increases in the level of nitrogen in the pasture as a result of top-dressing were similar to those found in hay which had received the same rate of application 2 weeks before cutting (Ferguson, 1948). It was also noted that top-dressing had no effect on the protein-N/NPN ratio.

In spite of a lower intake of nitrogen on the first day of experiment (ii), cow A, fed on high-N pasture, had higher concentrations of NH_3 in the rumen. This suggests that, either the protein in the top-dressed pasture was more readily hydrolysed, or the higher level of soluble

carbohydrate in the untreated pasture (approximately 2.2% of dry matter) increased protein synthesis sufficiently to utilize the additional nitrogen ingested.

The effect of starch in lowering ammonia concentrations in the rumen of pasture-fed animals is in agreement with observations of Annison (1956) and of Lewis and McDonald (1958) after the dosage of animals on hay and concentrate diets with starch. In so far as ammonia levels in the portal blood are related to ammonia levels in the rumen, (Lewis, Hill & Annison, 1957), starch supplementation would seem to improve the utilization of dietary nitrogen in ruminants grazing on pasture high in protein.

The lack of any consistent difference in the levels of total VFA as a result of either the difference in the level of nitrogen in the feed or the addition of starch was unexpected in view of the findings of Lewis and McDonald that casein added to the rumen of sheep at the same time as starch resulted in higher concentrations of total VFA. In addition, the present _in vitro_ experiments show that there is a direct relationship between the effect of added sugar on ammonia utilization and the formation of VFA. Reduction in ammonia concentration, without an accompanying increase in VFA concentration when starch was added, may well indicate that starch was being utilized as an energy source for protein synthesis by rumen bacteria. An increase in the protein level of rumen contents after the addition of starch has been observed _in vitro_ by Phillipson, Dobson & Blackburn (1959).

The variation in the effect of supplementary carbohydrate on VFA production _in vivo_ and _in vitro_ could also have been due to differences between the proportions of the various carbohydrate fractions available for fermentation. Cellulose would have been a major constituent of the

mixed pasture fed during the *in vivo* experiment but would not have constituted a significant proportion of the carbohydrate fraction in the plant juice extract. Burroughs, Gerlaugh, Edgington & Bethke (1949), Bonsembiante (1958) and Shazly, Dehority & Johnson (1961) found that the digestibility of cellulose decreased in the presence of supplementary soluble sugars and/or starch. Therefore, in the present *in vivo* experiments, the production of VFA from supplementary starch may have been offset by a reduced production from cellulose with the result that no net change in total concentration occurred. *In vitro*, fermentation of the added sugars and the absence of cellulose in the extract probably accounted for the observed increase in concentrations of total VFA.

With the exception of ribose, the sugars present in ryegrass juice extract are among those commonly found in grasses (Mackenzie & Wylam, 1957). The absence of sucrose in the extract was likely due to enzymic hydrolysis during extraction and subsequent storage. Similarly, arabinose and galactose are usually present in polymeric form in the hemicellulose and pectic fractions respectively (Bailey, 1962) and their occurrence as free sugars was probably due to enzymic degradation (Wylam, 1953).

The reduction in ammonia concentration in rumen liquor when soluble sugars were added to the incubation mixture agrees with observations made previously by Pearson and Smith (1943) and McNaught (1951) when studying the *in vitro* conversion of NPN to protein-N. The rapid fermentation of galacturonic acid, in the present work, indicates that the presence of a primary alcohol group is not essential to the utilization of a carbohydrate as was suggested by McNaught. Lewis and McDonald (1958) found that the greatest increases in protein utilization occurred when the food material acting as an energy source was attacked at a rate comparable to the rate

at which non-protein nitrogen becomes available in the rumen. In the present work, the very high rate of ammonia formation during the first half-hour of incubation indicated that the nitrogen in the grass juice extract was readily metabolized and in these circumstances readily metabolized sugars should be effective in facilitating the incorporation of ammonia into bacterial protein.

The considerable reduction in ammonia formation brought about by supplementation of soluble sugars in ryegrass extract with galactose, glucose, sucrose and lactose, stresses the importance of the concentrations of readily fermented carbohydrate in pasture grasses and clovers. This will apply to sucrose and mannose in particular and to a lesser extent ribose. Hemicelluloses and pectic substances probably would also be broken down sufficiently quickly to enhance ammonia utilization. Thus, provided the amount of protein being ingested by an animal is not in excess of the protein requirements of that animal, the availability of soluble sugars and other readily hydrolysed carbohydrates is likely to be the main factor limiting the utilization of pasture protein when carbohydrate/protein ratios are low, as occurs in grass and clover pastures in early spring and late autumn. The chemical composition of pasture as affected by season requires further investigation before the most efficient utilization of grassland by ruminants is likely to be achieved.

Summary

1. Paired feeding of rumen-fistulated cows showed that 7.4 - 14.8% increases in nitrogenous constituents in pasture led to higher concentrations of ammonia in the rumen after feeding. Infusion of 500 g. of starch into the rumen was not always effective in lowering ammonia content to the control levels when additional nitrogen (28 - 64 g. at each feeding) was ingested as a pasture constituent. In general, 900 g. starch in similar conditions led to ammonia levels of the same order as recorded in the controls. Infusion of starch did not increase VFA concentrations.

2. Incubation of rumen liquor with ryegrass extract gave rapid production of ammonia. This could be largely prevented by the addition of galactose, sucrose, lactose and glucose. The results suggest that one of the factors that can limit the utilization of pasture protein is likely to be the amount of soluble sugars and other readily hydrolysed carbohydrate available in the pasture.

The material presented in this section has been published in the Journal of the Science of Food and Agriculture, 1965, 16, 268.

GENERAL DISCUSSION

The results of the present work indicated that addition of lipid under both *in vivo* and *in vitro* conditions affected the metabolism of protein and carbohydrates by rumen micro-organisms.

An increase in the level of NH_3 in rumen liquor was the major change noted in protein metabolism. The increase did not occur in all experiments and its occurrence may have been related to the nitrogen content of the ration.

The main effect on carbohydrate metabolism was a reduction in cellulose digestion. In the initial experiments, it was found that the addition of lipid to the diet of cows fed freshly-cut pasture reduced the concentration of acetic acid in the rumen. This observation suggested that the digestibility of the carbohydrate fraction was being affected. Further experiments indicated that lipid had no apparent effect on the rate of digestion of soluble carbohydrate *in vitro* but did reduce the digestion of cotton thread and grass fibre under *in vivo* and *in vitro* conditions, respectively.

These findings are in agreement with results from other laboratories which indicated that the effect of lipid on carbohydrate metabolism, under conditions of hay and concentrate feeding, depended on the nature of the diet. Little effect was found when high energy fattening rations were fed (Jordan *et al*, 1958) but marked reductions in cellulose digestion occurred when high fibre rations were fed (Buysse, 1962). However, the reason for this effect on cellulose digestion is not clear. Brooks *et al*, (1954) suggested that bacterial action on cellulose could be retarded as a result of the coating of fibrous material with lipid. The present

finding, that a large percentage of added lipid became occluded to the particulate material in the rumen, strengthens this possibility. White *et al.*(1958) noted that cellulose digestion did not return to normal until 17 days after the removal of oil from the ration implying that bacterial activity must be affected. Although no reduction in the metabolic activity of the micro-organisms digesting the soluble carbohydrate consitituents was found in the present short-term incubation experiments, reductions did occur when grass fibre was incubated for 48 hr. in the presence of lipid. A third possible reason for the reduction in cellulose digestion, which was not investigated in the present work, was also suggested by White *et al.*(1958). These workers found that the addition of calcium restored cellulose digestion and proposed that a calcium-lipid complex was formed, as had previously been shown to occur in rats fed corn oil or olive oil (Swell *et al.*, 1956), with the result that the calcium requirement in the rumen was increased. However, Davison and Woods (1963) found no improvement in the performance of lambs receiving supplementary fat when calcium was added to the ration. Phillips & Roberts (1966) reported that the digestibility of crude fibre was reduced significantly following oral administration of sunflower seed oil, even though the dietary calcium level of 1% was somewhat greater than has been reported to alleviate this effect of oil.

Increases in the concentration of propionic acid following addition of lipid were another consistent feature of both *in vivo* and *in vitro* experiments. Shaw and Ensor (1959) noted that the inclusion of 300 ml./day of cod-liver oil, oleic acid or linoleic acid in a dairy cattle ration decreased the molar proportion of acetic acid and increased the molar proportion of propionic acid. Linoleic acid had the greatest effect,

decreasing acetic acid proportions from 78.9 to 52.2% and increasing propionic acid proportions from 9.9 to 29.9%. These changes in rumen VFA proportions were accompanied by a depression in the percentage of milk fat. Hilditch (1956) noted this effect of cod-liver oil on milk fat and proposed that the unsaturated C_{20} and C_{22} fatty acids were responsible since hydrogenation was known to eliminate the effect. He suggested that these acids blocked the synthesis of several of the short-chain fatty acids characteristic of butterfat. It would appear however, in view of the findings of Shaw and Ensor (loc. cit.), that the effect is related more to the degree of unsaturation than to the length of the carbon chain. Furthermore, the high correlation between the molar percent of ruminal acetic acid and the fat content of milk and an equally high negative correlation between the molar percent of ruminal propionic acid and the fat content of milk (Shaw, Robinson, Senger, Lakshamanan & Lewis, 1960) indicate that the effect was probably due to altered acid proportions in the rumen.

The explanation for the increase in the concentration of propionic acid remains largely speculative. As has been pointed out previously, part of this increase was undoubtedly due to fermentation of the glycerol released on hydrolysis of the added oil (Garton *et al*, 1961). However, in the present *in vitro* experiments in particular, the observed increases in propionic acid exceeded the theoretical values and it appears probable that the increases were due to alterations in the metabolism of the dietary carbohydrate. In an interesting review paper, Van Soest (1963) noted that the effect of unsaturated oils on rumen propionate concentrations was similar to that produced by a variety of other compounds all of which are known to affect oxidation-reduction processes. For example, Jamieson (1959)

found that nitrate administered directly into the rumen of sheep resulted in an increase in the proportion and, in most cases, in the concentration of propionic acid. An increase in the oxidation-reduction potential was also reported. Potassium chlorate has also been shown to increase the proportion of propionate and has been used successfully for the treatment of ketosis (Blackburn, Castle & Drysdale, 1959). The only apparent characteristic common to the metabolism of these compounds in the rumen is a requirement for hydrogen. In the present experiments, the proportion of linolenic acid in the rumen had returned to about the pre-feeding level within 6.5 hr. after the infusion of 500 g. of linseed oil indicating that hydrogenation was occurring rapidly in vivo. It can be calculated that complete hydrogenation of 500 g. of linseed oil would utilize approximately 7 g. of hydrogen. These observations suggest that hydrogenation may be the key to an understanding of the differential effect of various lipids on the proportions and concentrations of volatile fatty acids in the rumen. Shaw and Ensor (1959), after a study of the effect of feeding cod-liver oil and unsaturated fatty acids on rumen VFA and milk fat, concluded that:

> "Oils containing long-chain, highly unsaturated acids, unsaturated fatty acids per se and possibly certain unsaturated hydrocarbons when added to rations for ruminants would be expected to produce the above noted results (altered VFA proportions and depressed milk fat) in greater or lesser degree, depending on the amount and degree of unsaturation of the additive."

Degree of unsaturation may also explain the difference between the effects of linseed and whale oil (750 g.) on feed intake, and on the total

and individual concentrations of VFA in rumen liquor which were observed in the present *in vivo* experiments. Approximately 91% of the linseed oil infused consisted of unsaturated fatty acids, principally linolenic. Only 60% of the fatty acids of whale oil were unsaturated of which about 96% consisted of monounsaturated C_{14}, C_{16} and C_{18} acids.

Any substantial demand for hydrogen would be expected to alter the redox potential of rumen contents. As yet, little information is available concerning the effects of variations in potential on the metabolism of different nutrients. Marston (1948) and Hungate (1960) obtained values of approximately -380 mv. at pH 7.0. Broberg (1957) noted that wide variations in redox potential occurred in several cases of bloat and suggested that enzymatic processes other than normal govern the course of events in some cases of bloat. The activity of certain of the rumen protozoa and bacteria is known to be affected by changes in redox potential (Smith & Hungate, 1958; Quinn *et al.*, 1962). Therefore, the effect of linseed oil on the concentration of propionic acid may have been due to an alteration in carbohydrate metabolism either directly, as a result of the increased requirement for hydrogen, or indirectly as a result of alterations in redox potential and hence, in the relative proportions of microbial species present in the rumen.

The inappetence observed in the present experiments and in the work of Titchen *et al.*, (1966) following the infusion of lipid may have been an indirect result of a change in the rate of passage of digesta from the reticulorumen. In the present work, the extensive change in the volume and consistency of the digesta which normally occurred following feeding was not observed after the infusion of oil. The difference was most evident before the morning feeding period. Therefore, although not

measured, there appears little doubt that rate of passage was decreased considerably.

Many workers, including Crampton, Donefer & Lloyd (1960), Milford (1960), Minson, Raymond & Harris (1960), Blaxter (1961) and Blaxter, Wainman & Wilson (1961), have found that when cattle and sheep are given either pasture or dried roughages, there was a close relation between the amounts they consumedand the rate of passage of a specific food. Rate of passage has been shown to be directly related to the rate at which food was fermented and left the rumen (Blaxter, Graham & Wainman, 1956). Factors such as the specific gravity of digesta particles and the particle size, which are related to rate of fermentation, have also been found to alter rate of passage (Campling & Freer, 1962; O'Dell, King, Cook & Moore, 1963). Blaxter et al.(1956) showed that sheep tended to eat to constant "fill" regardless of the ration they were given. These results indicate that there is a physical limit to the amount of roughage which an animal can eat and that any factor which results in an increased rate of passage will allow the animal to increase intake.

In the present experiments, the reduction in the rate of cellulose digestion would have reduced the rate of passage of digesta from the rumen, thereby reducing intake at subsequent feedings.

Rate of passage and intake were probably also affected by other factors.

An association between rate of flow through the reticulo-omasal orifice and motility of the forestomach has been recognized (Stevens, Sellers & Spurrell, 1960). Although the exact nature of this relationship is not well understood, the following facts are relevant:

(1) The contents of the ventral sac of the rumen are generally more liquid than those of the dorsal sac. Most of the material

entering the omasum probably originates in the ventral sac (Cuthbertson, 1959).

(2) The transfer of digesta through the reticulo-omasal orifice occurs mainly at the time of the second contraction of the reticulum during the "A" sequence. At this time, the orifice is open and pressure is low in the omasum (Balch, Kelly & Heim, 1951).

(3) The final phase of both "A" and "B" sequences, either of which can precede the reticular contractions of another "A" sequence, forces the semi-liquid contents into the reticulum (Reid & Cornwall, 1959). They are then in position for transfer to the omasum.

(4) The flow of digesta from the reticulorumen is greatest during feeding (Balch, 1958).

(5) The frequency of coordinated contractions is also greatest during feeding (Reid & Cornwall, 1959). Considerable circumstantial evidence would therefore indicate that rumen motility is also a factor governing the rate of passage of digesta to the omasum and, by implication, is related to intake.

The presence of lipid in the duodenum, in addition to reducing abomasal and forestomach motility, has been shown to delay the emptying time of the abomasum (Bath & Hill, umpublished, cited by Hill, 1965). Distension of the abomasum has been found to have a similar effect on movement of digesta through the omasum (Phillipson & Ash, 1965).

Rate of passage can therefore be retarded at any of several points in the digestive tract with the result that retention time of digesta anterior to that point will be increased and intake decreased.

Mayer (1955) suggested that a chemostatic mechanism, based on arterio-venous differences in blood glucose levels, was responsible for controlling

the intake of simple-stomached animals. In ruminants, blood glucose levels are low and intravenous infusions of glucose have not effected changes in intake (Dowden & Jacobson, 1960). However, other metabolites may be involved. For example, there is an appreciable difference in arterio-venous concentrations of acetic acid after feeding (Reid, 1950). Rook, Balch & Campling (1960) and Ulyatt (1964) found that the intrarumenal infusion of acetic acid led to significant reductions in intake. In the present experiments, reductions in intake generally occurred at the feeding following the first period of oil infusion. Changes in the relative concentrations of acetic and propionic acids in rumen liquor also occurred soon after infusion. As a result, levels of acetic acid in the blood would have been altered (Annison, Hill & Lewis, 1957) and could have been a factor controlling intake.

Inappetance and diarrhea also occurred following the intraduodenal infusion of lipids (Titchen et al, 1966; Philips & Roberts, 1966). This suggests that factors other than those related to metabolism in the rumen may also be partly responsible for the low tolerance of ruminants for fats. Philips (1965) reported that 10% fat in the ration is approximately the upper limit which can be tolerated by ruminants. Monogastrics, on the other hand, can readily metabolize much higher levels. For example, the suckling calf thrives on a milk diet which, on a dry basis, contains approximately one-third fat. Levels of up to 30% fat, incorporated into rations for swine, have increased average daily gain and improved feed efficiency (Kuryvial, Bowland & Berg, 1962). In addition to those factors already discussed, it is suggested that the difference between ruminants and monogastrics, in the degree of hydrolysis before absorption, also acts

to limit the utilization of lipids by ruminants. In the simple-stomached animal, hydrolysis occurs mainly in the small intestine by the action of pancreatic lipase and is less extensive than in the ruminant. Consequently, in man, lipids are believed to be absorbed mainly as monoglycerides rather than as free fatty acids (Benson & Rampone, 1966). Monoglycerides, fatty acids and bile salts combine to produce a stable, lipid emulsion of small particle size (micelle). If, as Garton (1965) has concluded, the absorption of lipid from the duodenum of the ruminant follows a pattern similar to that in other animals, micelle formation and therefore absorption could be limited by the amount of monoglyceride and/or glycerol available. The diarrhea which occurred in both the present experiments and following the intraduodenal infusion of lipid (Titchen et al., 1966; Phillips & Roberts, 1966) could probably then be related to the presence of hypertonic material in the lower digestive tract. Titchen et al. (1966) have discussed the possible inhibiting effect on appetite of stimulation of osmoreceptors located in the duodenum by lipids.

Energy is generally the major factor limiting production by ruminants fed primarily on roughage. As has been noted previously, the addition of lipid to rations for simple-stomached animals, in addition to increasing the energy level of the diet has improved rate of gain and feed efficiency (Kuryvial et al., 1962; Lewis, 1965). Consequently, the level added to a specific ration has depended on considerations such as the effect of dietary fat on carcass composition or the price of fat relative to alternate sources of energy. The present in vitro results suggest that supplementary lipid would also be a valuable source of energy for ruminants. For example, when ryegrass fibre was incubated with lipid, the decrease in the

concentration of acetic acid which occurred was accompanied by a similar increase in the concentration of propionic acid. The total concentration of VFA was not affected. Since the heat of combustion of propionic acid is considerably higher than that of acetic acid (367.2 and 209.4kcal./mole, respectively) more energy would be available for productive purposes despite the depression in cellulose digestion. The fermentation of rye-grass juice with either linseed or whale oil produced a similar effect although the change in the relative concentrations of acetic and propionic acids was less extensive.

Blaxter (1962) has discussed the relationship between the molar proportions of VFA in rumen liquor and the efficiency of energy utilization for synthesis of body fat. In general, efficiency of fat synthesis increased as the proportion of propionic acid relative to acetic acid increased. For example, he found that when the total VFA of rumen liquor contained 68% acetic acid, the absorbed products of digestion were used with an efficiency of 32%. When only 50% of the VFA in the rumen was acetic, utilization of the absorbed end-products for fattening was 54%. Therefore, under the present *in vitro* conditions, addition of lipid not only increased the gross energy of the total VFA but improved the efficiency with which body fat could be synthesized from the mixture.

The infusion of oil in the present *in vivo* experiments had a similar effect on the molar proportions of acetic and propionic acids. Therefore, provided the total concentration of VFA was not markedly reduced, lipogenesis should have increased. Indeed, this may have occurred in experiment i, Part 1, when 500 g. of linseed oil was infused into the rumen. This level represented approximately 5.5 - 6.0% of the daily weights of dry matter consumed before the infusions began. However, intake and

total VFA concentrations were reduced appreciably when 750 g. of either linseed or whale oil were given to animals fed freshly-cut herbage and when 500 g. of linseed oil was given to animals fed ryegrass hay. These doses amounted to 7.5 - 8.0, 13.5 - 16.2 and 7.8 - 11.1% respectively of the daily weights of dry matter ingested before infusion. The addition of lipid to rations for ruminants would only be of value, as a source of supplementary energy, if the combined effects of the lipid on intake, cellulose digestibility and therefore, on the energy represented by the total VFA concentration were of less magnitude than the increased energetic efficiency for lipogenesis represented by the change in the proportional concentrations of individual acids in the mixture. The results of both the present experiments and those reported previously by others (loc. cit.) suggest that this condition would only be met when the added lipid represented such a small fraction of the dry matter intake that any improvement in energetic efficiency would be of little practical consequence. Therefore, supplementary lipid has a very limited potential as a source of additional energy for ruminants given rations high in roughage.

The present work also provided further evidence of the inter-relationship of carbohydrate and protein. Lewis & McDonald (1958) found that the greatest increases in protein utilization occurred when the food material acting as an energy source was attacked at a rate comparable to that at which non-protein nitrogen became available in the rumen. This is not surprising when it is considered that:

(1) Dietary protein is degraded by the rumen organisms to non-protein nitrogen, principally amino acids and ammonia.

(2) There is a close correlation between the solubility of a protein and its rate of degradation in the rumen.

(3) Rumen organisms resynthesize non-protein nitrogen to microbial protein - a process requiring energy.

(4) The efficiency of protein synthesis is largely dependent on the presence of an adequate supply of readily available carbohydrate which furnishes the necessary energy.

(5) Excess ammonia is absorbed from the rumen. Most of this excess is subsequently excreted thereby resulting in a loss of potential protein for productive purposes.

Under practical conditions a wide variety of protein and carbohydrate sources are available for use in ruminant rations. In order to achieve maximum feed efficiency it would appear that, in addition to formulating rations to contain recommended levels of protein and carbohydrate, the selection of ingredients would also need to be based on a knowledge of the probable inter-relationship, during fermentation in the rumen, of the protein and carbohydrate contained in a specific combination of ingredients. The lack of sufficient information would generally preclude the use of the latter consideration at the present time although exceptions do exist. For example, it has been shown that the utilization of nitrogen, by steers fed rations in which urea furnished approximately 33% of the nitrogen, was not influenced by the carbohydrates of various cereal grains, but was depressed when cane molasses was substituted (Bell, Gallup & Whitehair, 1951). Nitrogen balance data obtained by Smith, McLaren, Anderson, Welch & Campbell (1957, 1958) with lambs fed corn cobs, wheat straw or oat-mill feed in rations in which 67% of the nitrogen was furnished by urea indicated that nitrogen utilization was increased by corn cobs and decreased by oat-mill feed when compared with the results

obtained with lambs fed wheat straw. These workers also showed that replacement of various amounts of wheat straw with equal quantities of a mixture of dextrose and starch improved nitrogen utilization. Therefore, in rations in which a substantial portion of the nitrogen is present as urea, starch is the most suitable carbohydrate and is probably best supplied by one or more of a variety of cereal grains.

Chalmers and Synge (1954) noted that herring meal protein was superior to casein as a supplement for sheep and related this superiority to a lower rumenal ammonia formation and a consequent lower nitrogen excretion. Henderickx and Martin (1963) found that the rate of breakdown of each of a wide variety of proteins was directly proportional to solubility in a mineral buffer solution. These workers also tested the effect of a number of carbohydrates on the rate of protein degradation and resynthesis in vitro. They found that those carbohydrates which were most effective in stimulating synthesis of a microbial protein were also most effective in inhibiting the breakdown of food proteins. The effectiveness of each carbohydrate in altering rates of degradation and resynthesis varied depending on the particular protein being tested.

Consideration of protein-carbohydrate inter-relationships would also appear to be necessary for animals on pasture, particularly those consuming herbage high in nitrogen. Waite (1958) demonstrated that nitrogen top-dressing had an adverse effect on the soluble carbohydrate content of grass and Barnett (1955) suggested that during periods of rapid growth the supply of readily available carbohydrate might not be adequate to provide the necessary energy for the rumen organisms concerned in the utilization of nitrogen. Johns (1955 b) observed that the concentration of ammonia in the rumen of sheep on a high protein pasture ranged from

35 mg. of NH_3 - N/100 ml. (summer) to 130 mg./100 ml. (winter). In the present work, maximum concentrations of NH_3 varied from approximately 15 to 65 mg./100 ml. depending on the maturity and crude protein content of the pasture fed. Considerable variation in the ratio of protein to soluble carbohydrate was also found in the pasture. It would appear desirable, therefore, to improve the balance of nutrients by supplementing pasture high in protein with a source of readily fermentable carbohydrate. However, in practice, a response in level of production has seldom been obtained by supplementary feeding except under conditions of intensive grazing or during prolonged periods of wet weather (Conway, 1963; Musangi, Holmes & Jones, 1965; Forbes, Raven & Robinson, 1966). It can also be postulated, on the basis of work by Blaxter & Wilson (1963) that cattle or sheep grazing relatively mature stands of grass would respond to supplementary feeding.

The lack of response by animals grazing herbage at a highly digestible stage is due, in part, to the effect of the supplement on the intake of herbage. Forbes et al.(1966) found that the intake of a group of steer calves, measured as starch equivalent, increased by less than 1 lb./head/day when 4 lb. of barley was fed with a freshly-cut, mixed stand of timothy and meadow fescue. When barley was given ad libitum, the steers consumed an average of 7.2 lbs. of grain/day. Due to the decline in the intake of grass, the additional 3.2 lbs. of barley had a negligible effect on intake of starch equivalent. Blaxter et al.(1961) and Blaxter & Wilson (1963) found that when restricted amounts of concentrate were given to sheep, the extent of depression in voluntary intake of hay varied inversely with the quality of the hay. A similar effect probably occurs when concentrates are given to animals at grass.

The effect of supplementary soluble carbohydrates on intake is probably partially due to their well-known effect on cellulose digestibility (Burroughs et al., 1949; Head, 1953; Bonsembiante, 1958; el Shazly et al., 1961) and hence, to the rate of disappearance of digesta from the rumen. This relationship has been discussed previously in connection with the effect of supplementary lipid on voluntary intake.

Although the loss of digestible nutrients resulting from the depression in cellulose digestibility might be expected to affect animal performance, a compensatory increase in digestibility of the soluble carbohydrate fraction also occurs when supplementary starch or soluble sugars are given (Forbes et al., 1966). The amount of concentrate given appears to be a major factor governing the degree of compensatory digestion achieved.

The feeding of a supplementary source of readily fermented carbohydrate would also be expected to alter the proportions of VFA present in rumen liquor. In some cases, the change would probably not be extensive since relatively large proportions of propionic acid have been found in the rumen of animals grazing highly digestible herbage (Bath & Rook, 1961; Terry & Tilley, 1961; Bath, Rook & Rowland, 1962). However, wide variations in proportions of VFA were found by these authors. Part of this variation was attributed to a species effect and, although all the herbage tested was described as being leafy and of good quality, differences in chemical composition within species were probably also involved. These variations in VFA ratios suggest that, even for good quality pasture, situations may exist where supplementary feeding would be a practical means of improving animal performance.

Many unanswered questions, concerning factors which affect the performance of ruminants fed young, highly-digestible forage, remain.

Further knowledge of the relationship of protein and carbohydrate in the diet on biochemical and physiological changes occurring in the animal may well provide answers to some of these questions.

REFERENCES

Abou Akkada, A.R. & El Shazly, K. (1958). J. Agric. Sci., 51, 157.

Agrawala, D., Duncan, C.W. & Huffman, C.F. (1953). J. Nutr., 49, 29.

Allen, R.S., Hill, F.O., Hammond, E.G. & Jacobson, N.L. (1959). Report 5th Conf. Rumen Function. Ed. by Ellis, N.R., U.S. Agric. Res. Centre, Beltsville, Md.

Allison, M.J., Bryant, M.P. & Doetsch, R.N. (1962). J. Bacteriol., 83, 523.

Annison, E.F. (1954). Biochem. J., 57; 400.

Annison, E.F. (1956). Biochem. J., 64; 705.

Annison, E.F., Chalmers, M.I., Marshall, S.B.M. & Synge, R.L.M. (1954). J. Agric. Sci., 44, 270.

Annison, E.F., Hill, K.J. & Lewis, D. (1957). Biochem. J., 66, 592.

Arias, C., Burroughs, W., Gerlaugh, P. & Bethke, R.M. (1951). J. Animal Sci., 10, 683.

Ash, R.W. & Dobson, A. (1963). J. Physiol., 164, 39.

Association of Official Agricultural Chemists. (1960). Official Methods of Analysis, 9th Edn. Washington, D.C.: A.O.A.C.

Bailey, R.W. (1958). J. Sci. Food Agric., 9, 748.

Bailey, R.W. (1962). Proc. N.Z. Soc. Animal Prod., 22, 99.

Balch, C.C. (1949). Ph.D. Thesis; Reading University. Cited by Balch, C.C. (1961). In Digestive Physiology and Nutrition of the Ruminant. p. 26. Ed. D. Lewis. London. Butterworths.

Balch, C.C. (1950). Brit. J. Nutr., 4, 361.

Balch, C.C. (1958). Brit. J. Nutr., 12, 330.

Balch, C.C. (1960). Proc. 8th Int. Grassland Cong., Reading. p. 528.

Balch, C.C. (1961). In Digestive Physiology and Nutrition of the Ruminant. p. 26. Ed. D. Lewis. London: Butterworths.

Balch, C.C., Balch, D.A., Bartlett, S., Bartrum, M.P., Johnson, V.W., Rowland, S.J. & Turner, J. (1955). J. Dairy Res., 22, 270.

Balch, C.C., Kelly, A. & Heim, G. (1951). Brit. J. Nutr., 5, 207.

Balch, D.A. & Rowland, S.J. (1957). Brit. J. Nutr., 11, 288.

Barcroft, J., McAnally, R.A. & Phillipson, A.T. (1944). J. Exp. Biol., 20, 120.

Barnett, A.J.G. (1955). Suomen Kemistilehti, 28, 3.

Barnett, A.J.G. & Reid, R.L. (1961). Reactions in the Rumen. p. 110. London: Arnold.

Bath, I.H. (1958). Analyst, 83, 451.

Bath, I.H. & Rook, J.A.F. (1961). Proc. Nutr. Soc., 20, XV.

Bath, I.H., Rook, J.A.F. & Rowland, S.J. (1962). Int. Dairy Congr. XVI. Copenhagen, 1, 49.

Bathurst, N.O. & Allison, R.M. (1949). N.Z.J. Sci. Technol., 31B, (2) 1.

Belasco, I.J. (1954). J. Animal Sci., 13, 739.

Belasco, I.J. (1956). J. Animal Sci., 15, 496.

Bell, D.J. (1955). In Modern Methods of Plant Analysis, Vol. 2, p. 1. Ed. by Paech, K. & Tracey, M.V. Berlin: Springer - Verlag.

Bell, M.C., Gallup, W.D. & Whitehair, C.K. (1951). J. Animal Sci., 10, 1037 (Abstract).

Benson, A.A., Wintermans, J.F. & Weiser, R. (1959). Plant Physiol., 34, 315.

Benson, J.A., & Rampone, A.J. (1966). Ann. Rev. Physiol., 28, 201.

Bentley, O.G., Lehmkuhl, A., Johnson, R.R., Hershberger, T.V. & Moxon, A.L. (1954). J. Am. Chem. Soc., 76, 5000.

Bentley, O.G., Johnson, R.R., Hershberger, T.V., Cline, J.H. & Moxon, A.L. (1955). J. Nutr., 57, 389.

Best, C.H. & Taylor, N.B. (1961). The Physiological Basis of Medical Practise. 7th Edn. Baltimore, Md.; Williams & Wilkins.

Blackburn, P.S., Castle, M.W. & Drysdale, A.D. (1959). Vet. Record, 71, 665.

Blackburn, T.H. & Hobson, P.N. (1960). Brit. J. Nutr., 14, 445.

Blaxter, K.L. (1961). Federation Proc., 20, 268.

Blaxter, K.L. (1962). In The Energy Metabolism of Ruminants. Hutchison: London.

Blaxter, K.L., Graham, N.M. & Wainman, F.W. (1956). Brit. J. Nutr., 10, 69.

Blaxter, K.L., Wainman, F.W. & Wilson, R.S. (1961). Animal Prod., 3, 51.

Blaxter, K.L. & Wilson, R.S. (1963). Animal Prod., 5, 27.

Boda, J.M. & Johns, A.T. (1962). Nature, Lond., 193, 195.

Bǒda, K., Tomas, J. & Kona, E. (1961). Vet. Cas., 10, 548. Abstract, Nutr. Abstr. Rev. 1963. 33, 84.

Bonsembiante, M. (1958). Riv. Zootec., 31, 23. Abstracted 1958. Nutr. Abstr. Rev., 28, 862.

Bouckaert, J.H. & Oyaert, W. (1952). Zootechnia, 1, 21.

Brethour, J.R., Sirny, R.J. & Tillman, A.D. (1958). J. Animal Sci., 17, 171.

Briggs, H.M., Gallup, W.D., Heller, V.G. & Darlow, A.E. (1948). J. Animal Sci., 7, 35.

Briggs, P.K., Hogan, J.P. & Reid, R.L. (1957). Austral. J. Agric. Res., 8, 674.

Broberg, G. (1957). Nord. Vet.-Med., 9, 931.

Brooks, C.C., Garner, G.B., Gehrke, C.W., Muhrer, M.E. & Pfander, W.H. (1954). J. Animal Sci., 13, 758.

Bryant, A.M. (1961). M. Agr. Sc. Thesis, University of New Zealand.

Bryant, A.M. (1964). N.Z. J. Agric. Res., 7, 694.

Bryant, M.P. (1959). Bacteriol. Revs., 23, 125.

Bryant, M.P. (1963). J. Animal Sci., 22, 801.

Bryant, M.P. & Doetsch, R.N. (1955). J. Dairy Sci., 38, 340.

Bryant, M.P., Doetsch, R.N. & Robinson, I.M. (1962). J. Bacteriol., 84, 605.

Bryant, M.P. & Robinson, I.M. (1961). Appl. Microbiol., 9, 96.

Bryant, M.P., Robinson, I.M. & Chu, H. (1959). J. Dairy Sci., 42, 1831.

Burroughs, W., Gall, L.S., Gerlaugh, P. & Bethke, R.M. (1950). J. Animal Sci., 9, 214.

Burroughs, W., Gerlaugh, P., Edgington, B.H. & Bethke, R.M. (1949). J. Animal Sci., 8, 9.

Buysse, F. (1962). Rev. Agric. Brussels. 15, 343.

Campling, R.C. & Freer, M. (1962). Brit. J. Nutr., 16, 507.

Carroll, E.J. & Hungate, R.E. (1954). Appl. Microbiol., 2, 205.

Chalmers, M.I. (1960). Abstracts - 5th Int. Congress on Nutrition. (Washington, D.C.)

Chalmers, M.I. (1961). In Digestive Physiology and Nutrition of the Ruminant. p. 205. Ed. D. Lewis. London: Butterworths.

Chalmers, M.I., Cuthbertson, D.P. & Synge, R.L.M. (1954). J. Agric. Sci., 44, 254.

Chalmers, M.I. & Synge, R.L.M. (1954). Advanc. Protein Chem., 9, 93.

Clark, R. & Quin, J.I. (1951). Onderstepoort J. Vet. Sci., 25, 93.

Cline, J.H., Hershberger, T.V. & Bentley, O.G. (1958). J. Animal Sci., 17, 284.

Conchie, J. & Levvy, G.A. (1957). Biochem. J., 65, 389.

Conway, A. (1963). Res. Rep. Animal Prod. Div., The Agric. Inst., Dublin. p. 5.

Conway, E.J. & O'Malley, E. (1942). Biochem. J., 36, 655.

Coombe, J.B., Tribe, D.E. & Morrison, J.W.C. (1960). Austral. J. Agric. Res., 11, 247.

Coop, I.E. (1949). N.Z. J. Sci. Technol., 31A, 1.

Crampton, E.W., Donefer, E. & Lloyd, L.E. (1960). J. Animal Sci., 19, 538.

Cuthbertson, D.P. (1959). Proc. Nutr. Soc., 18, 97.

Dahl, O. (1958). Acta. Agric. Scand., 8, 106.

Davey, A.W.F. (1965). Proc. N.Z. Soc. Animal Prod., 25, 106.

Davison, K.L. & Woods, W. (1960). J. Animal Sci., 19, 54.

Davison, K.L. & Woods, W. (1963). J. Animal Sci., 22, 919.

Dehority, B.A., Bentley, O.G., Johnson, R.R. & Moxon, A.L. (1957). J. Animal Sci., 16, 502.

Doetsch, R.N. & Jurtshuk, P. (1957). Md. Agric. Expt. Sta. Misc. Publ. No. 291, 8.

Dowden, D.R. & Jacobson, D.R. (1960). Nature, Lond., 188, 149.

Duncan, W.R.H. & Garton, G.A. (1962). J. Lipid Res., 3, 53.

El Shazly, K. (1952). Biochem. J., 51, 640.

El Shazly, K., Dehority, B.A. & Johnson, R.R. (1961). J. Animal Sci., 20, 268.

Elam, C.J. & Davis, R.E. (1962). J. Animal Sci., 21, 568.

Elam, C.J., Gutierrez, J. & Davis, R.E. (1960). J. Animal Sci., 19, 1089.

Ellis, W.C. & Pfander, W.H. (1958). J. Nutr., 65, 235.

Elsden, S.R. (1945). J. Exp. Biol., 22, 51.

Elsden, S.R. (1946). Biochem. J., 40, 252.

Emery, E. & Koerner, W.E. (1961). Anal. Chem., 33, 146.

Engelhardt, W.V., Hoeller, H. & Hoernicke, H. (1963). J. Dairy Sci, 46, 65.

Erwin, E.S., Dyer, I.A. & Ensminger, M.E. (1956). J. Animal Sci., 15, 710.

Farrell, J.I. & Ivy, A.C. (1926). Am. J. Physiol., 76, 227.

Ferguson, W.S. (1948). J. Agric. Sci., 38, 33.

Flatt, W.P., Warner, W.G. & Loosli, J.K. (1959). Cornell Agric. Expt. Sta. Memoir, No. 361.

Fontenot, J.P., Gallup, W.D. & Nelson, A.B. (1955). J. Animal Sci., 14, 807.

Forbes, T.J., Raven, A.M. & Robinson, K.L. (1966). J. Br. Grassld. Soc., 21, 167.

Garton, G.A. (1960). Nature, Lond., 187, 511.

Garton, G.A. (1965). In Physiology of Digestion in the Ruminant. p. 390. Ed. R.W. Dougherty. Butterworths: Washington.

Garton, G.A., Hobson, P.N. & Lough, A.K. (1958). Nature, Lond., 182, 1511.

Garton, G.A., Lough, A.K. & Vioque, E. (1959). Biochem. J., 73, 46P.

Garton, G.A., Lough, A.K. & Vioque, E. (1961). J. Gen. Microbiol., 25, 215.

Gray, F.V., Jones, G.B. & Pilgrim, A.F. (1960). Austral. J. Agric. Res., 2, 382.

Gray, F.V., Pilgrim, A.F., Rodda, H.J. & Weller, R.A. (1952). J. Exp. Biol., 29, 57.

Gray, F.V., Pilgrim, A.F. & Weller, R.A. (1953). Nature, Lond., 172, 347.

Gutierrez, J., Williams, P.P., Davis, R.E. & Warwick, E.J. (1962). Appl. Microbiol., 10, 548.

Hale, W.H., Mueller, R., Culbertson, C.C. & Hammond, W.E. (1956). A.H. 212. Iowa State College, Ames. Cited by Jordan, R.M., Croom, H.G. and Hanke, H. (1958). J. Animal Sci., 17, 819.

Harris, L.E. & Mitchell, H.H. (1941 a). J. Nutr., 22, 183.

Harris, L.E. & Mitchell, H.H. (1941 b). J. Nutr., 22, 167.

Harris, L.E., Work, S.H. & Henke, L.A. (1943). J. Animal Sci., 2, 238.

Hawke, J.C. (1957). J. Dairy Res., 24, 366.

Hawke, J.C. (1963). J. Dairy Res., 30, 67.

Hawke, J.C., Hansen, R.P. & Shorland, F.B. (1959). J. Chromatogr., 2, 547.

Head, M.J. (1953). J. Agric. Sci., 43, 281.

Head, M.J. & Rook, J.A.F. (1955). Nature, Lond., 176, 262.

Henderickx, H. & Martin, J. (1963). Compt. Rend. Rech, Inst. Rech. Sci. Ind. Agr. Bruxelles, No. 31.

Henneberg, W. & Stohmann, F. (1864). Beitr, zur. Begrundung einer rationellen Futterung der Weiderkauer, No's. 1 & 2. Braunschweig.

Hilditch, T.P. (1956). The Chemical Constitution of Natural Fats, 3rd. Ed., p. 581. London: Chapman and Hall.

Hilditch, T.P. & Thompson, H.M. (1936). Biochem. J., 30, 677.

Hill, F.D., Saylor, J.H., Allen, R.S. & Jacobson, N.L. (1960). J. Animal Sci., 19, 1266.

Hill, K.J. (1965). In Physiology of Digestion in the Ruminant. p. 221. Ed. R.W. Dougherty. Butterworths: Washington.

Hirsch, J. & Ahrens, E.H. (1958). J. Biol. Chem., 233, 311.

Hirst, E.L., Mackenzie, D.J. & Wylam, C.B., (1959). J. Sci. Food. Agric., 10, 19.

Hobson, P.N. & Mann, S.O. (1961). J. Gen. Microbiol., 25, 227.

Hoflund, S., Holmberg, J. & Sellmann, G. (1956 a). Cornell Vet., 46, 51.

Hoflund, S., Holmberg, J. & Sellmann, G. (1956 b). Cornell Vet., 46, 51.

Hoflund, S., Quin, J.I. & Clark, R. (1948). Onderstepoort J. Vet. Sci., 23, 395.

Holmes, P., Moir, R.J. & Underwood, E.J. (1953). Austral J. Biol. Sci., 6, 637.

Hough, L., Jones, J.K.N. & Wadman, W.H. (1950). J. Chem. Soc., 1702.

Houpt, T.R. (1959). Amer. J. Physiol., 197, 115.

Hungate, R.E. (1960). Bacteriol. Rev., 24, 353.

Hungate, R.E., Bryant, M.P. & Mah, R.A. (1964). Ann. Rev. Microbiol., 18, 131.

Hungate, R.E., Fletcher, D.W., Dougherty, R.W. & Barrentine, B.F. (1955). Appl. Microbiol., 3, 161.

Hydén, S. (1956). Ann. Agric. Coll. Sweden, 22, 139.

Hydén, S. (1958). Ann. Agr. Coll. Sweden. 24, 55.

Hydén, S. (1961). In Digestive Physiology and Nutrition of the Ruminant. p. 35. Ed. D. Lewis. Butterworths: London.

Ivy, A.C. & Gray, J.S. (1937). Cold Spring Harbor Symp., 5, 405.

James, A.T. & Martin, A.J.P. (1952). Biochem. J., 50, 679.

Jamieson, N.D. (1959). N.Z. J. Agric. Res., 2, 314.

Jarrige, R., (1960). Proc. 8th Int. Grassland Cong., Reading. p. 628.

Jayasinghe, J.B. (1961). Ceylon Vet. J., 9, 135.

Johns, A.T. (1953). N.Z. J. Sci. Technol., 35A, 262.

Johns, A.T. (1955 a). N.Z. J. Sci. Technol., 37A, 301.

Johns, A.T. (1955 b). N.Z. J. Sci. Technol., 37A, 323.

Jordan, R.M., Croom, H.G. & Hanke, H. (1958). J. Animal Sci., 17, 819.

Kabot, F.J. & Ettre, L.S. (1963). J. Gas Chromatogr., 1, 17.

Kameoka, K., Morimoto, H., Takahashi, S. & Kubota, D. (1953). Bull. Nat. Inst. Agric. Sci., Japan, Series G., 7, 169.

Kuryvial, M.S., Bowland, J.P. & Berg, R.T. (1962). Can. J. Animal Sci., 42, 23.

Lampila, M. (1955). Maatalaust. Aikakausk., 27. 142.

Lampila, M. (1960). Maatalaust. Aikakausk., 32, 169.

Lampila, M. & Poijärvi, I. (1959). Maataloust. Aikakausk., 31, 315.

Lees, T.M. & DeMuria, P.J. (1962). J. Chromatogr., 8, 108.

Lewis, A.H. (1941). Emp. J. Exp. Agric., 9, 43.

Lewis, D. (1951). Biochem. J., 48, 175.

Lewis, D. (1961). In Digestive Physiology and Nutrition of the Ruminant. p. 127. Ed. by Lewis, D. London: Butterworths.

Lewis, D. (1962). J. Agric. Sci., 58, 73.

Lewis, D. (1965). Feedstuffs, 37, No. 21, 18.

Lewis, D., Hill, K.J. & Annison, E.F. (1957). Biochem. J., 66, 587.

Lewis, D. & McDonald, I.W. (1958). J. Agric. Sci., 51, 108.

Lewis, D. & McDonald, I.W. (1962). J. Dairy Sci., 45, 765.

Lofgreen, G.P., Loosli, J.K. & Maynard, L.A. (1951). J. Animal Sci., 10, 171.

Long, J.F. & Brooks, F.P. (1965). Amer. J. Physiol., 209, 447.

Louw, J.G. & van der Wath, J.G. (1943). Onderstepoort J. Vet. Sci., 18, 177.

McAnally, R.A. & Phillipson, A.T. (1942). J. Physiol., 101, 13P.

McDonald, I.W. (1948). Biochem. J., 42, 584.

McDonald, I.W. (1952). Biochem. J., 51, 86.

McDonald, I.W. (1954). Biochem. J., 56, 120.

McDonald, I.W. (1962). Proc. N.Z. Soc. Animal Prod., 22, 79.

McDonald, I.W. & Hall, R.J. (1957). Biochem. J., 67, 400.

McDougall, E.I. (1948). Biochem. J., 43, 99.

Mackenzie, D.J. & Wylam, C.B. (1957). J. Sci. Food Agric., 8, 38.

MacLeod, R.A. & Murray, J.F. (1956). J. Nutr., 60, 245.

McNaught, M.L. (1951). Biochem. J., 49, 325.

McNaught, M.L., Owen, E.C., Henry, K.M. & Ken, S.K. (1954). Biochem. J., 56, 151.

Malins, D.C. & Mangold, H.K. (1960). J. Amer. Oil Chem. Soc., 37, 576.

Mangold, H.K. (1961). J. Amer. Oil Chem. Soc., 38, 708.

Marston, H.R. (1948). Biochem. J., 42, 564.

Mayer, J. (1955). Nutr. Abstr. & Rev., 25, 597.

Milford, R. (1960). Austral. J. Agric. Res., 11, 138.

Mills, R.C., Booth, A.N., Bohstedt, G. & Hart, E.B. (1942). J. Dairy Sci., 25, 925.

Minson, D.J. & Pigden, W.J. (1961). J. Animal Sci., 20, 962.

Minson, D.J., Raymond, W.F. & Harris, C.E. (1960). Proc. 8th Int. Grassland Cong., Reading. p. 470.

Moir, R.J. & Williams, V.J. (1950). Austral. J. Sci. Res., B3, 381.

Morrison, F.B. (1956). Feeds and Feeding. 22nd Edn. Clinton, Iowa: Morrison Pub. Co.

Musangi, R.S., Holmes, W. & Jones, J.G.W. (1965). Proc. Nutr. Soc., 24, i.

Nichols, R.E. (1954). Vet. Med., 49, 91.

O'Dell, G.D., King, W.A., Cook, W.C. & Moore, S.L. (1963). J. Dairy Sci., 46, 38.

Orth, A., Kaufmann, W. & Rohr, K. (1966). Ztschr. Tierphysiol. Tierernaabrung Futtermittelk., 21, 83. (Abstract, 1966, Nutr. Abstr. & Rev., 36, 1147).

Pearson, R.M. & Smith, J.A.B. (1943 a). Biochem. J., 37, 153.

Pearson, R.M. & Smith, J.A.B. (1943 b). Biochem. J., 37, 148.

Pfander, W.H. & Verma, I.S. (1957). J. Animal Sci., 16, 1087.

Phillips, G.D. (1965). In Physiology of Digestion in the Ruminant. p. 435. Ed. R.W. Dougherty. Butterworths: Washington.

Phillips, G.D. & Roberts, W.K. (1966). Can. J. Animal Sci., 46, 59.

Phillipson, A.T. (1952). Brit. J. Nutr., 6, 190.

Phillipson, A.T. & Ash, R.W. (1965). In Physiology of Digestion in the Ruminant. p. 97. Ed. R.W. Dougherty. Butterworths: Washington.

Phillipson, A.T. & Cuthbertson, D.P. (1956). Proc. 7th Int. Cong. Animal Husbandry, Madrid. Subject, 6; p. 7.

Phillipson, A.T., Dobson, M.J. & Blackburn, T.H. (1959). Nature, Lond., 183, 402.

Polan, C.E., McNeill, J.J. & Tove, S.B. (1964). J. Bacteriol., 88, 1056.

Preston, R.L., Breuer, L.H. & Pfander, W.H. (1961). J. Animal Sci., 20, 947.

Preston, T.R., Whitelaw, F.G. & MacLeod, N.A. (1963). Anim. Prod., 5, 147.

Pridham, J.B. (1956). Anal. Chem., 28, 1967.

Quin, J.I., Oyaert, W. & Clark, R. (1951). Onderstepoort J. Vet. Res., 25, 51.

Quinn, L.Y., Burroughs, W. & Christiansen, W.C. (1962). Appl. Microbiol., 10, 583.

Reed, F.M., Moir, R.J. & Underwood, E.J. (1949). Austral. J. Sci. Res., B2, 304.

Reid, C.S.W. (1963). Proc. N.Z. Soc. Animal Prod., 23, 169.

Reid, C.S.W. (1965). Proc. N.Z. Soc. Animal Prod., 25, 65.

Reid, C.S.W., Bailey, R.W. & Glenday, A.C. (1967). N.Z. J. Agric. Res., 10, 1.

Reid, C.S.W. & Cornwall, J.B. (1959). Proc. N.Z. Soc. Animal Prod., 19, 23.

Reid, C.S.W. & Johns, A.T. (1957). N.Z. J. Sci. Technol., 38A, 908.

Reid, C.S.W., Melville, A.W. & Cornwall, J.B. (1960). N.Z. J. Agric. Res., 3, 41.

Reid, R.L. (1950). Austral. J. Agric. Res., 1, 338.

Reid, R.L., Hogan, J.P. & Briggs, P.K. (1957). Austral. J. Agric. Res., 8, 691.

Reiser, R. (1951). Fed. Proc., 10, 236.

Reiser, R. & Reddy, H.G.R. (1956). J. Amer. Oil Chem. Soc., 33, 155.

Richards, E.L. (1963). J. Dairy Res., 30, 223.

Rook, J.A.F., Balch, C.C. & Campling, R.C. (1960). Proc. Nutr. Soc., 19, i.

Routley, D.G. & Sullivan, J.T. (1958). J. Agric. Food. Chem., 6, 687.

Shaw, J.C. (1961). Proc. 8th Int. Cong. Animal Prod., Hamburg, 29.

Shaw, J.C. & Ensor, W.L. (1959). J. Dairy Sci., 42, 1238.

Shaw, J.C., Ensor, W.L., Tellechea, H.F. & Lee, S.D. (1960). J. Nutr., 71, 203.

Shaw, J.C., Robinson, R.R., Senger, M.E., Lakshamanan, S. & Lewis, T.R. (1960). J. Nutr., 69, 235.

Shorland, F.B. (1961). J. Sci. Food. Agric., 12, 39.

Shorland, F.B., Weenink, R.O. & Johns, A.T. (1955). Nature, Lond., 175, 1129.

Shorland, F.B., Weenink, R.O., Johns, A.T. & McDonald, I.R.C. (1957). Biochem. J., 67, 328.

Smith, G.S., Anderson, G.C., Welch, J.A., McLaren, G.A. & Campbell, C.D. (1958). J. Animal Sci., 17, 1198. (Abstract).

Smith, G.S., McLaren, G.A., Anderson, G.C., Welch, J.A. & Campbell, C.D. (1957). J. Animal Sci., 16, 1052, (Abstract).

Smith, V.R. (1941). J. Dairy Sci., 24, 659.

Smith, P.H. & Hungate, R.E. (1958). J. Bacteriol., 75, 713.

Smith, P.H., Sweeney, H.C., Rooney, J.R., King, K.W. & Moore, W.E.C. (1956). J. Dairy Sci., 39, 598.

Snedecor, G.W. (1956). In Statistical Methods. p. 251. Fifth Edn., Iowa State College Press: Ames.

Stevens, C.E., Sellers, A.F. & Spurrell, A. (1960). Amer. J. Physiol., 198, 449.

Stickland, L.H. (1934). Biochem. J., 28, 1746.

Sullivan, J.T., Phillips, T.G. & Routley, D.G. (1960). J. Agric. Food Chem., 8, 152.

Sutherland, T.M., Ellis, W.C., Reid, R.S. & Murray, M.G. (1962). Brit. J. Nutrition, 16, 603.

Swell, L., Trout, Jr., E.C., Field, Jr., H. & Treadwell, C.R. (1956). Proc. Soc. Exp. Biol. Med., 92, 613.

Swift, R.W., Thacker, E.J., Black, A., Bratzler, J.W. & James, W.H. (1947). J. Animal Sci., 6, 432.

Sym, E.A. (1938). Acta. Biol. Exptl. (Varsovie)., 12, 192.

Tappeiner, H. (1883). Ber. dtsch. chem. Gas., 16, 1734.

Terry, R.A. & Tilley, J.M.A. (1961). Exp. Grassld. Res. Inst., Hurley. No. 13, p. 79.

Titchen, D.A. & Reid, C.S.W. (1965). In Physiology of Digestion in the Ruminant. p. 68. Ed. R.W. Dougherty. Butterworths: Washington.

Titchen, D.A., Reid, C.S.W. & Vleig, P. (1966). Proc. N.Z. Soc. Animal Prod., 26, 36.

Tove, S.B. & Mochrie, R.D. (1963). J. Dairy Sci., 46, 686.

Ulyatt, M.J. (1964). Proc. N.Z. Soc. Animal Prod., 24, 43.

Van Soest, P.J. (1963). J. Dairy Sci., 46, 204.

Waite, R. (1958). J. Sci. Food Agric., 9, 39.

Waite, R. & Boyd, J. (1953). J. Sci. Food Agric., 4, 257.

Wakil, S.J. (1961). J. Lipid Res., 2, 1.

Ward, J.K., Tefft, C.W., Sirny, R.J., Edwards, H.N. & Tillmann, A.D. (1957). J. Animal Sci., 16, 633.

Weenink, R.O. (1959). N.Z. J. Sci., 2, 273.

Weenink, R.O. (1961). J. Sci. Food Agric., 12, 34.

Weller, R.A., Gray, F.V. & Pilgrim, A.F. (1958). Brit. J. Nutr., 12, 421.

White, T.W., Grainger, R.B., Baker, F.H. & Stroud, J.W. (1958). J. Animal Sci., 17, 797.

Wicke, A. & Weiske, J. (1896). Z. phys. Chem., 22, 137. Cited by Lewis, D. & McDonald, I.W. (1958). J. Agric. Sci., 51, 108.

Willey, N.B., Riggs, J.K., Colby, R.W., Butler, Jr., O.D. & Reiser, R. (1952). J. Animal Sci., 11, 705.

Williams, V.J. & Christian, K.R. (1956). N.Z. J. Sci. Technol, 38A, 403.

Williams, V.J., Nottle, M.C., Moir, R.J. & Underwood, E.J. (1953). Austral. J. Biol. Sci., 6, 142.

Wilson, M.K. & Briggs, C.A.E. (1955). J. Appl. Bacteriol., 18, 294.

Wise, C.S., Dimler, R.J., Davis, H.A. & Rist, C.E. (1955). Anal. Chem., 27, 33.

Wood, R.D., Bell, M.C., Grainger, R.B. & Teekell, R.A. (1963). J. Nutr., 79, 62.

Wright, D.E. (1959). Nature, Lond., 184, 875.

Wright, D.E. (1960). Nature, Lond., 185, 546.

Wright, D.E. (1961). N.Z. J. Agric. Res., 4, 216.

Wylam, C.B. (1953). J. Sci. Food Agric., 4, 527.

Wylam, C.B. (1954). J. Sci. Food Agric., 5, 167.

APPENDIX I

Proportions of individual volatile fatty acids in duplicate samples removed from each of three different regions of the rumen

Date of Sampling		30 . 7 . 64				31 . 7 . 64				1 . 8 . 64			
Location of sample		Time of sampling after commencement of feeding (hr.)				Time of sampling after commencement of feeding(hr.)				Time of sampling after commencement of feeding(hr.)			
		0	2	4.5	6.5	0	2	4.5	6.5	0	2	4.5	6.5
	A*	74.4	75.1	71.5	71.7	67.0	70.5	68.6	68.8	70.9	75.2	70.3	69.9
		76.5	76.7	72.0	70.1	67.8	69.7	66.9	68.4	71.6	73.9	70.7	70.1
Reticulum	P	16.4	17.4	19.7	18.8	19.6	19.9	20.5	19.5	17.1	17.7	20.8	19.7
		14.9	16.9	20.0	19.6	19.4	20.2	21.2	19.9	17.7	18.9	21.3	21.3
	B	9.0	7.5	8.8	9.5	13.4	8.9	10.9	11.8	12.0	7.1	8.9	10.4
		8.5	6.4	7.8	10.2	12.9	10.0	11.8	11.8	10.7	7.2	8.0	8.6
	A	75.1	75.3	69.1	70.5	65.9	70.0	67.3	69.8	72.1	74.6	68.1	70.4
		74.1	75.6	68.9	71.3	65.6	70.1	70.9	69.9	71.9	75.5	69.3	70.7
Mid region	P	16.4	17.4	20.5	19.4	20.2	19.9	21.2	19.1	16.9	18.6	21.1	18.9
		15.6	17.6	21.1	18.8	20.2	20.1	19.8	19.6	17.9	17.9	20.3	19.5
	B	8.6	7.2	10.2	10.1	13.9	10.0	11.4	11.1	11.0	6.8	10.8	10.7
		8.3	6.8	10.0	9.9	14.1	9.8	9.3	10.4	10.2	6.6	10.4	9.8
	A	69.4	74.6	69.7	71.1	67.0	72.6	67.4	65.2	70.3	73.3	69.7	69.5
		74.7	74.0	70.0	71.1	71.3	71.1	66.1	65.8	71.4	73.7	69.0	70.7
Posterior dorsal blind	P	20.6	18.0	20.4	19.3	19.4	18.1	21.2	21.8	18.3	17.9	20.6	19.9
		16.3	18.2	20.3	18.9	18.2	19.3	21.7	21.1	17.9	19.2	20.8	18.8
	B	9.9	7.4	9.0	9.7	13.5	9.3	11.5	13.0	11.4	8.8	9.7	10.6
		9.0	7.7	9.7	10.0	10.4	9.6	12.4	13.1	10.7	7.1	10.2	10.5

* A - acetic acid P - propionic acid B - butyric acid

APPENDIX II

Concentrations of individual volatile fatty acids (m-mole/l.) in duplicate samples removed from each of three different regions of the rumen

Date of Sampling		30 . 7 . 64				31 . 7 . 64				1 . 8 . 64			
Location of sample		Time of sampling after commencement of feeding(hr.)				Time of sampling after commencement of feeding(hr.)				Time of sampling after commencement of feeding(hr.)			
		0	2	4.5	6.5	0	2	4.5	6.5	0	2	4.5	6.5
	A*	42.5	72.1	85.8	67.9	56.4	65.2	74.9	65.4	45.5	71.2	84.1	68.2
		43.2	75.4	88.3	77.7	56.5	61.2	75.0	55.5	47.8	66.4	88.7	73.1
Reticulum	P	9.4	16.7	23.7	17.8	16.5	18.4	22.4	18.5	11.0	16.8	24.9	19.2
		8.6	16.6	24.6	21.7	16.2	17.8	23.7	16.1	11.8	17.0	26.7	22.2
	B	5.1	7.5	10.6	9.0	11.3	8.9	11.9	11.2	7.7	6.7	10.7	10.2
		4.8	6.5	9.6	11.3	10.7	8.8	13.2	9.6	7.1	6.5	10.0	9.0
	A	46.4	73.0	101.5	72.7	62.4	61.8	79.5	69.0	53.3	72.8	94.8	76.2
		50.1	78.6	98.9	79.1	59.5	66.0	77.5	77.1	49.2	77.1	94.7	73.5
Mid region	P	10.2	16.8	30.1	20.1	19.1	17.6	25.0	18.9	12.5	18.1	29.4	20.4
		10.5	18.3	30.3	20.8	18.3	18.9	21.6	21.6	12.2	18.3	27.7	20.3
	B	5.3	7.0	14.9	10.5	13.2	8.8	13.4	11.0	8.1	6.6	15.0	11.6
		5.6	7.1	14.3	11.0	12.8	9.3	10.1	11.4	7.0	6.7	14.2	10.2
	A	48.9	71.0	91.3	78.5	57.5	66.4	84.5	74.7	50.1	69.4	93.6	74.1
		49.5	68.1	95.9	76.9	61.1	68.3	83.7	74.5	52.7	71.0	88.6	80.8
Posterior dorsal blind	P	14.6	17.2	26.7	21.4	16.6	16.6	26.6	25.1	13.0	17.0	27.7	21.2
		10.9	16.8	27.8	20.4	15.6	18.5	27.5	23.9	13.2	18.5	26.7	21.5
	B	7.0	7.0	11.8	10.7	11.6	8.6	14.4	14.9	8.1	8.3	13.0	11.3
		6.0	7.1	13.3	10.8	8.9	9.2	15.7	14.9	7.9	6.8	13.1	12.0

* A - Acetic acid P - Propionic acid B - Butyric acid

APPENDIX III

Variations in rumen ammonia concentrations (mg./100 ml.) in twin cows fed perennial ryegrass hay with and without the addition of 500 g. linseed oil

Experiment(v)

Date of sampling	Cow c					Cow C				
	Treatment	Time of sampling after commencement of feeding(hr.)				Treatment	Time of sampling after commencement of feeding(hr.)			
		0	2	4	6		0	2	4	6
Part (i)										
18/9/63	(no oil)	4.4	14.9	4.8	1.6	(no oil)	3.9	14.1	4.9	1.6
19/9/63	(linseed oil 500 g.)	4.7	11.5	6.2	2.5	(no oil)	5.1	11.9	5.0	2.1
20/9/63	(linseed oil 500 g.)	4.0	8.5	3.7	3.7	(no oil)	3.7	12.9	3.4	1.7
Part (ii)										
25/9/63	(no oil)	3.8	4.9	2.1	1.6	(no oil)	4.2	7.2	1.8	1.6
26/9/63	(no oil)	3.6	10.6	1.6	1.0	(linseed oil 500 g.)	2.6	8.8	3.7	2.2
27/9/63	(no oil)	2.9	5.1	1.4	3.7	(linseed oil 500 g.)	3.3	6.8	3.3	3.3

APPENDIX IV

Variation in rumen volatile fatty acid concentration (m-mole/l.) in twin cows fed perennial ryegrass hay with and without the addition of 500 g. linseed oil

Experiment v

Date of sampling	Cow c					Cow C				
	Treatment	Time of sampling after commencement of feeding(hr.)				Treatment	Time of sampling after commencement of feeding(hr.)			
		0	2	4	6		0	2	4	6
Part (i)										
18/9/63	(no oil)	62.9	93.7	62.0	67.2	(no oil)	72.2	99.1	60.7	64.9
19/9/63	(linseed oil 500 g.)	69.2	88.9	71.5	64.7	(no oil)	72.2	92.5	77.1	69.5
20/9/63	(linseed oil 500 g.)	68.0	80.8	77.1	78.9	(no oil)	62.8	84.3	81.0	67.4
Part (ii)										
25/9/63	(no oil)	50.8	89.7	71.1	71.3	(no oil)	72.5	109.0	79.5	75.7
26/9/63	(no oil)	56.1	81.3	70.8	67.7	(linseed oil 500 g.)	76.2	101.6	78.6	65.5
27/9/63	(no oil)	64.7	100.7	68.7	62.0	(linseed oil 500 g.	86.2	90.9	71.0	70.7

APPENDIX V

Concentrations of the individual volatile fatty acids (m-mole/l.) in twin cows fed perennial ryegrass hay with and without the addition of 500 g. linseed oil

Experiment v

Date of sampling		Time of sampling after commencement of feeding(hr.) Cow c 0	Cow c 6	Cow C 0	Cow C 6
Part (i)					
18/9/63	A[1]	48.9	49.2	53.5	47.5
	P	10.3	11.6	13.1	11.7
	B	3.7	6.4	5.6	5.7
19/9/63*	A	50.1	48.5	53.2	51.8
	P	11.8	10.8	13.0	11.5
	B	7.3	5.4	6.0	6.2
20/9/63*	A	45.0	49.8	46.1	47.9
	P	17.1	22.0	10.4	12.4
	B	5.9	7.1	6.3	7.1

Date of sampling		Time of sampling after commencement of feeding(hr.) Cow c 0	Cow c 6	Cow C 0	Cow C 6
Part (ii)					
25/9/63	A	37.2	49.1	51.2	51.6
	P	11.0	16.0	12.2	14.7
	B	2.6	6.2	9.1	9.4
26/9/63**	A	43.1	52.0	55.4	45.8
	P	10.3	11.6	13.0	12.0
	B	2.7	4.1	7.8	7.7
27/9/63**	A	52.0	48.3	60.6	46.2
	P	9.7	9.1	18.9	15.8
	B	3.0	4.6	6.7	8.7

* cow c received 500 g. linseed oil between 0 - 2 hr.

** cow C received 500 g. linseed oil between 0 - 2 hr.

[1] A - acetic acid P - propionic acid B - butyric acid

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STUDIES ON RUMEN METABOLISM. I.—Effect of Lipids on the Concentration of Ammonia, Total and Individual Volatile Fatty Acids in the Rumen

By J. A. ROBERTSON and J. C. HAWKE

The infusion of linseed oil or whale oil into the rumen of pasture-fed identical twin cows resulted in marked reductions in intake and in the concentrations of acetic and butyric acids in the rumen. Concentrations of propionic acid however remained at or above those found during the pre-treatment periods. Added linseed oil also resulted in an increase in the concentration of ammonia in the rumen.

Introduction

Until recently very little was known concerning the metabolism of dietary lipid in the ruminant. This was due, in part, to the limitations of the techniques available for lipid analysis and also to a lack of interest in lipids because of their relatively small contribution to the intake of dry matter by ruminants. The recent inclusion of animal fats and vegetable oils in animal rations[1] and the use of oils and tallow in the prevention and treatment of bloat[2] have served to accentuate the need for more information on lipid metabolism in the rumen, and in particular, the effect of lipids on protein and carbohydrate metabolism.

Several workers[3-6] have reported that the addition of fat in excess of 5% of dry matter to rations high in fibre reduced the digestibility of dry matter, organic matter, cellulose and protein. Brooks *et al.*[4] suggested that the coating of feed particles by fat could account for part of the decrease in digestibility. Chalmers,[7] investigating the effects of added oil on ammonia-levels in the rumen, found that when nitrogen levels in the feed were 3% of dry matter, the addition of oil resulted in a depression in the concentration of ammonia in the rumen, whereas at a nitrogen level of 4·5% the added oil resulted in an increased concentration.

Little information is available concerning the effect of lipids on rumen metabolism in animals fed on pasture. Furthermore, the observed fluctuations in the lipid content of pasture species with stage of growth[8] could result in a considerable variation in lipid intake and it was decided therefore to investigate the effect of oil on protein and carbohydrate metabolism in animals consuming freshly-cut pasture. Because the lipids of grasses and clovers contain predominantly linolenic and linoleic acids, linseed and whale oil were used in these experiments.

Experimental

Treatment of animals

Two pairs of identical-twin, non-lactating Jersey cows with rumen fistulas were used in the experiments. The animals were stall-fed on freshly-cut pasture, consisting of a perennial ryegrass and white clover mixture, and were trained to consume their feed in two periods of approximately 2 h. beginning at 9 a.m. and 4 p.m. Water and iodised salt were available *ad libitum*. Before the beginning of each series of experiments, the animals were stall-fed for a period of at least 7 days. The oils were administered as oil/water emulsions 50 : 50 (w/v) with 1% Lissapol NX (Imperial Chemical Industries Ltd.) as emulsifying agent, over the morning feeding period.

Experiment (i).—The animals (pair *a* A) weighed about 900 lb. Initially, a single dose of an emulsion containing 500 g. of linseed oil was infused into the rumen of cow *a* during one morning feeding period. A week later cow A received the same dosage on each of four consecutive mornings. Rumen samples were taken from each animal just before the morning feed and 2, 4 and 6·5 h. later.

Experiment (ii).—The animals (pair *b* B) weighed about 600 lb. An emulsion containing 750 g. of linseed oil was given to each animal on two consecutive days. The rumen of both animals were sampled on the day prior to dosage as well as on the two days of oil infusion.

Experiment (iii).—An emulsion containing 750 g. of whale oil (Perano Bros. Ltd., Picton, New Zealand) was infused into the rumen of cow B on two consecutive days. Rumen samples

were taken from both animals on the day prior to treatment and on each of the two days of dosage. One week later cow *b* received the same amount of emulsion on two consecutive days and both animals were sampled as before.

Rumen sampling

Preliminary experiments were made to determine if one region of the rumen could be selected for sampling purposes. Samples, to give approximately 200 ml. of liquor after being strained through muslin, were removed from the reticulum, the dorsal posterior blind sac and an area intermediate between the dorsal and ventral positions which was directly below the fistula. The results are presented in Fig. 1 and indicate that intermediate values for total volatile fatty acids (VFA) and ammonia were obtained from the position below the fistula and the lowest values for samples taken from the reticulum. These findings are in agreement with those of Bryant[9] and in subsequent trials it was decided to sample from the intermediate position only.

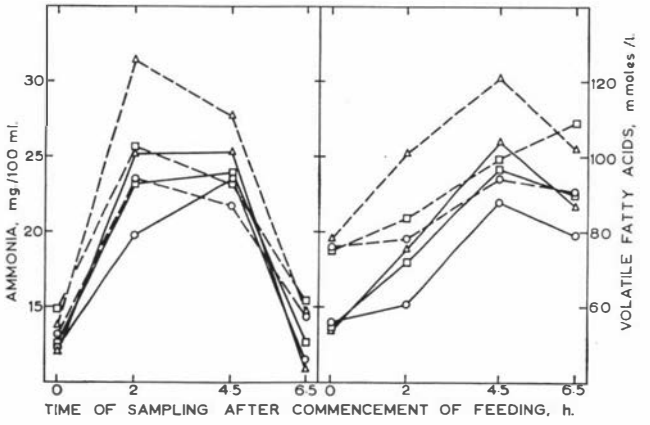

FIG. 1.—*Concentrations of ammonia and VFA in different regions of the rumen*

- - - - - - - Cow *a* ——— Cow A

○ Anterior dorsal region
□ Midway between dorsal and ventral regions
△ Posterior dorsal blind sac

Analytical methods

Pasture analysis.—Dry matter was determined by drying a sample for 16 h. in a forced-draught oven at 100° and total nitrogen by the Kjeldahl method modified for samples containing nitrate.[10] Methods for the extraction of soluble sugars and the hydrolysis of the hemicellulose and cellulose fractions were as described by Jarrige.[11] Soluble sugars and products of hydrolysis were determined by the method of Bath.[12] All analyses were carried out on samples obtained at the beginning of each experiment.

pH measurement.—Determinations were made on rumen liquor immediately after sampling, using a glass electrode (Radiometer pH Meter 23).

Ammonia was determined in 1 ml. of rumen liquor by the boric acid–hydrochloric acid procedure of Conway & O'Malley.[13]

Total volatile fatty acids.—Separation of VFA from 5 ml. of rumen liquor was effected by steam-distillation in a Markham still using the method of Johns,[14] 60 ml. of distillate being collected, followed by the collection of a further 60 ml. to serve as a blank. The distillates were then titrated with 0·05N-sodium hydroxide and phenolphthalein indicator. After addition of a small excess of alkali, the salts of the fatty acids were concentrated *in vacuo* to a suitable volume (0·5–1·0 ml.) for gas–liquid chromatographic analysis.

Individual volatile fatty acids.—The proportions of acetic, propionic and butyric acids in the volatile fraction were determined by gas–liquid chromatography[15] at 137° with the column packing described by Hawke.[16] The concentrated salts of the fatty acids were taken up in

a syringe and approximately 0·05 ml. was acidified in a platinum boat containing a mixture of equal parts by weight of Celite 545 and dry potassium bisulphate. The boat was then pushed into the heated region of the column and the nitrogen supply connected.

Fluid volume of the rumen.—The method of Hydén[17, 18] was used to determine fluid volume. A solution of 100 g. of polyethylene glycol (P.E.G.) (mol. wt. 4000) in 2 l. of water was sprayed into the rumen of cow *b* and cow B immediately before the morning feed, on two consecutive days. Rumen samples were taken at 2-h. intervals over a 12-h. period. The samples, which could not be clarified by filtration after the addition of barium chloride, barium hydroxide and zinc sulphate, were centrifuged at 10,000 *g* for 15 min. in a Spinco ultra-centrifuge.[19] The average values for the two days were 54 and 57 l. for cow *b* and cow B respectively.

Results

Pasture composition

Marked changes in the chemical composition of the pasture occurred between experiments (Table I). Soluble sugars were in highest concentration during late winter (August) when growth was slow and lowest during the period of rapid growth in the autumn (May). Conversely, protein was highest in the autumn sample and lowest in the late winter sample. Values for hemicellulose were similar to those for soluble sugars while cellulose remained relatively constant at about 18% of dry matter.

Table I

Chemical composition of pasture for the period of the experiments in which linseed oil and whale oil were infused into the rumen of twin fistulated cows

(results as % of dry weight)

Date of sampling	Dry matter	Soluble sugars	Hemi-cellulose	Cellulose	Total carbo-hydrate	Protein (N × 6·25)	Protein/ soluble carbo-hydrate ratio
Expt. (i) (30.8.61)	14·1	18·4	19·9	17·7	56·0	21·0	1·14 : 1
Expt. (ii) (13.11.61)	20·7	16·1	16·2	18·0	50·3	18·2	1·13 : 1
Expt. (iii) (7.5.62)	10·6	9·0	9·8	18·9	37·7	28·7	3·2 : 1

Feed intake

The infusion of 500 g. of emulsified linseed oil into the rumen of cow *a* in the first series of experiments appeared to depress intake slightly, although intake levels each day were sufficiently variable to make this uncertain (Table II). The treatment of cow A with the same weight of emulsified linseed oil produced a reduction in intake to about 70% of the pre-treatment level. The control animals maintained their intake close to the pre-treatment levels with the exception of a low intake for cow *a* on one day.

In experiment (ii) a marked drop in intake occurred with both animals (pair *b* and B) at the feeding period which followed the infusion into the rumen of 750 g. of emulsified linseed oil (Table II). Despite negligible intakes during some subsequent feeding periods considerable amounts of solid ingesta remained in the rumen.

A decreased feed intake also resulted when animals *b* and B were given 750 g. of emulsified whale oil. The drop in intake was 40–50% of the pre-experimental level which was not as great as that brought about by the infusion of the same weight of linseed oil (Table II).

Ammonia concentration in the rumen

The addition of oil would appear to affect nitrogen metabolism in the rumen since levels of ammonia were altered appreciably in all experiments (Fig. 2). In cow *a* (experiment i) the concentration rose from a normal maximum of 20–25 mg./100 ml. rumen liquor (at 2 h. after the commencement of feeding) to about 50 mg./100 ml. of rumen liquor after the addition of oil. At 4 h. the ammonia-concentration had dropped considerably but was still well above that for the control animal. After the infusion of oil into cow A the ammonia level was similar to that for the control animal until the afternoon of the third day of treatment when it rose

Table II

Intake of fresh pasture by stall-fed twin cows with and without added oil (lb.)

Experiment (i) Date	Oil added	Morning		Afternoon		Total	
		Cow *a*	Cow A	Cow *a*	Cow A	Cow *a*	Cow A
28.8.61	—	78	72	84	77	162	149
29.8.61	—	69	70	70	64	139	134
30.8.61	linseed, 500 g.	55*	80	71	81	126	161
11.9.61	—	66	54	71	84	137	138
12.9.61	linseed, 500 g.	54	63*	73	29	127	102
13.9.61	,, ,,	49	54*	54	37	103	91
14.9.61	,, ,,	70	50*	68	49	138	99
15.9.61	,, ,,	76	57*	64	45	140	102
Experiment (ii)							
		Cow *b*	Cow B	Cow *b*	Cow B	Cow *b*	Cow B
13.11.61	—	44	37	64	64	108	101
14.11.61	linseed, 750 g.	37	34*	53	9	90	43
15.11.61	,, ,,	31	2*	61	28	92	30
16.11.61	,, ,,	24*	0	9	9	33	9
17.11.61	,, ,,	3*	3	8	13	11	16
Experiment (iii)							
7.5.62	—	42	56	57	60	99	116
8.5.62	whale, 750 g.	55	56*	64	41	119	97
9.5.62	,, ,,	36	20*	54	28	90	48
16.5.62	—	56	44	51	44	107	88
17.5.62	whale, 750 g.	64*	44	41	43	105	87
18.5.62	,, ,,	29*	44	34	49	62	93

* animal received oil

to 50 mg./100 ml. of rumen liquor. The concentration in the sample taken prior to feeding on the fourth morning was 80 mg./100 ml. after which it dropped to a level below that found in samples from the control animal.

It was thought that increasing the amount of linseed oil from 500 g. to 750 g. would establish whether the irregular increases in ammonia concentrations in experiment (i) were due to the addition of oil. However, although the ammonia concentrations in the rumen of the experimental animals were higher than those in the controls, the very large increases which were found occasionally in the first experiment were not obtained. In experiment (ii) the concentrations of ammonia in the rumen were lower in the control animals than in the previous experiment (Fig. 3) and this was probably due to the lower nitrogen levels in the pasture (Table I). The values for ammonia for both animals on the day before the addition of oil were almost identical, rising to a peak of about 15 mg./100 ml. of rumen liquor at 2 h. and decreasing slowly over the next few hours. On the first day of treatment the initial rise at 2 h. was followed by further increases at 4 and 6 h. reaching a maximum of about 20 mg./100 ml. of rumen liquor at 6 h. The level the following day remained almost constant at 13–15 mg./100 ml. despite a much lower feed intake.

It was thought that the severe effect of the larger dose of linseed oil on intake might have been due to a characteristic of the oil such as the presence of a cyanogenetic glucoside. Consequently whale oil, another highly unsaturated oil, was used in experiment (iii). The effect of ammonia concentration after addition of 750 g. whale oil to cows *b* and B is shown in Fig. 4. Concentrations for both animals immediately before treatment were almost identical (max. 60–65 mg./100 ml. of rumen liquor) and followed very similar patterns subsequent to treatment. The oil had no effect until the second day when concentrations remained almost constant at between 30–40 mg./100 ml. of rumen liquor.

pH measurements of rumen liquor

In general, pH and total VFA concentrations showed an inverse relationship. In experiment (ii), rumen pH for the control animals varied from a maximum of approximately 7·0 to a minimum of approximately 6·7. Little change from the control values was found on the

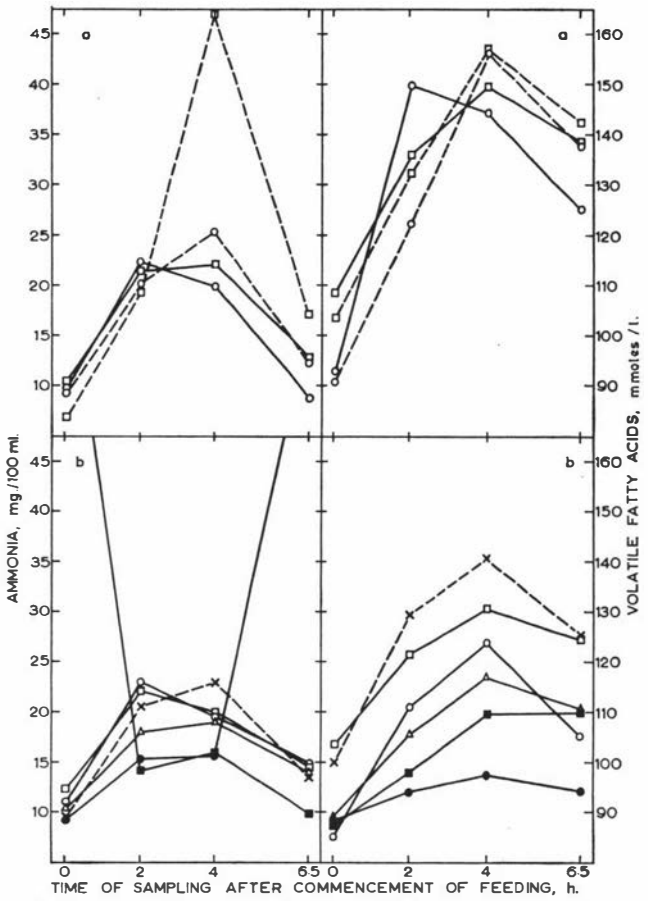

FIG. 2.—*Variations in rumen ammonia and VFA concentrations in twin cows stall-fed on freshly-cut mixed pasture with and without* 500 *g. of added linseed oil*

a. 29.8.61 Cow *a* (without oil) ○ - - - - ○ Cow A (without oil) ○——○
30.8.61 Cow *a* (with oil) □ - - - - □ Cow A (,, ,,) □——□

b. 11–15.9.61 Cow *a* (without oil) × - - - - × (average for 5 days)
11.9.61 Cow A ,, ,, (without oil)" ○——○
12.9.61 ,, ,, ,, ,, □——□
13.9.61 ,, ,, ,, ,, △——△
14.9.61 ,, ,, ,, ,, ●——●
15.9.61 ,, ,, ,, ,, ■——■

first day of oil addition, but on the second day the pH in the samples from both treated animals rose to maxima of about 7·6 immediately after feeding and remained relatively constant throughout the day. The pH of samples from control animals in the experiment with whale oil varied from 7·1 to 6·2. The addition of oil appeared to decrease this variation of pH throughout the hours of sampling.

Concentration of total volatile fatty acids in the rumen

No apparent change occurred in the concentration of volatile acids in samples taken from cow *a* after infusion of 500 g. linseed oil (Fig. 2). Values for both animals varied from a minimum of approximately 90 mmoles/l. of rumen liquor before feeding, to a maximum of 145–160 mmoles/l. 4 h. after the commencement of feeding. The maximum concentrations in cow A decreased slightly on each successive day of treatment. This decrease was paralleled by a tendency for intake to fall on successive days of treatment (Table II).

VFA concentrations in cows *b* and B were similar before treatment in experiment (ii) and were well below those found during the pre-treatment period in experiment (i). The concentration of VFA in samples from both animals on the first day of oil dosage, followed the same

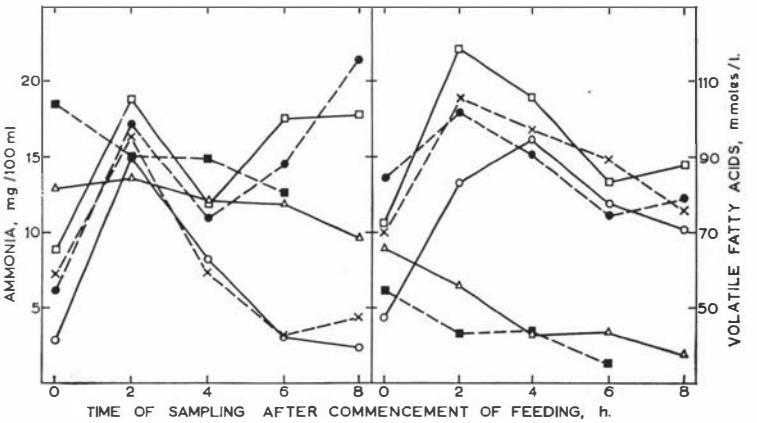

FIG. 3.—*Variations in rumen ammonia and VFA concentrations in twin cows stall-fed on freshly-cut mixed pasture with and without 750 g. of added linseed oil*

13–15.11.61 cow *b* (without oil) ×----× (average for 3 days)
16.11.61 ,, ,, (with oil) ●----●
17.11.61 ,, ,, ,, ,, ■----■

13.11.61 cow B (without oil) ○——○
14.11.61 ,, ,, (with oil) □——□
15.11.61 ,, ,, ,, ,, △——△

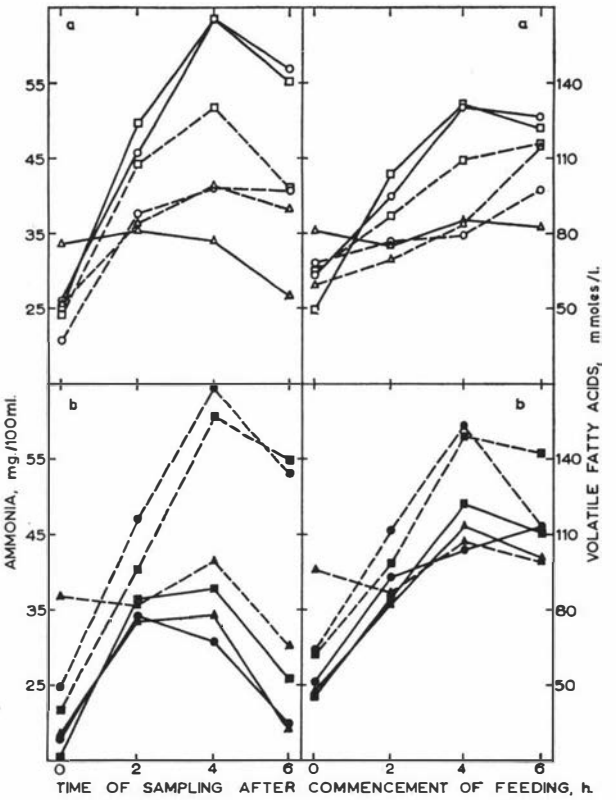

FIG. 4.—*Variation in rumen ammonia and VFA concentrations in twin cows stall-fed on freshly-cut mixed pasture with and without addition of 750 g. of whale oil*

a. 7.5.62 Cow *b* (without oil) ○----○ Cow B (without oil) ○——○
8.5.62 ,, ,, ,, ,, □----□ ,, ,, (with oil) □——□
9.5.62 ,, ,, ,, ,, △----△ ,, ,, ,, ,, △——△

b. 16.5.62 Cow *b* (without oil) ●----● Cow B (without oil) ●——●
17.5.62 ,, ,, (with oil) ■----■ ,, ,, ,, ,, ■——■
18.5.62 ,, ,, ,, ,, ▲----▲ ,, ,, ,, ,, ▲——▲

pattern and were about the same level as during the control period but decreased in subsequent samples on the second day.

The administration of 750 g. of emulsified whale oil had an effect on total VFA concentrations similar to that produced by the same weight of linseed oil. On the first day of treatment the concentrations of VFA in samples from animals *b* and B followed those found on the pre-treatment day. Concentrations obtained the following day showed much less overall variation and were lower than those found in the earlier experiment.

Individual volatile fatty acids

The concentrations of acetic, propionic and butyric acids (mmoles/l. of rumen liquor) were altered appreciably by the addition of 500 g. of linseed oil to the rumen (Table III). In cow *a*, there was an almost immediate decrease in the concentration of acetic acid to about half that present in the pre-experimental period, and a considerable increase in the concentrations of propionic and butyric acids. The oil had no effect on the concentrations of the acids in cow A until the third day of infusion when the amount of propionic was well above previous levels despite a decrease in total concentration of acids. By the fourth day, the concentrations of acetic and propionic acids were approximately equal whereas prior to oil infusion the acetic/propionic ratio had been approximately 4·5 : 1.

Changes in concentration of the fatty acids after infusion of 750 g. of emulsified linseed oil were observed on the second day. Although the total concentration of VFA decreased to about half, there was almost no change in the concentration of propionic acid ; acetic and butyric acids were both reduced to between $\frac{1}{3}$ and $\frac{1}{2}$ of the former levels.

When the same weight of whale oil was introduced into the rumen, no obvious effect on the concentration of individual acids occurred until the second day of infusion. As in the previous experiment, the concentration of propionic acid remained at, or was higher than, the control level despite a decrease in the concentration of total fatty acids in the rumen.

Discussion

The addition of linseed oil resulted generally in increased concentrations of ammonia in the rumen. Unless the oil acted as a physical barrier to absorption, it is unlikely that these increases were due to decreased absorption from the rumen since the higher pH values in the animals given oil compared with those for the controls, especially in experiment (ii), would have favoured an increase in the absorption rate.[20] Therefore, the increases in the concentration of ammonia appear to have been due to an effect of oil on protein metabolism and would have occurred as a result of either increased deamination of the amino-acids in the rumen or reduced utilisation of ammonia by the rumen micro-organisms. Davison & Woods[6] found that the addition of maize oil to rations high in roughage resulted in a decreased retention of nitrogen and, from the present work, it appears that increased ammonia levels in the rumen might have caused higher losses of urinary and faecal nitrogen. On the other hand, a decrease in ammonia concentration occurred after the addition of whale oil. This was possibly due to the decrease in feed intake and hence protein intake, although decreases in intake did not have this depressing effect on the ammonia concentration when linseed oil was used. The relationship between ammonia production and the amount of oil and protein ingested appears to be rather complex since Chalmers[7] has found that when nitrogen levels in the feed were 3% of the intake of dry matter, the addition of oil to the ration depressed rumen-ammonia concentrations, but when nitrogen levels were 4·5% of the dry matter intake, increased ammonia concentrations occurred. In the present experiments, the nitrogen levels in the pasture were approximately 3 and 4·5% of the dry matter for the linseed and whale oil experiments, respectively.

The high protein levels found in New Zealand pastures, particularly in late autumn and early spring,[21] might be expected to lead to conditions of ammonia toxicity. In the present experiments, maximum ammonia concentration in the rumen (60–65 mg./100 ml.) occurred at the time when pasture levels of protein were highest and of carbohydrate lowest. Although no toxic symptoms were noticed, these levels were of the magnitude found by other workers[22, 23] to cause an increase in the level of ammonia in peripheral blood with eventual toxic effects on the animal.

Table III

Concentration of the individual volatile fatty acids (mmoles/l.) in the rumen of twin cows stall-fed on freshly-cut mixed pasture with and without added oil

Experiment (i)

Date			Time of sampling after commencement of feeding (h.) 0	2	4	6·5
	Cow *a*	A*	67·4	89·1	102·6	93·7
	(no oil)	P	8·5	19·4	28·5	21·4
		B	14·8	13·7	25·3	22·5
29.8.61						
	Cow A	A	62·2	100·9	93·3	84·2
	(no oil)	P	16·0	28·0	28·6	22·3
		B	13·8	20·8	22·5	18·9
	Cow *a*	A	72·9	53·8	56·8	45·2
	(linseed	P	13·8	45·2	67·5	59·2
	oil, 500 g.)	B	17·0	33·3	32·9	38·2
30.8.61						
	Cow A	A	73·7	84·5	92·7	101·9
	(no oil)	P	17·7	31·0	27·7	21·8
		B	16·5	20·7	29·2	14·9
	Cow *a*	A	69·9	75·7	100·0	95·5
	(no oil)	P	15·1	24·9	22·6	20·0
		B	17·0	26·4	20·2	15·5
11.9.61						
	Cow A	A	—	83·9	89·1	63·5
	(no oil)	P	—	16·2	17·9	24·0
		B	—	10·9	17·4	17·8
	Cow *a*	A	—	86·0	98·3	87·7
	(no oil)	P	—	29·1	27·2	24·0
		B	—	21·1	20·0	17·2
12.9.61						
	Cow A	A	78·1	72·3	84·1	79·6
	(linseed	P	13·2	26·4	23·8	22·5
	oil, 500 g.)	B	12·3	22·8	22·7	22·5
	Cow *a*	A	57·2	74·5	83·1	77·4
	(no oil)	P	12·1	22·6	28·6	17·2
		B	11·9	19·7	14·7	13·9
13.9.61						
	Cow A	A	54·1	61·8	68·2	65·0
	(linseed	P	21·7	28·5	32·0	29·6
	oil, 500 g.)	B	13·3	15·4	16·8	16·1
	Cow *a*	A	76·6	90·5	92·8	83·6
	(no oil)	P	16·7	22·8	27·6	25·3
		B	11·3	20·8	22·5	18·5
14.9.61						
	Cow A	A	46·3	55·4	51·2	45·9
	(linseed	P	29·8	31·2	34·7	41·6
	oil, 500 g.)	B	11·9	7·5	11·6	6·6
	Cow *a*	A	76·6	87·2	95·6	87·1
	(no oil)	P	19·4	25·3	27·4	25·4
		B	14·9	20·5	19·6	16·9
15.9.61						
	Cow A	A	35·3	38·1	44·1	52·0
	(linseed	P	42·8	42·6	48·8	49·2
	oil, 500 g.)	B	9·1	17·2	16·7	9·2

Experiment (ii)

Date			Time of sampling after commencement of feeding (h.) 0	2	4	6	8
	Cow *b*	A	45·5	73·5	69·0	61·6	58·7
	(no oil)	P	8·3	18·9	16·8	14·3	9·2
		B	5·8	6·5	12·4	11·8	6·9
13.11.61							
	Cow B	A	37·2	63·9	71·9	55·3	53·2
	(no oil)	P	5·7	10·9	13·2	14·1	10·6
		B	4·6	8·2	9·3	7·9	6·6
	Cow *b*	A	59·9	76·2	75·7	63·1	59·1
	(no oil)	P	12·3	23·1	19·6	13·3	12·5
		B	9·8	13·1	15·1	16·6	9·3
14.11.61							
	Cow B	A	52·9	80·6	74·8	52·5	52·7
	(linseed	P	11·7	22·8	17·3	18·6	23·3
	oil, 750 g.)	B	6·8	15·0	13·7	12·0	12·0
	Cow *b*	A	45·7	69·9	58·3	62·4	49·5
	(no oil)	P	11·8	23·2	14·0	13·4	12·4
		B	11·0	11·6	9·5	10·5	9·2
15.11.61							
	Cow B	A	35·6	31·6	21·3	24·3	21·3
	(linseed	P	25·8	18·9	18·4	14·8	12·9
	oil, 750 g.)	B	4·1	5·4	2·9	4·0	3·2
	Cow *b*	A	64·7	74·6	75·0	52·1	51·6
	(linseed	P	12·4	16·9	12·1	11·6	15·8
	oil, 750 g.)	B	7·2	10·4	3·4	10·7	11·5
16.11.61							
	Cow *b*	A	—	27·5	26·8	24·0	—
	(linseed	P	—	11·2	12·4	8·7	—
	oil, 750 g.)	B	—	4·2	4·1	2·5	—
17.11.61							

Experiment (iii)

Date			Time of sampling after commencement of feeding (h.) 0	2	4	6
	Cow *b*	A	46·4	51·6	49·8	61·3
	(no oil)	P	11·1	15·9	19·7	19·4
		B	10·7	9·0	9·8	16·7
7.5.62						
	Cow B	A	44·3	66·3	85·7	85·3
	(no oil)	P	10·5	16·8	23·1	18·1
		B	8·8	11·7	21·5	23·1
	Cow *b*	A	45·0	61·2	73·3	73·6
	(no oil)	P	11·0	17·6	21·8	22·7
		B	9·1	8·2	14·0	19·3
8.5.62						
	Cow B	A	37·6	71·2	81·6	70·4
	(whale oil,	P	10·2	18·8	26·7	26·6
	750 g.)	B	11·6	13·4	23·4	25·0
	Cow *b*	A	14·1	45·9	57·3	76·1
	(no oil)	P	11·0	12·4	16·0	23·4
		B	7·8	11·5	10·9	15·8
9.5.62						
	Cow B	A	43·0	36·2	40·1	42·2
	(whale oil,	P	26·6	27·7	33·6	29·4
	750 g.)	B	11·5	11·9	11·5	11·4
	Cow *b*	A	44·2	80·3	104·7	72·9
	(no oil)	P	9·9	20·6	29·6	19·7
		B	9·9	10·3	19·5	20·3
16.5.62						
	Cow B	A	36·6	60·9	71·0	76·1
	(no oil)	P	8·6	19·1	19·3	21·8
		B	5·9	12·9	12·9	14·4
	Cow *b*	A	54·1	64·4	99·3	93·1
	(whale oil,	P	7·2	20·5	27·7	26·2
	750 g.)	B	9·9	13·7	21·9	22·9
17.5.62						
	Cow B	A	31·6	60·0	84·0	77·3
	(no oil)	P	7·6	17·7	24·5	21·4
		B	6·3	7·6	13·9	12·0
	Cow *b*	A	49·2	46·8	57·3	55·2
	(whale oil,	P	26·9	26·7	34·5	32·7
	750 g.)	B	19·8	12·7	15·4	12·9
18.5.62						
	Cow B	A	33·2	57·0	73·3	70·0
	(no oil)	P	7·8	18·2	23·6	18·6
		B	6·6	7·7	17·1	11·7

* A = acetic acid P = propionic acid B = butyric acid

In considering the effect of the metabolism of glycerides on the amounts of VFA produced, it can be calculated that complete hydrolysis of 500 g. of linseed oil and the quantitative fermentation of glycerol to propionic acid could account for an increase in the concentration of propionic acid in the rumen of about 10 mmoles/l. (fluid volume as determined by the P.E.G. method was approximately 55 l.). In experiments (i) and (iii) increases in propionic acid concentration equalled or exceeded the calculated value. Moreover, other workers[24, 25] have shown that glycerol is fermented relatively slowly in the rumen and that the volatile acids, predominantly propionic, did not account for more than 50% of the glycerol metabolised.[26] Therefore very little of this increase can be attributed to the oil itself and appears likely to have been brought about by the effects of oil on the digestion of carbohydrates.

The most pronounced effect of added oil on the metabolism of carbohydrates was a decrease in concentration of acetic acid in the rumen. Some of this decrease was undoubtedly due to the reduction in intake which occurred in varying degrees following oil infusion. However, in experiment (i) in particular, large reductions in the concentration of acetic acid occurred and were accompanied by only small reductions in feed intake. It is therefore probable that the oil had a direct effect on the metabolism of the carbohydrate constituents in the pasture. Other workers[4, 6, 27] found that cellulose digestion was reduced when maize oil was added to low-quality rations for sheep, and Brooks *et al.*[4] postulated that this resulted from the cellulose becoming coated with oil which reduced its availability to cellulolytic enzymes. The present work would appear to support this hypothesis, since rations high in cellulose are known to result in high proportions of acetic acid being formed in the rumen and any decrease in cellulose digestion should therefore result in reductions in acetic acid concentrations. *In vitro* experiments to determine the effect of oil on cellulose digestion are in progress.

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References

[1] Ault, W. C., Riemenschneider, R. W., & Saunders, D. H., *U.S. Dept. Agric., Agric. Res. Service, Utilisation Res. Rep.*, 1960, No. 2
[2] Reid, C. S. W., & Johns, A. T., *N.Z. J. Sci. Tech.*, 1957, **A38,** 908
[3] Swift, R. W., Cowan, R. L., Barron, G. P., Maddy, K. H., & Grose, E. C., *J. Anim. Sci.*, 1951, **10,** 434
[4] Brooks, C. C., Garner, G. B., Gehrke, C. W., Muhrer, M. E., & Pfander, W. H., *J. Anim. Sci.*, 1954, **13,** 758
[5] Brethour, J. R., Sirny, R. J., & Tillman, A. D., *J. Anim. Sci.*, 1958, **17,** 171
[6] Davison, K. L., & Woods, W., *J. Anim. Sci.*, 1960, **19,** 54
[7] Chalmers, M. I., *Abstr. 5th Int. Congr. on Nutrition* (Washington, D.C.), 1960
[8] Hawke, J. C., *J. Dairy Res.*, 1963, **30,** 67
[9] Bryant, A. M., M. Agr. Sc. Thesis, Univ. of New Zealand, 1961
[10] 'Official Methods of Analysis', 9th edn, 1960 (Washington, D.C.: Ass. off. Agric. Chemists)
[11] Jarrige, R., *Proc. 8th Int. Grassland Congr.*, 1960, 628
[12] Bath, I. H., *Analyst*, 1958, **83,** 451
[13] Conway, E. J., & O'Malley, E., *Biochem. J.*, 1942, **36,** 655
[14] Johns, A. T., *N.Z. J. Sci. Tech.*, 1955, **A37,** 323
[15] James, A. T., & Martin, A. J. P., *Biochem. J.*, 1952, **50,** 679
[16] Hawke, J. C., *J. Dairy Res.*, 1957, **24,** 366
[17] Hydén, S., *Ann. Agric. Coll. Sweden*, 1956, **22,** 139
[18] Hydén, S., *Ann. Agric. Coll. Sweden*, 1958, **24,** 55
[19] Ullyatt, M. J., personal communication
[20] Coombe, J. B., Tribe, D. E., & Morrison, J. W. C., *Aust. J. agric. Res.*, 1960, **11,** 247
[21] Johns, A. T., *N.Z. J. Sci. Tech.*, 1955, **A37,** 301
[22] Head, M. J., & Rook, J. A. F., *Nature, Lond.*, 1955, **176,** 262
[23] Lewis, D., Hill, K. J., & Annison, E. F., *Biochem. J.*, 1957, **66,** 587
[24] Johns, A. T., *N.Z. J. Sci. Tech.*, 1953, **A35,** 262
[25] Garton, G. A., Hobson, P. N., & Lough, A. K., *Nature, Lond.*, 1958, **182,** 1511
[26] Garton, G. A., Lough, A. K., & Viogue, E., *J. gen. Microbiol.*, 1961, **25,** 215
[27] White, T. W., Grainger, R. B., Baker, F. H., & Stroud, J. W., *J. Anim. Sci.*, 1958, **17,** 797

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STUDIES ON RUMEN METABOLISM. II.*—*In vivo* Hydrolysis and Hydrogenation of Lipid

By J. C. HAWKE and J. A. ROBERTSON

The hydrolysis and hydrogenation of grass and clover lipids and linseed oil have been followed in fistulated twin cows. Mono- and di-glycerides, in addition to fatty acids, were found in the rumen liquor after infusion of linseed oil into the rumen. The proportions of linoleic acid found in rumen liquor 6·5 h. after infusion of linseed oil were above normal, but the proportions of linolenic acid had returned to about the pre-feeding level in this time. The free fatty acids were more saturated than the unhydrolysed triglycerides, but the proportions of unsaturated fatty acids in the partially hydrolysed glycerides were not greatly different to those of the triglycerides.

Introduction

The modification of dietary lipids in the rumen is of interest in so far as it influences the composition of depot and milk fats of ruminants.[1] One of the important activities of rumen micro-organisms is the hydrogenation of unsaturated fatty acids, a process which has been followed by *in vitro* incubation experiments[2, 3] and by the post-slaughter analysis of rumen contents.[4] In a detailed analysis, Shorland *et al.*[5] have shown that *trans*- and positional isomers of unsaturated acids are among the products of hydrogenation of highly unsaturated fatty acids.

More recently Garton *et al.*[6] found that lipolysis, resulting in the liberation of free fatty acids from neutral lipids, also occurs when neutral lipids are incubated with rumen contents, and Hobson & Mann[7] isolated lipolytic bacteria from the rumen. Garton *et al.*[8] and Wright,[9] in comparing the hydrolysis of a number of triglycerides, found extreme variations in the amounts of free fatty acids released after 22 or 24 h. (15–90% for linseed oil). It appears that the triglycerides of low molecular weight are the most rapidly hydrolysed and the saturated triglycerides of high molecular weight the least rapidly hydrolysed. Garton *et al.*[8] could find no evidence for the presence of mono- and di-glycerides in their incubation experiments. The free fatty acids released were more saturated than the corresponding triglycerides which indicated that unsaturated fatty acids are more rapidly hydrogenated in the free state than when combined with glycerol.

Almost all the information on the hydrogenation of lipids has been obtained from *in vitro* and post-slaughter experiments. In one of the few *in vivo* experiments reported, Hoflund *et al.*[10] obtained evidence for the reduction of linolenic acid to linoleic acid by following changes in the iodine value of lipids in the rumen after the introduction of emulsified linseed oil through rumen fistulas.

In the following *in vivo* experiments, the relationship between the hydrolysis and hydrogenation of lipids, and the rates of hydrogenation of grass lipids and linseed oil in the rumen have been followed. Also the degree of hydrogenation of free fatty acids, partial hydrolysis products of triglycerides and unhydrolysed triglycerides has been compared.

Experimental

Treatment of animals and collection of rumen samples

Two pairs of non-lactating identical twin Jersey cows with rumen fistulas (denoted pair *a* A and *b* B) were used at different stages of the experiments. The animals were stall-fed on a freshly-cut mixed pasture of grass and clover approximately 12 in. long.[11] After a pre-experimental period of at least 7 days, linseed oil (500 g.) in the form of an oil–water emulsion (50 : 50) with 1% Lissapol NX (Imperial Chemical Industries Ltd.) as emulsifying agent, was infused through the middle section of the closed fistula over the 2-h. morning feeding period which followed an overnight fast.

Samples were obtained from the area of the rumen just below the fistula, prior to and following the morning feeding period. Rumen fluids were separated from the solid ingesta by straining the rumen samples through two layers of muslin.

* Part I : preceding paper

Extraction of lipid from rumen fluids

The lipid concentrations and compositional changes in the lipids were followed in total rumen fluids as obtained above, and in the clear supernatant which was obtained by centrifugation of a further portion of the sample for 30 min. at 14,600 *g*. Both the rumen fluids and the supernatant fraction were diluted with an equal volume of absolute ethanol and evaporated to dryness at approximately 60° *in vacuo* in a rotary evaporator. The residue was extracted with diethyl ether and the extract washed several times with water to remove non-lipid impurities. The ether extract was then evaporated to dryness, the final stages being carried out *in vacuo*. The extraction procedure described by Hilditch[12] was used to separate fatty acids from the neutral lipid.

Separation of lipids by thin-layer and column chromatography on silicic acid

Qualitative separations of rumen lipids into fatty acids, di- and tri-glycerides were made on thin layers of Silica Gel G (Merck, Germany). Slurries of silicic acid were prepared by the usual technique[13] and applied to glass plates by the method of Lees & De Muria.[14] Light petroleum–diethyl ether–acetic acid (90 : 10 : 1, by vol.), light petroleum–diethyl ether–acetic acid (60 : 40 : 1, by vol.) and toluene–ethyl acetate–ethanol–acetic acid (10 : 5 : 5 : 0·1, by vol.) solvent mixtures were used to develop the chromatograms and the lipids were detected under ultra-violet light after spraying with 0·2% 2′,7′-dichlorofluorescein in ethanol.[15]

Rumen lipids were also chromatographed on columns of silicic acid (Mallinckrodt) by the procedure of Hirsch & Ahrens[16] except that smaller columns were used. These columns (12 cm. × 1 cm.) of silicic acid (7 g.) did not separate triglycerides and fatty acids but enabled monoglycerides to be eluted in less than 500 ml. of solvent. Triglycerides and fatty acids were separated after column chromatography by the usual solvent extraction procedures.[12]

Separation and determination of fatty acids

The lipid fractions were saponified by refluxing with ethanolic potash for 3 h. and the non-saponified material was then removed by ether extraction. The fatty acids were then converted to methyl esters by refluxing with 1% sulphuric acid in methanol[12] and these were analysed by gas–liquid chromatography in a Pye Argon Chromatograph. To identify the methyl esters, R_F values were determined on 4 ft. × 4 mm. i.d. columns of two types, (*a*) 20% Apiezon M on Celite 545 (48–85 mesh) held at 200° and (*b*) 20% succinic acid/diethylene glycol on Celite 545 (48–85 mesh) held at 150°.[17] The proportions of each component were determined by measurement of peak areas.

Results

Variations with feeding in the amounts of lipid in rumen liquor

The experiments described in the previous paper[11] showed a regular pattern of changes in the levels of ammonia and volatile fatty acids in the rumen following stall-feeding on fresh pasture. Increases in the lipid content also occurred soon after feeding commenced (Table I) in both the total rumen liquor and in the supernatant fraction. The lipid of the total rumen liquor would have included lipid from bacteria, protozoa, partly digested plant material and the rumen fluid. The rumen fluid did make some contribution to this increase but, soon after the commencement of feeding, it is assumed that partly digested plant material would have been a major contributor. The lipid levels returned to approximately the pre-feeding levels in 6 h. even when linseed oil was infused into the rumen. A large proportion of this linseed oil probably adhered to the solid ingesta, since, in later *in vitro* experiments in which 4 g. of linseed oil were added to 100 ml. of rumen liquor, it was found that approximately 80% of the oil became adhered to the solid material.

The proportions of free fatty acids in the total lipid of the supernatant varied considerably. High values (>35%) were obtained on two days and on one day very much lower values (8–28%) were obtained. In general, the free fatty acid levels increased in the period of feeding and a high level of lipase activity in the rumen was evident.

Table I

Lipid levels in rumen liquors obtained from twin cows fed on fresh pasture with and without the addition of linseed oil

(Lipid as mg./100 ml. of rumen liquor)

Sample time after commencement of feeding, h.	31.7.61		29.8.61		30.8.61*		12.9.61†	
	Total liquor	Supernatant‡	Total liquor	Supernatant	Total liquor	Supernatant‡	Total liquor	Supernatant‡
Cow A								
0	72	11 (61)	71	11	50	20 (8)	173	38 (74)
2	108	19 (75)	132	23	464	148 (22)	216	42 (69)
4·5	83	5 (40)	132	31	151	14 (27)	261	42 (71)
6·5	55	7 (69)	100	18	—	10 (10)	105	34 (71)
Cow *a*								
0	68	7 (45)	66	14	34	12 (14)	165	30 (41)
2	126	14 (57)	122	55	192	16 (28)	684	138 (69)
4·5	53	16 (35)	96	14	135	19 (14)	104	48 (75)
6·5	47	4 (50)	52	10	—	13 (10)	104	42 (86)

* 500 g. of linseed oil added to the rumen of Cow A after taking the first sample (0 h.)
† 500 g. of linseed oil added to the rumen of Cow *a* after taking the first sample (0 h.)
‡ % free fatty acids in brackets

Fatty acid composition of rumen liquor

Palmitic, stearic, oleic, linoleic and linolenic acids were usually the major fatty acid constituents of rumen liquor obtained from cows feeding on fresh pasture. Appreciable proportions of myristic acid were found on one occasion (Table II) but otherwise 1·0–3·0% was the more usual proportion of this acid. In addition to myristic, palmitic and stearic acids the remaining saturated fatty acids from C_{12} to C_{20} were present as minor constituents. Small proportions (<3·5%) of C_{15} and C_{17} branched-chain acids were also present in rumen liquor. In order to simplify the comparisons of major constituents, values for the minor constituents have not been presented in the tables. Fatty acids above C_{20} were not determined.

The proportions of the major fatty acid components after an overnight fast varied considerably (Tables II and III). Stearic acid was quantitatively the most important fatty acid, followed by palmitic and oleic acids. The unsaturated fatty acids did not make a large contribution to the total fatty acids present and again appreciable fluctuations in the proportions of these acids occurred.

Despite the considerable variability in the relative proportions of the major components, a distinct pattern of changes in composition was apparent with increased time after the feeding of fresh grass and clover. These changes were consistent with the known hydrogenation reactions in the rumen, and, after a rise in the proportions of the unsaturated fatty acids after feeding, there was a progressive shift from linoleic to oleic. The data obtained from cow *a* (Table III) showed that there may be some delay in release of plant lipid as there were considerable increases in the proportions of palmitic acid 6·5 h. after the commencement of feeding.

Four and a half and six hours after the infusion of linseed oil into the rumen there was a very rapid decrease in the proportions of linolenic acid, accompanied by increases in the proportions of linoleic, oleic, and stearic acids as a result of *in vivo* hydrogenation. A decrease in the amount of lipid present in the rumen liquors accompanied these changes (Table I).

Table II

Changes in the fatty acid composition of total rumen liquor after feeding fresh pasture

(54 lb. to Cow A—31.7.61)

Sampling time after commencement of feeding, h.	Fatty acids (moles-%)					
	C_{14}	C_{16}	C_{18}	$C_{18}{}^{1}$-	$C_{18}{}^{2}$-	$C_{18}{}^{3}$-
0	9·2	27·4	41·6	21·6	1·5	—
2	23·1	16·3	24·7	20·7	7·8	7·3
4·5	6·0	16·7	49·2	21·0	6·4	0·8
6·5	—	39·1	30·5	15·6	10·9	3·9

Table III

Comparison of changes in the fatty acid composition of total rumen liquor from twin cows

[Cow A fed on fresh pasture (55 lb.) and linseed oil (500 g.) added to the rumen. Cow *a* fed on fresh pasture (80 lb.) 30.8.61]

	Fatty acids (moles-%)				
	C_{16}	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
Composition of linseed oil	6·1	2·9	16·7	17·6	56·7
Sampling time after commencement of feeding, h.					
Cow A (linseed oil added)					
0	23·8	32·9	26·4	12·2	4·7
2	6·7	3·4	17·0	18·7	54·2
4·5	8·0	9·6	33·9	29·2	19·3
6·5	10·6	18·3	37·9	28·9	3·3
Cow *a*					
0	23·0	54·5	6·1	12·3	4·1
2	17·0	57·2	13·8	4·5	7·5
4·5	18·1	44·7	19·1	8·6	9·5
6·5	31·2	45·2	17·2	6·2	0·2

Fatty acid composition of rumen supernatant

Only small amounts of lipid were released into the supernatant after feeding on fresh forage alone (Table I). At the conclusion of the feeding period, i.e., at 2 h., increased proportions of oleic acid were present and at 4·5 h. some linoleic acid also appeared (Table IV, cow *a*). In addition appreciable proportions of myristic acid and some unidentified fatty acids of lower molecular weight (C_{12}–C_{14}) were present.

Even a short time after the infusion of linseed oil, only about one-third of the total lipid in the rumen liquor was in the supernatant fraction, and the degree of unsaturation of the lipid in this fraction decreased rapidly, very little unsaturated fatty acid remaining at 6·5 h. (Table IV, cow A).

Further examination of the lipids in the rumen supernatant 4·5 h. after infusion of linseed oil into the rumen, was carried out after fractionation into free fatty acid, triglyceride, diglyceride and monoglyceride on silicic acid columns (Table V). Only 41% of the lipid eluted from these columns was triglyceride and hydrolysis products of triglyceride. The remainder was green and yellow pigment which was eluted with light petroleum, and phosphorus-containing lipid mixed with green pigment which was eluted with methanol. Compared with the results of a similar experiment given in Table IV, the lipids showed a greater overall hydrogenation at 4·5 h. The free fatty acid fraction was more saturated than the triglyceride fraction, while the partially hydrolysed glycerides were of intermediate saturation. Despite being the least

Table IV

Comparison of changes in the fatty acid composition of the major constituents of the supernatant obtained from rumen liquor

(Samples corresponding to those in Table III for total rumen liquor)

Sampling time after commencement of feeding, h.	Fatty acids (moles-%)										
	C_{14}	C_{15iso}	C_{15}	C_{16}	$C_{16}^{1=}$	C_{17iso}	C_{17}	C_{18}	$C_{18}^{1=}$	$C_{18}^{2=}$	$C_{18}^{3=}$
Cow A (linseed oil added)											
0	2·2	2·2	1·6	35·5	—	0·5	1·6	51·6	3·2	1·6	tr.
2	tr.	—	—	14·0	—	—	—	12·0	22·0	16·0	36·0
4·5	2·9	—	1·9	23·3	—	—	—	16·9	12·3	39·8	2·9
6·5	6·4	3·8	5·1	35·9	—	—	—	46·2	tr.	tr.	tr.
Cow *a*											
0	7·3	5·2	3·1	46·9	—	—	—	37·5	—	—	—
2	14·8	2·9	3·4	41·2	1·0	1·0	1·5	31·3	2·9	—	—
4·5	5·3	4·4	2·2	38·7	2·2	0·9	1·8	26·7	13·8	4·0	—
6·5	9·4	4·7	4·0	43·7	tr.	—	1·3	27·5	9·4	tr.	—

Table V

Fatty acid composition of triglycerides and the hydrolysis products of triglycerides in the supernatant of rumen contents four and half hours after infusion of linseed oil

(total lipid = 106 mg./100 ml. of supernatant rumen fluid)

Lipid fraction	Amount, wt.-%	Fatty acids (moles-%)					
		C_{16}	$C_{16}{}^{1=}$	C_{18}	$C_{18}{}^{1=}$	$C_{18}{}^{2=}$	$C_{18}{}^{3=}$
Triglyceride	24·2	37·3	2·6	20·5	18·2	15·0	6·4
Diglyceride	13·3	29·1	2·5	30·4	24·0	14·0	tr.
Monoglyceride	27·3	28·7	3·4	31·6	16·1	19·1	1·0
Fatty acid	35·2	29·1	2·1	49·0	14·6	4·9	2·3

saturated of the four lipid components examined, the composition of the triglycerides was very different from that of the linseed oil infused into the rumen (Table III), the most abundant fatty acids being palmitic and stearic acids with oleic, linoleic and linolenic acids in decreasing order of abundance.

Qualitative separation of lipid by thin-layer chromatography

The presence of various lipids and hydrolysis products in the rumen supernatant obtained from cows on a grass and clover diet, with and without added linseed oil, was demonstrated by chromatographing lipid extracts on thin layers of silicic acid and spraying with 2′,7′-dichlorofluorescein. Fatty acids were found to be present before and after feeding grass and clover and diglycerides only after feeding. Triglycerides could not be detected, but unidentified fluorescent spots ran ahead of triglyceride, at or near the solvent front in light petroleum–diethyl ether–acetic acid (60 : 40 : 1 by vol.). Fluorescent material which remained at the origin in this solvent was shown, by developing chromatograms with toluene–ethyl acetate–ethanol–acetic acid (10 : 5 : 5 : 0·1 by vol.), to include mono- and di-galactolipid. Slower-moving spots also present were probably partial hydrolysis products of galactolipids.

Fluorescent compounds corresponding to triglycerides and diglycerides were found in the rumen supernatant, in addition to fatty acids, after linseed oil had been added (Fig. 1). The intensity of the diglyceride spots increased with time over the 6·5 h. sampling period. Monoglycerides could not be detected, but green pigmented material which ran at the same rate as monoglyceride standard could have masked the monoglycerides if they were present.

Discussion

The wide variations in lipolytic activity of rumen fluids reported by other workers[8, 9, 18] have been found to occur in the present *in vivo* experiments and may be similarly attributed to variations in the microbial population and in the ability of the micro-organisms to metabolise lipid. Reasonable agreement was obtained between pairs of animals at the one time, but, despite a similar feeding regime, appreciable differences in the lipid levels and in the proportions of non-esterified fatty acids occurred between experiments. This may indicate that compositional changes in pasture plants influence lipid metabolism as well as affecting protein and carbohydrate metabolism.[11]

The rapid return of the lipid content of rumen liquor and rumen supernatant to pre-feeding levels, even when emulsified linseed oil was introduced, was also observed by Hoflund *et al.*[10] It is unlikely that passage of the emulsion from the rumen would occur so rapidly and *in vitro* experiments showed that the rapid decrease in lipid levels in rumen liquor is probably due to the occlusion of lipid on particulate material in the rumen.

The presence of mono- and di-glycerides in the rumen after the infusion of linseed oil was of particular interest : Garton *et al.*[8] could not detect these intermediates of triglyceride hydrolysis after incubation of linseed oil with rumen liquor obtained from sheep being fed on hay and concentrates. Furthermore, diglycerides were also detected in rumen liquor obtained from a fistulated cow fed on fresh pasture alone ; this may indicate a difference in metabolism in the rumen of animals fed on fresh pasture and on hay and concentrates. Since mono- and di-galactolipids are the most abundant lipids in grasses and clovers,[19] and α- and β-galactosidases

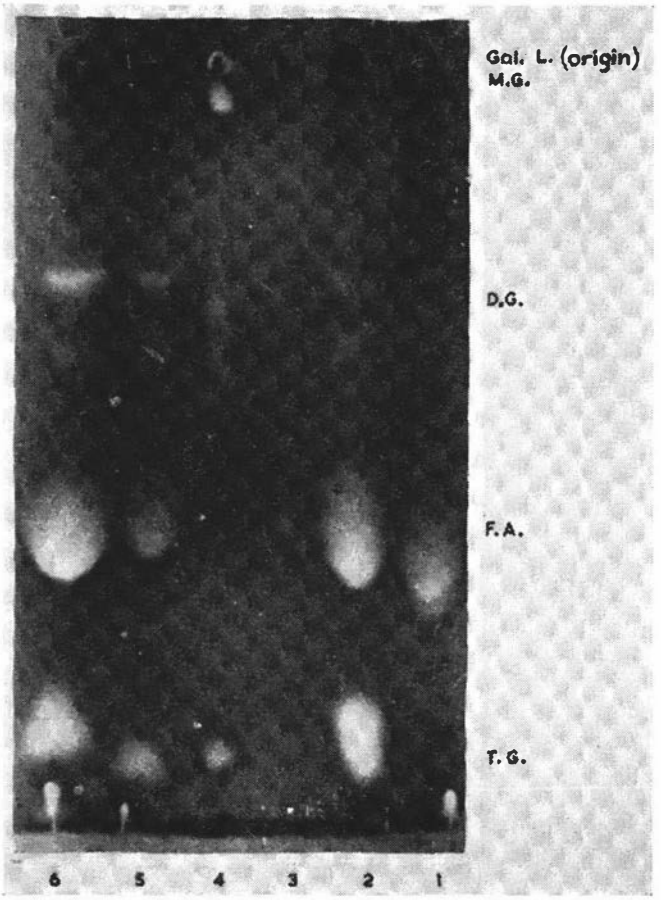

FIG. 1.—*Separation of neutral lipids from rumen liquor by thin-layer chromatography on silicic acid*

From right to left: 1. pre-feeding sample; 2. sample 2 h. after commencement of stall-feeding on mixed pasture and addition of 500 g. linseed oil; 3. oleic acid; 4. (in descending order) galactolipid, monopalmitin, α,α′-distearin, trimyristin; 5. 4 h. sample; 6. 6·5 h. sample.

Developing solvent: light petroleum–diethyl ether–acetic acid (60 : 40 : 1, v/v). Detection with 0·2% 2′,7′-dichlorofluorescein in ethanol and photographed under ultra-violet illumination.

have been found in rumen micro-organisms,[20] these lipids would seem to be the most likely precursors of diglycerides. However, in more recent *in vitro* experiments,[21] diglycerides could not be found among the hydrolysis products when galactolipids were incubated with rumen liquor.

In vitro experiments[3, 8] have shown that linolenic acid, the most abundant fatty acid constituent in pasture grasses and clovers,[19] is very rapidly hydrogenated by rumen micro-organisms. Because changes are being followed in a dynamic system, *in vivo* experiments are less satisfactory for following quantitative transformations between fatty acids. Nevertheless, the information obtained here does show that, in cows on a diet of fresh grass and clover, there is rapid hydrogenation in the rumen. Six and a half hours after the addition of 500 g. of emulsified linseed oil, above-normal proportions of linoleic acid persisted in the rumen but the proportions of linolenic acid had returned to about the pre-feeding level. Thus it can be readily appreciated why variations in the nature and the amount of lipid in the normal diet of ruminants is not likely to affect greatly the composition of the depot and milk fat. However if, as suggested by Shaw *et al.*,[22] dietary changes can markedly alter hydrogenation activity, variations in the composition of pastures at different stages of growth could still be one of the factors responsible for the regular seasonal changes in the proportions of unsaturated fatty acids, principally oleic acid, in the

milk fats of grazing cows.[23] Furthermore, in view of this rapid hydrogenation within the rumen, the presence of 11·5% of linolenic acid in plasma lipids of pasture-fed heifers[24] is unexplained.

The present work has confirmed the finding of Garton *et al.*[8] that fatty acids released from triglycerides by lipolysis undergo more extensive hydrogenation than triglyceride fatty acids. Compared with the composition of linseed oil, however, the triglycerides extracted from rumen liquor 4·5 h. after the addition of linseed oil, were much more saturated ; the proportions of the unsaturated fatty acids in partially hydrolysed lipid were not greatly different to those of the triglycerides. The presence of large proportions of palmitic acid lends support to the suggestion[8] that this fatty acid might be derived from the C_{18} fatty acids of linseed oil. Increases in the proportions of palmitic acid during the post-feeding period were also observed when cows were receiving a grass and clover diet only. However, Gray *et al.*[25] could find no evidence for the synthesis of palmitic acid from short-chain fatty acids in rumen contents although this is a well-established route to palmitic acid in other biological systems.[26]

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References

[1] Willey, N. B., Riggs, J. K., Colby, R. W., Butler, O. D., & Reiser, R., *J. Anim. Sci.*, 1952, **11,** 705
[2] Reiser, R., *Fed. Proc.*, 1951, **10,** 236
[3] Shorland, F. B., Weenink, R. O., & Johns, A. T., *Nature, Lond.*, 1955, **175,** 1129
[4] Reiser, R., & Reddy, H. G. R., *J. Amer. Oil Chem. Soc.*, 1956, **33,** 155
[5] Shorland, F. B., Weenink, R. O., Johns, A. T., & McDonald, I. R. C., *Biochem. J.*, 1957, **67,** 328
[6] Garton, G. A., Hobson, P. N., & Lough, A. K., *Nature, Lond.*, 1958, **182,** 1511
[7] Hobson, P. N., & Mann, S. O., *J. gen. Microbiol.*, 1961, **25,** 227
[8] Garton, G. A., Lough, A. K., & Vioque, E., *J. gen. Microbiol.*, 1961, **25,** 215
[9] Wright, D. E., *N.Z. J. agric. Res.*, 1961, **4,** 216
[10] Hoflund, S., Holmberg, J., & Sellmann, G., *Cornell Vet.*, 1956, **46,** 53
[11] Robertson, J. A., & Hawke, J. C., *J. Sci. Fd Agric.*, 1964, **15,** 274
[12] Hilditch, T. P., ' The Chemical Constitution of Natural Fats ', 3rd edn, 1956, p. 581 (London : Chapman & Hall)
[13] Mangold, H. K., *J. Amer. Oil Chem. Soc.*, 1961, **38,** 708
[14] Lees, T. M., & De Muria, P. J., *J. Chromatogr.*, 1962, **8,** 108
[15] Malins, D. C., & Mangold, H. K., *J. Amer. Oil Chem. Soc.*, 1960, **37,** 576
[16] Hirsch, J., & Ahrens, E. H., *J. biol. Chem.*, 1958, **233,** 311
[17] Hawke, J. C., Hansen, R. P., & Shorland, F. B., *J. Chromatogr.*, 1959, **2,** 547
[18] Allen, R. S., Hill, F. D., Hammond, E. G., & Jacobson, N. L., in *Rep. 5th Conf. Rumen Function*, ed. by N. R. Ellis (Beltsville, Md., U.S.A. : U.S. Agric. Res. Centre)
[19] Weenink, R. O., *N.Z. J. Sci.*, 1959, **2,** 273
[20] Conchie, J., & Levvy, G. A., *Biochem. J.*, 1957, **65,** 389
[21] Hawke, J. C., & Weenink, R. O., unpublished
[22] Shaw, J. C., Ensor, W. L., Tellechea, H. F., & Lee, S. D., *J. Nutrit.*, 1960, **71,** 203
[23] Hawke, J. C., *J. Dairy Res.*, 1963, **30,** 67
[24] Duncan, W. R. H., & Garton, G. A., *J. Lipid Res.*, 1962, **3,** 53
[25] Gray, F. V., Pilgrim, A. F., Rodda, H. J., & Weller R. A., *J. exp. Biol.*, 1952, **29,** 57
[26] Wakil, S. J., *J. Lipid Res.*, 1961, **2,** 1

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STUDIES ON RUMEN METABOLISM. III.*—Effect of Lipids *in vitro* and *in vivo* on Microbial Activity

By J. A. ROBERTSON and J. C. HAWKE

The addition of lipids increased the formation of ammonia when strained rumen liquors were incubated anaerobically at 39° with ryegrass juice extract high in protein, but decreased the formation of ammonia when fibrous substrates low in protein were used instead of ryegrass juice extract. The addition of lipid did not affect ammonia levels in the rumen of cows on a hay diet. Lipid decreased the digestion of fibrous ryegrass constituents and cotton thread by rumen micro-organisms *in vitro* and *in vivo* respectively. Decreased formation of acetic acid and increased formation of propionic acid also occurred in the presence of lipid. High rates of fermentation of the constituents of ryegrass juice were not affected by lipid and were accompanied by rapid hydrogenation of the constituent unsaturated fatty acids of the lipid itself.

Introduction

Earlier work[1, 2] showed that the infusion of lipid into the rumen brought about a decrease in total concentrations of volatile fatty acids (acetic, propionic and butyric acids) but had a variable effect on NH_3 concentrations in the rumen although an accompanying decrease in intake made interpretation difficult. These observations, and the depressing effect of lipid on the digestibility of cellulose, protein and nitrogen-free extract found by other workers,[3–5] suggest that lipids bring about a general decrease in the metabolic activity of the rumen micro-organisms.

The effect of lipids on metabolic activity was investigated further by the *in vitro* incubation of rumen liquors with varying amounts of linseed or whale oil in the presence of soluble or fibrous ryegrass constituents and measuring gas production, hydrogenation activity and the formation of the volatile acids and ammonia. These two ryegrass fractions were chosen to permit the separation of the effect of lipid on the readily fermentable constituents including soluble sugars and on the more slowly fermented fibrous material such as cellulose. Furthermore the *in vitro* technique obviated difficulties in interpretation due to absorption and intake fluctuations.

The results of the *in vitro* experiments with cellulose substrates suggested further *in vivo* experiments on the influence of lipid on the relationship between metabolic activity and cellulose digestion.

Experimental

Materials

Non-lactating fistulated cows, which were fed indoors and fasted overnight before samples were taken, provided the source of rumen liquor. Rumen samples were strained through muslin and the liquor held in a vacuum flask (previously warmed to about 40°) during the setting up of the manometric apparatus.

Rumen liquors were obtained from cows fed on fresh grass when soluble ryegrass constituents were used in the incubation experiments, whereas the cows were on a hay diet in the experiments with ryegrass fibre and for the *in vivo* investigation of cellulose digestion. These feeding regimes were chosen following the suggestion of Boda & Johns[6] that selection of diet is necessary in order to decrease adaptation effects when investigating separately the fermentation of soluble and fibrous dietary constituents.

Plant juices and fibre were prepared by passing freshly-cut perennial ryegrass through a Bentall 'Protess' extraction plant. The juices, which contained 9·8% of dry matter, were frozen in 600-ml. batches and the fibre was washed in running tap-water for 3 h. before it was freeze-dried. Both fractions were held at −25° until required.

Raw linseed oil and whale oil emulsions in water (50 : 50, w/v) were used as substrates along with plant juices or plant fibre.

* Part II : *J. Sci. Fd Agric.*, 1964, **15**, 283

Measurement of fermentation rates

Incubations of rumen fluids were carried out at $39 \pm 0{\cdot}5°$ in bottles of 1 pint capacity which were connected through rubber stoppers to manometers (260 ml.) and to a valve to permit the addition of oil emulsion and plant juices and the acidification of the contents.[6] To ensure release of CO_2 in the form of bicarbonate, gas volumes were measured after acidification with 4 ml. of 10N-sulphuric acid at the end of the incubation period.

Incubation of rumen fluids with lipid and grass juice

Eight bottles were used in each experiment and were placed on shaker racks in the water-bath at 39°. Rumen fluid (100 ml.) obtained from a cow feeding on freshly-cut grass was added to each bottle and the bottles were gassed for 1 min. with a mixture of 95% N_2/5% CO_2. Ryegrass juice (20 ml.) was added to six bottles and water (20 ml.) to the remaining two bottles which acted as blanks. The appropriate volume of oil/water emulsion (2, 4 or 8 ml.) was added to three of the six bottles containing ryegrass juice and an equal volume of water added to each of the two blanks and to the remaining three bottles which contained ryegrass juice. The bottles were then shaken momentarily by hand to ensure adequate mixing and the manometers adjusted to zero.

One of the blanks was acidified at zero time and the other at 3 h. The difference in manometer readings between the two blanks measured the gas produced by fermentation of material present in the rumen liquor. Of the remaining bottles one with added oil and one without added oil were incubated at 1, 2 and 3 h. respectively. Any difference in these pairs measured the effect of added oil on the metabolic activity of the rumen micro-organisms.

Incubation of rumen fluids with lipid and grass fibre

The effect of lipid on the fermentation of plant fibre by rumen fluids was determined in a similar manner to that described above. Because the amounts of volatile fatty acids formed were likely to depress the pH of rumen liquor, a bicarbonate/phosphate buffer (pH 7·0) which was similar in composition to saliva[7] was used. One g. of washed ryegrass fibre, 100 ml. of rumen fluid, obtained from a cow on a hay diet, and 30 ml. of buffer were incubated with or without 4 ml. of linseed oil emulsion for 12, 24 and 48 h.

In vivo *cellulose digestion*

Non-lactating twin Jersey cows (Nos. 13 and 14) with rumen fistulae which were being stall-fed twice daily on ryegrass hay (at 9.30 a.m. and 3.30 p.m.) were used to measure the effect of lipid on cellulose digestion in the rumen. After an overnight fast and before the morning feeding period, twelve weighed strands of white cotton thread (Clark's No. 40) were suspended in the rumen of each cow,[8] six in the dorsal region and six in the ventral region. Two strands were removed from each region of the rumen after 10, 24 and 36 h., washed in water and alcohol and the dry weight determined.

During the first 2 days of each experiment the digestion of cotton thread was measured in both cows without added lipid. Another set of threads was then suspended in the rumen of each cow, and 500 g. of linseed oil perfused into the rumen of one cow on two consecutive days.

Analytical methods

Ammonia, total volatile fatty acids, proportions of individual volatile and non-volatile fatty acids in rumen liquor, and soluble sugars in the grass extracts were determined as described previously.[1, 2] A measure of the effect of lipid on fibre digestion in 48 h. under *in vitro* conditions was obtained by determining the residual crude fibre before and after fermentation.[8] Total nitrogen was determined by the Kjeldahl method.[9]

Results and discussion

Effect of lipid on the fermentation of soluble ryegrass constituents

The addition of ryegrass juice to rumen liquor considerably increased the amount of fermentation products (Table I). It is likely that the soluble sugars present in the ryegrass juice

(34 mg./ml. extract) were primarily responsible for the increases in the production of gas and volatile acids over the 3-h. incubation period. Similarly, the ryegrass juice which was high in crude protein (17·7 mg./ml.) produced approximately twofold increases in NH_3 concentration.

Despite standardisation of the feeding regime and of the sampling time, the rumen liquors before incubation contained between 4·0 and 6·7 mg. of ammonia/100 ml. of rumen liquor and 38·9–68·6 mmoles of volatile acids/l. of rumen liquor (Table I). Similarly, differences in the concentrations of ammonia and of total volatile acids were found on several occasions after incubation of rumen liquor with ryegrass extract. This would appear to be due to day-to-day variations in either the total number of organisms or in the relative proportions of different microbial species.[10]

Incubation of 1 and 2 g. of linseed or whale oil with liquor supplemented with ryegrass juice resulted in slight reductions in fermentation as measured by gas production compared with incubations without lipid (Table I). When 4 g. of either lipid was added, the amount of gas produced increased slightly. Thus the fermentation of soluble ryegrass constituents by rumen micro-organisms *in vitro* appeared to be little affected by lipid. Concentrations of ammonia in the rumen liquors incubated with lipid were generally higher than those for the controls. Increasing the amount of lipid from 1 to 4 g. did not appear to accentuate this effect.

The increases in ammonia concentration, although small, lend support to the findings of earlier *in vivo* work in which linseed oil produced irregular increases.[1]

After 1 h., the concentrations of total volatile fatty acids in the presence of lipid were from 0·8 to 10·2% higher than those for the controls although after 3 h. no differences were apparent. The effect of either linseed or whale oil on acetic acid and butryic acid concentrations was small and variable (Table II), but concentrations of propionic acid were from 2·2 to 9·4% higher when lipid was added. In the short incubation times used it is unlikely that glycerol, produced on hydrolysis of lipid, would make a significant contribution to the increase in propionic acid.

The addition of 2 g. of lipid to 100 ml. of rumen liquor and 20 ml. of ryegrass juice gave a lipid concentration comparable to the addition of about 900 g of lipid to a rumen with a fluid volume of 55 l.[1] Consequently, the absence of any marked differences in gas production, ammonia or total volatile acid concentrations in these short-term incubations suggests that the variations found previously when lipids were added to the rumen were not due to a decreased activity of the rumen micro-organisms in the fermentation of soluble constituents present in ryegrass juices.

Hydrogenation of linseed oil by rumen liquor

During the short-term incubation periods used it was found that the activity of the rumen micro-organisms changed the proportions of the fatty acids originally present in linseed oil

Table I

The formation of gas, NH_3, and total VFA from rumen liquors incubated with linseed oil or whale oil in the presence of ryegrass juice

Incubation time, h.	Linseed oil added									Whale oil added								
	—	—	1 g.	—	—	2 g.	—	—	4 g.	—	—	1 g.	—	—	2 g.	—	—	4 g.
	B[a]	C[b]	E[c]	B	C	E	B	C	E	B	C	E	B	C	E	B	C	E
	Gas production, ml.																	
0	75	75	75	165	165	165	156	156	156	103	103	103	135	135	135	168	168	168
1		128	130		229	224		220	—		157	161		201	200		210	212
2		164	159		261	256		253	254		183	182		224	223	223	223	228
3	90	194	192	184	227	286	167	272	278	116	215	215	153	248	246	178	252	—
	NH_3, mg./100 ml.																	
0	4·0	4·0	4·0	6·65	6·65	6·65	6·45	6·45	6·45	5·8	5·8	5·8	6·0	6·0	6·0	5·9	5·9	5·9
1		10·3	10·5		13·7	13·35		12·7	—		12·7	13·2		13·1	14·9		10·0	10·6
2		14·0	14·9		17·8	18·1		16·6	16·1		15·7	15·85		16·5	16·2		11·7	11·7
3	7·6	15·8	16·2	9·8	20·1	19·75	8·2	17·3	18·7	8·7	17·7	18·6	8·6	18·0	18·7	7·2	13·7	13·8
	Volatile fatty acids, mmole/l.																	
0	68·6	68·6	68·6	41·3	41·3	41·3	41·7	41·7	41·7	50·4	50·4	50·4	49·8	49·8	49·8	38·9	38·9	38·9
1		90·5	93·0		63·1	63·6		63·0	–		70·0	71·4		71·7	72·9		46·9	51·7
2		105·5	104·4		76·8	78·3		76·9	77·0		81·9	80·4		82·2	81·5		63·1	65·1
3	74·5	115·0	114·1	55·1	84·1	83·2	46·3	81·8	86·6	53·4	89·9	92·8	55·3	92·0	90·9	42·7	72·5	73·0

[a] B = blank, rumen liquor only; [b] C = control, rumen liquor and ryegrass juice; [c] E = experimental, rumen liquor, ryegrass juice and lipid

Table II

Concentrations of acetic, propionic and butyric acids (mmole/l.) formed on incubation of rumen liquor with linseed oil or whale oil

	Linseed oil added								
	—	—	1 g.	—	—	2 g.	—	—	4 g.
	B[a] (0 h.)	C[b] (3 h.)	E[c] (3 h.)	B (0 h.)	C (3 h.)	E (3 h.)	B (0 h.)	C (3 h.)	E (3 h.)
Acetic	51·4	79·6	77·1	30·6	61·7	61·7	31·1	60·0	61·2
Propionic	11·2	23·5	25·1	5·9	14·8	15·2	6·0	14·9	16·3
Butyric	6·0	10·1	11·9	4·8	7·6	6·3	4·6	6·9	7·1
	Whale oil added								
	—	—	1 g.	—	—	2 g.	—	—	—
Acetic	36·3	62·5	66·4	37·1	64·1	62·1			
Propionic	7·3	18·2	19·1	6·8	18·0	18·4			
Butyric	6·8	7·7	7·3	5·9	9·9	10·4			

[a] B = blank, rumen liquor ; [b] C = control, rumen liquor and ryegrass juice ;
[c] E = Experimental, rumen liquor and ryegrass juice + lipid

(Table III). Reductions of 11 and 5% in linolenic acid occurred in the supernatant and solid fractions of the rumen fluids, respectively, between 1 and 3 h. after the addition of linseed oil. The increases in the proportions of the dienoic and monoenoic acids indicate that the more unsaturated acids were preferentially hydrogenated. Incubations for 24 h. of linseed oil and rumen liquor carried out by Garton *et al.*[11] resulted in a much greater degree of hydrogenation, the proportion of C_{18}-trienoic acid decreasing from 54·5 to 5·1% of the total fatty acids. Hydrogenation appears to be more rapid *in vivo*, because very little C_{18}-trienoic acid remained 6·5 h. after infusion of linseed oil.[2]

Separation of the rumen liquor into a supernatant liquid and a residue showed that about four-fifths of the added lipid became occluded on the small particles present in the strained rumen liquor. It seems that a similar occlusion of oil to particulate material occurs in the rumen.[2]

Before the addition of lipid, rather more palmitic acid and less stearic and linolenic acids were found in the residue than in the supernatant liquid. However, after the addition of linseed oil the differences in the proportions of the major fatty acid components in these two fractions were small and largely within experimental error. The residual fractions, probably representative of the composition of the micro-organisms present, contained higher proportions of the

Table III

Amounts and composition of lipid in fractions of rumen liquor before and after incubation of strained rumen contents with linseed oil

Rumen contents (100 ml.) with and without 4 ml. of linseed oil*

Fatty acid	Supernatant liquid				Residue			
	Rumen contents alone (0·072 g.)	Time of incubation, h. 1 (0·706 g.)	2 (0·655 g.)	3 (0·711 g.)	Rumen contents alone (0·076 g.)	Time of incubation, h. 1 (2·718 g.)	2 (2·719 g.)	3 (2·343 g.)
C_{12}	2·1				1·3			
C_{13}^{br}	0·7				1·0			
C_{13}	1·3				0·4			
$C_{13}^{1=\text{ or br}}$	0·4				1·7			
C_{14}	2·9	tr	tr	0·8	4·8	0·4	tr	0·7
C_{15}^{br}	0·8				7·8			
C_{15}	0·6				5·4			
$C_{16}^{1=}$	0·2			0·8	2·5			
C_{16}	24·8	9·8	9·2	9·2	30·3	8·7	8·6	8·9
C_{17}^{br}	0·6				2·7			
C_{17}	1·0				2·7			
$C_{18}^{3=}$	13·0	47·4	45·8	42·3	5·6	49·0	48·8	46·6
$C_{18}^{2=}$	5·9	14·4	13·9	16·1	5·8	14·8	14·6	16·1
$C_{18}^{1=}$	11·8	22·6	24·8	24·6	11·3	21·5	23·3	22·2
C_{18}	33·7	5·8	6·3	6·2	16·7	5·6	5·7	6·5

* Fatty acid composition of linseed oil (moles-%) : C_{18} 6·1 ; $C_{18}^{3=}$ 56·7 ; $C_{18}^{2=}$ 17·6 ; $C_{18}^{1=}$ 16·7 ; C_{18} 2·9

br = branched tr = trace

branched chain and odd carbon fatty acids. In order to facilitate comparisons of the major fatty acid components, the amounts of these acids which were present only in traces in the samples incubated with linseed oil are not recorded in Table III.

Effect of lipid on the fermentation of grass fibre

Over a 48-h. period, gas production from 100 ml. of rumen liquor in the presence of 1 g. of ryegrass fibre was 2 to 3 times greater than endogenous fermentation. Ryegrass fibre produced very much slower rates of fermentation than ryegrass juice when incubated with rumen liquor (Tables I and IV).

Table IV

Formation of gas, ammonia and total volatile fatty acids from rumen liquor incubated with linseed oil in the presence of ryegrass fibre

Incubation time, h.	Experiment (i)			Experiment (ii)		
	B[a]	C[b]	E[c]	B	C	E
	Gas production, ml.					
0	124	124	124	104	104	104
12	—	191	205	—	166	179
24	—	252	261	—	228	236
48	186	294	272	165	287	252
	Ammonia, mg./100 ml.					
0	5·7	5·7	5·7	4·6	4·6	4·6
12	—	15·4	14·9	—	11·5	13·7
24	—	20·5	20·05	—	22·0	24·0
48	23·7	34·0	27·0	28·2	37·6	28·9
	Total volatile fatty acids, mmole/l.					
0	56·0	56·0	56·0	66·2	66·2	66·2
12	—	80·6	86·5	—	92·1	94·9
24	—	101·5	104·8	—	111·6	114·4
48	74·5	115·8	115·4	87·3	128·3	125·0

[a] B = blank, rumen liquor ; [b] C = control, rumen liquor + fibre ;
[c] E = experimental, rumen liquor + fibre + 2 g. of lipid

Gas production was slightly higher in the presence of 2 g. of linseed oil at 12 and 24 h. but was lower at 48 h. In two experiments, the respective weights of residual crude fibre after 48 h. incubations with lipid were 21·3 and 7·2% of the initial weight of crude fibre added compared with 16·9 and 1·6% without lipid respectively. Linseed oil slightly depressed ammonia production (Table IV). This effect is opposite to that found in the presence of soluble constituents (Table I).

Consistent with the differences in residual crude fibre, added lipid slightly depressed the levels of total volatile fatty acids produced over 48 h. (Table IV) although the formation of volatile acids was faster and produced slightly higher levels at 12 and 24 h. However, the concentrations of acetic and propionic acids at 48 h. were markedly affected (Table V) with decreases in acetic acid (10–12 mmoles/l.) being approximately balanced by increases in propionic acid (8–11 mmoles/l.). The concentrations of butyric acid were little affected.

The longer incubation periods used in these experiments probably allowed sufficient time for a substantial amount of hydrolysis of the linseed oil. Complete hydrolysis of 2 g. of lipid and a quantitative conversion of the glycerol moiety to propionic acid would account for an increase in the concentration of propionic acid of slightly less than 0·30 mmole/l. The observed increases (8·5 and 11·3 mmoles/l.) far exceeded that theoretical value and must therefore have been due to an effect of lipid on carbohydrate digestion.

Effect of lipid on in vivo *cellulose digestion*

The intakes of the twin cows in the first experiment were very similar both before and on the first day of the addition of lipid, but it was apparent that 500 g. of emulsified linseed oil added to the rumen depressed the intake of hay on the second day of infusion (Table VI). The second experiment, which commenced 4 days after the conclusion of the first, was less

Table V

Concentrations of acetic, propionic and butyric acids (mmoles/l.) formed on incubation of rumen liquor with 2 g. of linseed oil in the presence of ryegrass fibre

	Experiment (i)			
	B[a]	B[a]	C[b]	E[c]
Time of incubation, h.	0	48	48	48
Acetic	40·5	51·8	80·9	70·7
Propionic	9·2	13·0	20·0	31·3
Butyric	6·3	9·8	14·8	13·4
	Experiment (ii)			
Time of incubation, h.	0	48	48	48
Acetic	49·2	64·2	92·9	80·4
Propionic	9·7	12·6	20·0	28·5
Butyric	7·3	10·6	15·4	16·1

[a] B = blank, rumen liquor; [b] C = control, rumen liquor + ryegrass fibre;
[c] E = experimental, rumen liquor and ryegrass fibre + linseed oil

satisfactory because of a carry-over effect of lipid. As a result, the intake of the control animal (cow 13) was well below that of cow 14 when the second trial began. However, the effect of lipid may be assessed in part (ii) from the reduction in the dry-matter intake of cow 14 from 12·1 lb. on the first day of lipid addition to 9·2 lb. on the second day.

Added lipid depressed the digestion of cotton threads suspended in the rumen (Table VII). Part (i) shows this to better effect because of the carry-over effect of lipid from part (i). Judged by the loss of weight of the cotton thread, cellulytic activity was greater in the ventral than in the dorsal rumen. This is in agreement with observations made by other workers.[12–14] The residual weights of cotton threads were greater at 24 h. in the presence than in the absence of lipid, and at 36 h. the threads had remained as whole threads in 3 of the 4 determinations when lipid was added, but had disintegrated in the absence of lipid.

The pre-feeding concentration of ammonia in the rumen varied between 2·6 and 5·1 mg./100 ml.; peak levels recorded 2 h. after feeding began varied between 4·9 and 14·9 mg./100 ml. with common values between 9 and 12 mg./100 ml. Four h. after feeding began, the ammonia levels had returned to approximately the pre-feeding concentrations. These concentrations were low compared with those in samples taken at corresponding times from cows fed fresh forage high in crude protein (18·2–28·7% of dry matter).[1] No consistent differences in the concentrations of ammonia were apparent when lipid was added to the rumen of cows fed hay containing 7·5% of crude protein. Since somewhat irregular increases in ammonia concentrations were found in the rumen of cows fed on a high-protein diet, it is possible that under these latter conditions lipid had an effect on either formation or utilisation of ammonia which is not apparent when low-protein diets are fed.

As with ammonia, concentrations of volatile fatty acids in the rumen of cows fed on hay were much lower than on a diet of fresh fodder.[1] The pre-feeding levels were in the range

Table VI

Intake of ryegrass hay (lb. of dry matter) by stall-fed twin cows with and without added linseed oil

Date	Lipid added	Part (i) Morning		Afternoon		Total	
		Cow 13	Cow 14	Cow 13	Cow 14	Cow 13	Cow 14
17.9.63	—	4·2	4·3	5·1	4·8	9·3	9·1
18.9.63	—	5·2	5·6	4·9	5·1	10·1	10·7
19.9.63	Linseed oil, 500 g.	4·9*	5·0	5·4	4·9	10·3	9·9
20.9.63	,, ,, ,,	4·0*	4·7	1·2	2·3	5·2	7·0
		Part (ii)					
24.9.63	—	3·6	7·2	2·3	6·2	5·9	13·4
25.9.63	—	5·5	7·3	4·2	5·4	9·7	12·7
26.9.63	Linseed oil, 500 g.	4·8	5·7*	5·8	6·4	10·6	12·1
27.9.63	,, ,, ,,	6·1	4·6	4·3	4·6	10·4	9·2

* Cow received lipid

Table VII

Digestion of cotton thread (% of original wt. which remained undigested) in the rumen of cows fed on ryegrass hay with or without the addition of linseed oil

Date		Location of threads in rumen	Time in rumen, h. 10	24	36		Location of threads in rumen	Time in rumen, h. 10	24	36
			Part (i)							
17.9.63 to 18.9.63	Cow 13 (no oil)	Dorsal	97·4	78·4	—	Cow 14 (no oil)	Dorsal	96·5	69·4	—
			96·8	78·6	—			96·4	69·9	—
		Ventral	97·1	76·3	—		Ventral	97·7	57·8	—
			97·3	74·4	—			98·8	59·2	—
19.9.63 to 20.9.63	Cow 13 (linseed oil, 500 g.)	Dorsal	98·4	87·3	62·8	Cow 14 (no oil)	Dorsal	97·7	73·2	—
			98·1	85·5	59·1			97·3	73·1	—
		Ventral	100·0	71·2	47·8		Ventral	97·3	56·0	—
			99·1	73·3	44·8			98·3	51·7	—
			Part (ii)							
24.9.63 to 25.9.63	Cow 13 (no oil)	Dorsal	97·7	90·3	79·6	Cow 14 (no oil)	Dorsal	101·4	76·3	—
			98·1	90·6	76·1			99·9	69·8	—
		Ventral	101·7	85·3	55·7		Ventral	103·2	54·2	—
			98·0	79·7	46·6			95·2	53·8	—
26.9.63 to 27.9.83	Cow 13 (no oil)	Dorsal	99·6	78·6	56·4	Cow 14 (linseed oil, 500 g.)	Dorsal	97·5	84·5	53·7
			98·0	80·1	55·3			96·6	85·1	55·1
		Ventral	96·5	64·5	—		Ventral	97·1	71·9	—
			97·3	65·7	—			97·0	72·3	—

51–86 mmoles/l., increasing to peak levels of 81–109 mmoles/l. at 2 h. after feeding began. Maximum concentrations of total volatile acids over the period of sampling (2–6 h.) appeared to be slightly depressed by the addition of lipid, although the higher initial and final values obtained on the days after the addition of lipid may indicate that the fermentation was more prolonged. The only noticeable difference in the concentrations of the individual volatile fatty acids after the infusion of lipid into the rumen was the increase in propionic acid, particularly on the second day of infusion. Before the addition of lipid, the concentration of propionic acid in samples taken before feeding and 6 h. after feeding was generally between 10 and 13 mmoles/l. of rumen liquor. The levels increased to 17 and 22 and to 16 and 19 mmoles/l. on the second day of infusion for cows 13 and 14 respectively.

The results of the present *in vitro* experiments with linseed oil and ryegrass juice extracts high in protein-N (20 ml. ≡ 350 mg. of crude protein) are consistent with the results of earlier work from this laboratory which showed that the addition of linseed oil to cows feeding on pastures high in protein-N increased the ammonia concentrations in the rumen. However, linseed oil produced the opposite effect when juice extracts were replaced by ryegrass fibre (1 g. ≡ 200 mg. of crude protein). This relationship found *in vitro* between the level of substrate protein and the effect of lipids on ammonia concentration is similar to that reported by Chalmers[15] and Jayasinghe,[16] who found that, when nitrogen levels in the feed were 3% of the dry matter, the addition of lipid resulted in a depression in the concentration of ammonia in the rumen, whereas at nitrogen levels of 4·5%, the added lipid resulted in an increased concentration of ammonia. Despite the agreement between our *in vitro* experiments and the *in vivo* work of others, the present *in vivo* experiments with ryegrass hay containing 1·2% of protein-N (with and without lipid) did not show any consistent differences in the concentrations of ammonia.

That the utilisation of soluble plant constituents is little affected by lipid is confirmed by the *in vitro* experiments with plant juice extracts in which only very slight differences in the concentration of volatile fatty acids and in the volume of fermentation gases were observed in the presence and absence of lipid. On the other hand, *in vitro* and *in vivo* investigations with ryegrass fibre and cotton thread respectively showed that lipid (16·7 g./l. *in vitro* and 10·9 g./l. *in vivo*) depressed digestion of fibrous carbohydrates. *In vitro*, this effect was accompanied

by a decrease in the level of acetic acid which is normally the predominant acid formed by the fermentation in the rumen of feeds high in cellulose. From this and earlier work[3–5] it appears that reductions in the digestion of cellulose by rumen micro-organisms can be brought about by lipid under a variety of dietary conditions. Increased formation of propionate in the presence of lipid also appears to be related to the digestion of the fibrous rather than the soluble constituents of pasture species.

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References

[1] Robertson, J. A., & Hawke, J. C., *J. Sci. Fd Agric.*, 1964, **15,** 274
[2] Hawke, J. C., & Robertson, J. A., *J. Sci. Fd Agric.*, 1964, **15,** 283
[3] Brooks, C. C., Garner, G. B., Gehrke, C. W., Muhrer, M. E., & Pfander, W. H., *J. Animal Sci.*, 1954, **13,** 758
[4] Brethour, J. R., Sirny, R. J., & Tillman, A. D., *J. Animal Sci.*, 1958, **17,** 171
[5] Davison, K. L., & Woods, W., *J. Animal Sci.*, 1960, **19,** 54
[6] Boda, J. M., & Johns, A. T., *Nature, Lond.*, 1962, **193,** 195
[7] McDougall, E. I., *Biochem. J.*, 1948, **43,** 99
[8] Flatt, W. P., Warner, W. G., & Loosli, J. K., *Cornell agric. Exp. Sta. Memoir*, 1959, No. 361
[9] 'Official Methods of Analysis', 9th edn, 1960 (Washington, D.C.: Ass. Off. Agric. Chemists)
[10] Wilson, M. K., & Briggs, C. A. E., *J. appl. Bact.*, 1955, **18,** 294
[11] Garton, G. A., Lough, A. K., & Viogue, E., *J. gen. Microbiol.*, 1961, **25,** 215
[12] Balch, C. C., & Johnson, V. W., *Brit. J. Nutr.*, 1950, **4,** 389
[13] Miles, J. T., *J. Dairy Sci.*, 1951, **34,** 492
[14] Smith, P. H., Sweeney, H. C., Rooney, J. R., King, K. W., & Moore, W. E. C., *J. Dairy Sci.*, 1956, **39,** 598
[15] Chalmers, M. I., *Abstr. 5th Int. Congr. on Nutrition* (Washington, D.C.), 1960
[16] Jayasinghe, J. B., *Ceylon vet. J.*, 1961, **9,** 135

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STUDIES ON RUMEN METABOLISM. IV.*—Effect of carbohydrate on ammonia levels in the rumen of pasture-fed cows and in rumen liquors incubated with ryegrass extracts

By J. A. ROBERTSON and J. C. HAWKE

Paired feeding of rumen-fistulated cows showed that 7·4–14·8% increases in nitrogenous constituents in pasture led to higher maximum concentrations of ammonia in the rumen 2–4 h. and higher minimum values 6 h. after feeding. Infusion of 500 g. of starch into the rumen was not always effective in lowering ammonia content to the control levels when additional nitrogen, equivalent to about 130–160 g. of protein each feeding period, was included as a pasture constituent. In general, 900 g. of starch in similar conditions led to ammonia levels of the same order as recorded in the controls. Infusion of starch did not increase VFA concentrations.

Incubation of rumen liquor with ryegrass extract gave rapid production of ammonia. This could be largely prevented by the addition of galactose, sucrose, lactose and glucose. The results suggest that one of the factors that can limit the utilisation of pasture protein is likely to be the amount of soluble sugars and other readily hydrolysed carbohydrate available in the pasture.

Introduction

Because the type and the relative amounts of dietary protein and carbohydrate regulate the ammonia concentration and presumably the extent of microbial synthesis in the rumen,[1] the high concentrations of ammonia frequently found in the rumen of animals grazing on pasture has led to speculation on the efficiency of utilisation of nitrogenous constituents of pasture species.[2] The concentrations of sugars in grasses and clovers are influenced by factors such as stage of growth and climate,[3-5] and accompanying fluctuations in the levels of protein and other nitrogenous constituents result in considerable variation in the soluble carbohydrate/protein ratio throughout a growing season, with low values in early spring and in late autumn when growth is rapid.[6]

The significance of the variation in the protein level during a growing season is difficult to ascertain in a seasonal study because of the introduction of uncontrollable factors. Furthermore, the influence of the physical and chemical properties of a protein on its rate of utilisation makes supplementation of pasture protein with protein from another source an unreliable guide to the effects of variations in the level of protein in pasture on rumen digestion. Paired feeding experiments in which the protein intake was varied by growing fresh fodder with different nitrogen levels, provided a method for simulating natural variations in pasture protein and examining the effects of carbohydrate supplementation. Starch was infused into the rumen to examine carbohydrate supplementation, because it had been found greatly to increase the rate of disappearance of ammonia in the rumen of cows on hay and concentrate diets and to improve the conversion of urea to protein.[7]

The role of soluble sugars in the conversion of ryegrass protein to bacterial protein was followed *in vitro*. Ryegrass extract served as an appropriate protein substrate.

Experimental

Treatment of animals for in vivo *experiments*

Lactating twin Jersey cows (pair *a*, A) with rumen fistulas, which were being stall-fed twice daily (9.30 a.m. and 4.00 p.m.) on freshly cut clover–ryegrass pasture, were used to investigate the influence of readily fermentable carbohydrate on the utilisation of dietary protein as measured by ammonia levels in the rumen. One-half of the available pasture had previously been top-dressed with ammonium sulphate at the rate of 200 lb./acre. After a preliminary period of nine days, during which cow *a* was fed top-dressed pasture and cow A normal pasture, 500 g. of commercial wheat starch was infused, as a 50 : 50 aqueous suspension,

* Part III: *J. Sci. Fd Agric.*, 1964, **15**, 890

into the rumens of both animals during the morning (9.30–11.30 a.m.) and afternoon (4.00–6.00 p.m.) periods for two consecutive days (Experiment i). Rumen samples were collected immediately before the morning feeding period and 2, 4, 6, 8·5, 10·5, and 12·5 h. after feeding on the day before infusion of the starch and on the 2 days of infusion.

After the second day of infusion, cow *a* was fed normal pasture and cow A fed top-dressed pasture for a period of 14 days. Both animals then received 900 g. of starch twice daily for 2 consecutive days, with infusion and sampling times as described above (Experiment ii).

Treatment of animals for the in vitro *measurement of fermentation rates*

A non-lactating Jersey cow with a rumen fistula, which was being stall-fed on freshly cut red clover and fasted overnight before sampling, provided the source of rumen liquor. The collection of rumen fluids, preparation of ryegrass juice extract, and the measurement of fermentation rates at 39° were as described previously.[8]

Incubation of rumen liquor with carbohydrate

In the first series of experiments, the eight bottles of 1-pint capacity used as experimental vessels were arranged as follows: two blanks containing rumen liquor (100 ml.) and water (20 ml); two or three controls containing rumen liquor (100 ml.), perennial ryegrass juice (10 ml.) and water (10 ml.); three or four bottles containing rumen liquor (100 ml.), perennial ryegrass juice (10 ml.) and carbohydrate solution (10 ml.) which contained either 0·1 or 0·2 g. of carbohydrate. In order to determine gas formation, one of the blanks was acidified at zero time and the remaining bottles after incubation for 3 h.

In the second series of experiments, four bottles were used as controls (rumen liquor, 100 ml.; ryegrass juice, 10 ml.; water, 10 ml.) and three with 10 ml. water replaced by 10 ml. of a 10% solution of sugar. As the supply of α-D-galacturonic acid was limited, 10 ml. of a 5% solution were used. One of the controls was acidified at zero time and one control and one containing sugar after incubation for 0·5, 1·5 and 3 h.

Ammonia and VFA* concentrations were measured after acidification in all experiments.

Analytical methods

Moisture.—100 g. freshly cut herbage were chopped into 1–2 in. lengths and dried in a forced-draught oven on aluminium trays at 100° for 16 h.

Crude protein.—Crude protein was determined as described previously.[8]

Non-protein nitrogen.—5 g. of dried material were boiled with 250 ml. of 80% ethanol for 3 min. and filtered. The filtrate was evaporated to dryness on a water-bath and re-dissolved in 10 ml. of water.[9] Total nitrogen was determined on a 5-ml. aliquot by the Kjeldahl method.[10]

Ammonia and total VFA in the rumen liquors and total nitrogen and soluble sugars in the grass juice extracts were determined as described previously.[8]

Separation and identification of soluble sugars in grass juice extract

An aliquot (approx. 100 ml.) of the grass juice extract was centrifuged at 40,000 *g* for 30 min. to remove suspended particulate material.[4] Soluble sugars in the supernatant were then separated by chromatography on either Whatman No. 1 or No. 3 MM paper. The most useful eluting solvent for the separation of glucose, galactose, mannose, arabinose, ribose and fructose was ethyl acetate–acetic acid–water (3 : 1 : 3, by vol.). *p*-Anisidine hydrochloride was used as the spray reagent for pentoses and hexoses[11] and urea phosphate for ketoses.[12]

Quantitative estimation of sugars

The concentrations of the various sugars in ryegrass juice extract were estimated after purification by chromatography[13] on Whatman No. 3 MM paper. A volume of 0·003–0·015 ml. of supernatant prepared from ryegrass juice was applied to the paper as spots by an 'Agla'

* Acetic, propionic and butyric acids

micrometer syringe, and the papers (46 in. × 18 in.) eluted for 32–70 h. with ethyl acetate–acetic acid–water (3 : 1 : 3, by vol.). The papers were dried and, except for fructose determinations, dipped in aniline hydrogen phosphate[14] and heated at 105° for 10 min., 50–100 μg. of the sugars being estimated were used as standards throughout the procedure. Elution and the determination of the sugars was by the method described by Richards.[15]

For the determination of fructose, an additional marker strip containing the standard was chromatographed. This strip was cut off, sprayed with urea phosphate solution and heated. The areas on the main chromatogram corresponding to the position of fructose on the marker strip were cut out and eluted in 6 ml. of water. Fructose was estimated in 2 ml. of the eluted solution by the method of Bell,[16] and total soluble sugar by the method of Bath.[17]

Results

(1) In vivo *experiments*

Nitrogen levels in the feed and the level of intake

Total nitrogen in pasture top-dressed with ammonium sulphate at the rate of 200 lb./acre ranged from 3·18 to 3·72% of the dry matter and was 7·4–14·8% higher than the levels in untreated pasture obtained on corresponding days (Table I). Levels of non-protein N (NPN) were also higher in top-dressed pasture, and consequently the relative proportions of the NPN and the protein components in the two pastures were similar.

With the availability of pastures of different nitrogen levels, the two cows in paired feeding experiments were maintained on appreciably different nitrogen intakes except on the fourth day when cow *a*, feeding on pasture of lower nitrogen content, had an unusually high intake (Table I).

Table I

Nitrogen intake of twin cows fed on pasture containing different levels of nitrogenous constituents

		Pasture top-dressed with 200 lb. of $(NH_4)_2SO_4$/acre						Pasture without $(NH_4)_2SO_4$				
		N content of pasture, % of dry matter		Total N intake, lb.				N content of pasture, % of dry matter		Total N intake, lb.		
Date	Animal	Total-N	NPN	Morning	Afternoon	Total	Animal	Total-N	NPN	Morning	Afternoon	Total
31.10.62	Cow *a*	3·56	0·76	0·345	0·328	0·673	Cow A	3·28	0·71	0·266	0·289	0·554
1.11.62*	Cow *a*	3·41	0·74	0·351	0·368	0·719	Cow A	3·16	0·69	0·288	0·300	0·588
2.11.62*	Cow *a*	3·72	0·84	0·379	0·372	0·751	Cow A	3·16	0·72	0·237	0·303	0·540
15.11.62	Cow A	3·18	0·74	0·308	0·347	0·655	Cow *a*	2·96	0·64	0·355	0·355	0·710
16.11.62†	Cow A	3·25	0·79	0·361	0·397	0·758	Cow *a*	2·83	0·70	0·286	0·314	0·600
17.11.62†	Cow A	3·33	0·75	0·326	0·290	0·616	Cow *a*	2·94	0·66	0·303	0·285	0·588

* 500 g. of an aqueous suspension of starch infused into rumens of both animals during morning and afternoon feeding periods
† 900 g. of an aqueous suspension of starch infused into rumens of both animals during morning and afternoon feeding periods

Ammonia concentrations in the rumen and the effect of addition of starch

Changes in the ammonia concentration in the rumen followed much the same pattern throughout the 6 days of the experiment, with peak concentrations occurring 2–4 h. after the commencement of each feeding period (Fig. 1). Concentrations of ammonia were appreciably higher in the rumen when the cows were fed pasture with the higher nitrogen content. Without starch, the average ammonia concentration 6 h. after the start of feeding on high-N pasture was more than twice that for normal pasture (15·8 mg./100 ml. of rumen liquor compared with 6·9 mg./100 ml.).

The addition of 500 g. of starch twice daily had a variable effect on ammonia levels. On the second day of addition, together with a diet of high-N pasture, the recorded maxima and minima were similar to those for untreated pasture without starch. However, on the first day, when additional nitrogen equivalent to about 130–160 g. of protein was present in the high-N pasture given at each feeding period, this level of supplementation was insufficient to bring about a lowering of ammonia content. Increasing the dose to 900 g. of starch twice daily brought about appreciable and consistent reductions in ammonia level; in general, concentrations in the rumen of cows feeding on high-N pasture were of the same order as those

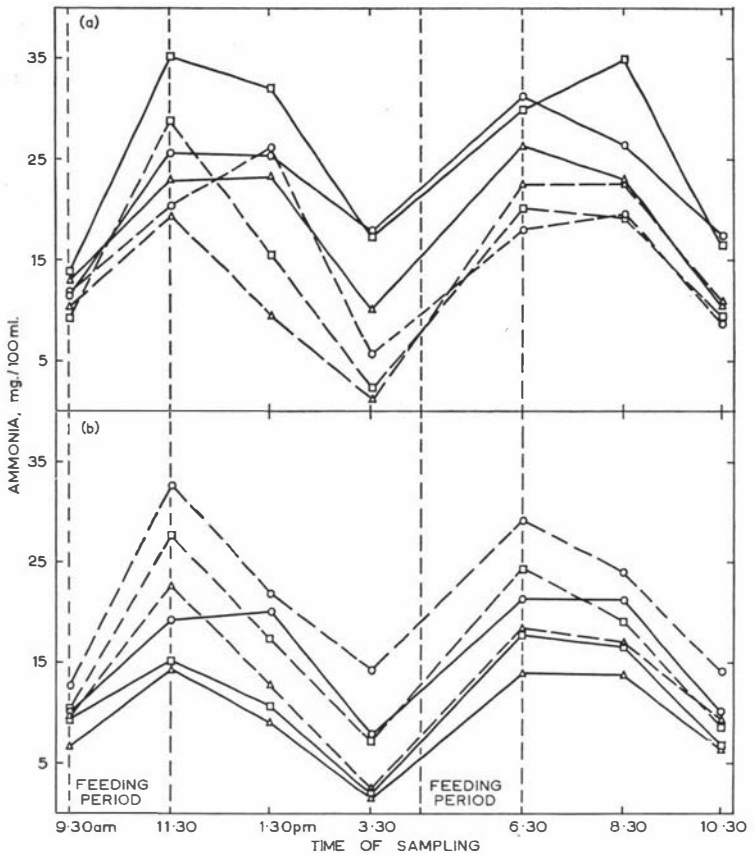

FIG. 1.—*Variations in rumen ammonia concentrations in twin cows fed on freshly cut mixed pasture with and without the addition of starch*

(a) —— Cow a—fed top-dressed pasture
--- Cow A—fed untreated pasture
○ 31/10/62—no starch
□ 1/11/62—500 g. starch added to rumen during each feeding period
△ 2/11/62—500 g. starch added to rumen during each feeding period

(b) —— Cow a—fed untreated pasture
--- Cow A—fed top-dressed pasture
○ 14/11/62—no starch
□ 15/11/62—900 g. of starch added to rumen during each feeding period
△ 16/11/62—900 g. of starch added to rumen during each feeding period

for normal pasture feeding without starch. The very low minima recorded 6 h. after feeding (< 5 mg./100 ml. on many occasions) was a further feature when starch was added.

VFA concentrations in the rumen

The concentrations of VFA generally rose steadily throughout the day, reaching maximum levels after the afternoon feeding period (Fig. 2). Values ranged from minima of 50–94 mmoles/l. to maxima of 124–162 mmoles/l. and, in general, the cow with the higher intake had slightly higher VFA levels. There was no apparent effect of either the nitrogen level in the feed or of added starch.

(2) In vitro *experiments*

Soluble sugars and crude protein in ryegrass juice extract

The concentrations (mg./ml.) of six sugars found in the grass juice extract as measured by quantitative paper chromatography were: galactose, 1·38; glucose, 4·00; mannose, 1·55;

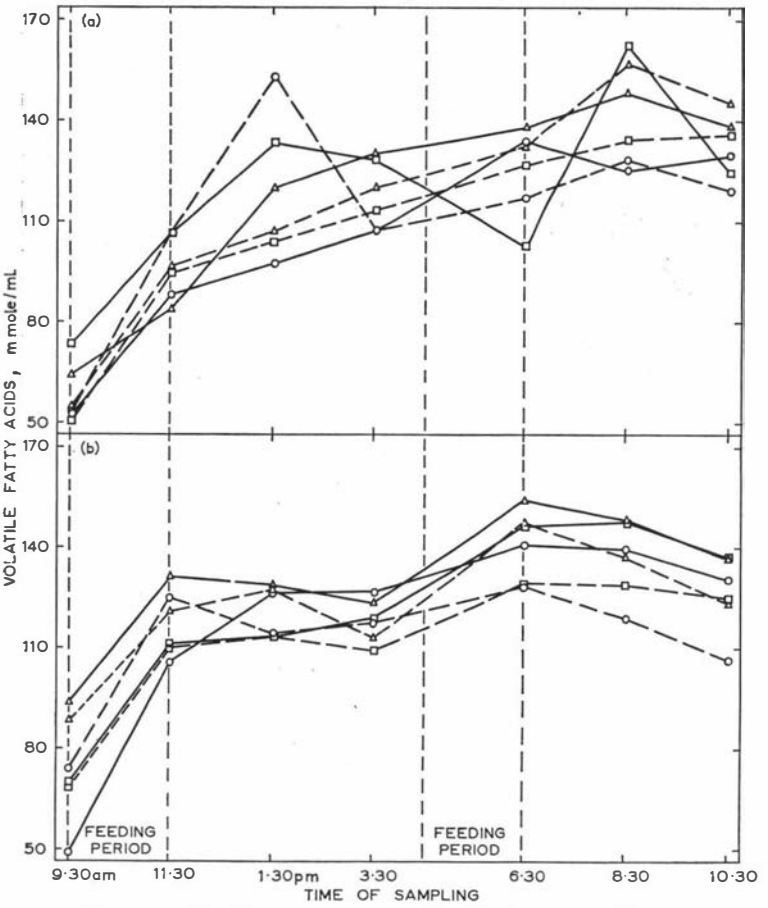

FIG. 2.—*Variations in VFA concentrations in the rumen of twin cows fed on freshly cut mixed pasture with and without the addition of starch*

(a) —— Cow *a*—fed top-dressed pasture
--- Cow A—fed untreated pasture
○ 31/10/62—no starch
□ 1/11/62—500 g. of starch added to rumen during each feeding period
△ 2/11/62—500 g. of starch added to rumen during each feeding period

(b) —— Cow *a*—fed untreated pasture
--- Cow A—fed top-dressed pasture
○ 14/11/62—no starch
□ 15/11/62—900 g. of starch added to rumen during each feeding period
△ 16/11/62—900 g. of starch added to rumen during each feeding period

fructose, 7·88; arabinose, 0·85; ribose, 0·29. Galacturonic acid was also identified. The total concentration of the individual sugars determined by this method (15·95 mg./ml. extract) was 0·7 mg./ml. higher than the value obtained for total soluble sugars by the method of Bath.[17] The ryegrass extract contained 4·4 mg. of crude protein/ml.

Effect of addition of 0·1 *and* 0·2 *g. soluble carbohydrate on metabolic activity* in vitro

In the absence of added substrate, small changes occurred in gas production, ammonia and VFA concentration after incubation of rumen liquors obtained from a cow fasted overnight for 3 h. (Table II). The considerable variations (55–155 ml.) in the gas released on acidification of collected rumen liquor was related to the initial level of VFA, and therefore to the pH—high VFA concentrations giving low pH values and hence low bicarbonate concentration in rumen liquor. Compared with values for the incubation of rumen liquor alone, the increases brought about by the addition of 10 ml. of ryegrass juice extract to 100 ml. of rumen liquor were as follows: gas formation, 33–76 ml.; ammonia concentration, 12·1–15·7 mg./100 ml.; VFA concentration, 18·1–32·9 m.moles/l.

Table II

Gas, ammonia and VFA formation in rumen liquor (100 *ml.*) *incubated with ryegrass juice alone* (10 *ml.*) *or with ryegrass juice* (10 *ml.*) *and soluble carbohydrates for* 3 *h. at* 39°

Exp. no.	Blank		Ryegrass juice alone	Ryegrass Juice and Glucose		Sucrose		L-Arabinose		Fructose	Galactose	Xylose
	0 h.	3 h.		0·1 g.	0·2 g.	0·1 g.	0·2 g.	0·1 g.	0·2 g.	0·2 g.	0·2 g.	0·2 g
Gas production, ml.												
1	155	176	209	222	—	220	—	—	—	—	—	—
2	122	138	180	192	—	193	—	188	—	—	—	—
3	67	82	151	—	170	—	170	—	158	—	—	—
4	126	142	205	—	226	—	225	—	—	225	—	—
5	55	77	153	—	175	—	168	—	—	—	173	167
NH_3 concentration, mg./100 ml.												
1	15·2	22·8	34·9	33·7	—	34·0	—	—	—	—	—	—
2	21·0	27·5	40·1	38·0	—	38·4	—	40·3	—	—	—	—
3	18·6	22·5	38·2	—	34·6	—	34·6	—	37·8	—	—	—
4	16·3	21·7	36·4	—	31·7	—	32·9	—	—	32·2	—	—
5	18·7	26·2	41·4	—	37·7	—	37·3	—	—	—	36·1	35·8
VFA, mmoles/l.												
1	—	—	—	—	—	—	—	—	—	—	—	—
2	56·5	53·5	76·4	85·6	—	84·3	—	84·4	—	—	—	—
3	76·1	76·0	94·1	—	110·2	—	110·7	—	109·5	—	—	—
4	52·7	53·4	79·5	—	85·8	—	85·7	—	—	85·6	—	—
5	81·1	79·2	112·1	—	117·5	—	117·8	—	—	—	118·5	117·8

It is apparent from Table II that at the levels investigated, glucose, sucrose, fructose, galactose and xylose produced very similar increases in the fermentation activity of the rumen micro-organisms in terms of gas and VFA formation and ammonia utilisation. Increasing the glucose or sucrose from 0·1 to 0·2 g. produced an almost proportional increase in this activity. Of the carbohydrates tested, L-arabinose was the least effective in increasing the utilisation of ammonia-N but increased gas formation and VFA levels by about the same as the other sugars.

Comparison of rates of fermentation of soluble carbohydrates

Preliminary experiments showed that the effects of glucose on gas production, ammonia and VFA concentrations increased as the levels of glucose were raised from 0·1 to 1·0 g. (Table III). Excepting for ammonia, these changes were approximately proportional to the increases in the amount of glucose added. Consequently, it was decided to test a series of carbohydrates at the 1·0 g. level since differences could then be expected to be more apparent than at the lower levels used above. Practical difficulties permitted rate measurements to be made using only one carbohydrate at a time: therefore direct comparisons of gas formation and VFA and ammonia concentrations are not valid because of the variations in the initial values for the different samples of rumen liquor.

A consistent feature of the incubation experiments was the high rate of gas, ammonia and VFA formation in the first half hour of incubation (Table IV). The increase in ammonia levels in the controls during this period was usually between one-half and three-fourths of the total increase over the 3-h. period, whereas increases in gas and VFA formation represented between one-third and one-half and between one-quarter and one-half of the 3-h. increases respectively. Although gas production and VFA formation in the controls increased at a slower rate between 30 min. and 3 h., the presence of certain sugars had the effect of sustaining the initial high rates.

Table III

Gas, ammonia and VFA formation in rumen liquor (100 *ml.*) *incubated with ryegrass juice alone* (10 *ml.*) *or with ryegrass juice* (10 *ml.*) *and glucose* (0·1–1·0 *g.*) *for* 3 *h. at* 39°

	Blank		Ryegrass juice alone	Ryegrass juice and glucose			
	0 h.	3 h.		0·1 g.	0·2 g.	0·5 g.	1·0 g.
Gas production, ml.	91	124	196	208	211	237	278
Ammonia, mg./100 ml.	28·1	33·9	50·6	48·6	46·9	44·7	43·6
VFA, m.moles/l.	66·0	73·8	85·0	87·5	92·7	103·6	111·5

Table IV

Gas, ammonia and VFA formation in vitro *with and without* 1 *g. of additional carbohydrate*

Carbohydrate		Gas production, ml. Incubation time (h.)				Ammonia, mg./100 ml. Incubation time (h.)				VFA, mmoles/l. Incubation time (h.)			
		0	0·5	1·5	3·0	0	0·5	1·5	3·0	0	0·5	1·5	3·0
Glucose	C*	150	183	201	218	23·8	34·8	36·7	37·9	54·2	61·3	70·0	81·0
	E†	150	200	249	296	23·8	32·1	31·6	26·9	54·2	75·0	92·5	103·0
Galactose	C	127	168	186	199	27·3	38·5	38·8	42·2	59·9	66·1	78·0	83·1
	E	127	174	238	285	27·3	37·1	32·4	26·1	59·9	73·4	96·6	113·8
Lactose	C	107	132	152	164	22·5	29·8	32·9	33·2	71·8	80·4	86·1	91·6
	E	107	144	179	239	22·5	30·5	26·1	24·2	71·8	87·0	96·9	113·8
Sucrose	C	129	164	185	195	21·0	30·0	32·4	33·2	62·0	72·4	80·3	85·7
	E	129	174	240	270	21·0	27·9	24·4	24·5	62·0	82·4	98·4	109·0
Xylose	C	175	200	218	233	17·9	24·6	38·8	30·9	45·3	52·9	61·9	63·8
	E	175	202	224	256	17·9	25·4	27·1	24·9	45·3	53·8	63·5	75·4
D-Arabinose	C	137	173	186	215	26·2	35·6	37·3	39·7	69·0	79·0	85·4	94·8
	E	137	176	187	208	26·2	34·1	37·7	40·5	69·0	76·5	87·2	99·2
α-D-galacturonic acid (0·5 g.)	C	170	194	219	240	18·6	28·3	31·9	33·7	49·7	56·4	67·7	69·9
	E	170	195	226	270	18·6	27·1	28·9	27·5	49·7	62·1	73·4	86·7

* C—control = rumen liquor (100 ml.) and ryegrass juice extract (10 ml.) and water (10 ml.)
† E—experimental = rumen liquor (100 ml.), ryegrass juice extract (10 ml.) and carbohydrate solution (10 ml.)

These same sugars had an even more marked effect on the levels of ammonia during the later stages of fermentation as shown by the subsequent decrease in ammonia concentration, whereas the levels continued to increase in the controls.

Comparison of the effect of sugars on gas production and ammonia formation shows that there is a close relationship between the fermentation of readily available substrate and the utilisation of nitrogen for protein synthesis (Fig. 3). Galactose, glucose, sucrose and lactose, which were fermented rapidly, brought about the greatest reduction in ammonia concentration. Galacturonic acid appears to behave in a similar way allowing for the lower concentration used. On the other hand, when D-arabinose was present, a negligible effect on fermentation rate was accompanied by practically no change in ammonia concentration.

Similarly, galactose, sucrose, lactose, glucose and galacturonic acid caused the greatest increases in VFA formation (16·8–30·7 mmoles/l. above the controls after incubation for 3 h.). Xylose was less effective (11·6 mmoles/l.), and D-arabinose had a very small effect (4·4 mmoles/l.).

Discussion

The higher ammonia levels present in rumen samples from animals fed top-dressed pasture illustrate a relationship between the nitrogen content of the pasture nitrogen intake and the concentration of ammonia in the rumen. Other workers[18–20] have shown that the time required for ammonia levels to reach a maximum after feeding, and the magnitude of the increases, depend largely on the solubility of the dietary protein. For example, a dose of 100 g. of casein gave a maximum ammonia concentration of 63·5 mmoles/l. 3 h. after administration, whereas zein, which is much less soluble, gave a peak of 11·0 mmoles/l. 10 h. after administration.[18] In the present work, maximum concentrations occurred 2–4 h. after feeding, which indicates that the plant protein was readily metabolised. McDonald[18] also observed lower ammonia concentrations a few hours after feeding than before feeding. It was suggested that, as a result of large amounts of energy becoming available for protein synthesis after feeding, ammonia utilisation would exceed formation. Later, when the rate of bacterial growth dropped, production could again exceed utilisation.

Increases in the level of nitrogen in the pasture as a result of top-dressing were similar to those found in hay which had received the same rate of application 2 weeks before cutting.[21] Ferguson[21] also noted that top-dressing had no effect on the protein-N/NPN ratio. In spite of a lower intake of nitrogen on the first day of Experiment (ii), cow *a*, fed on high-N pasture, had higher concentrations of ammonia in the rumen. This suggests that, either the protein in the top-dressed pasture was more readily hydrolysed, or the higher level of soluble carbohydrate

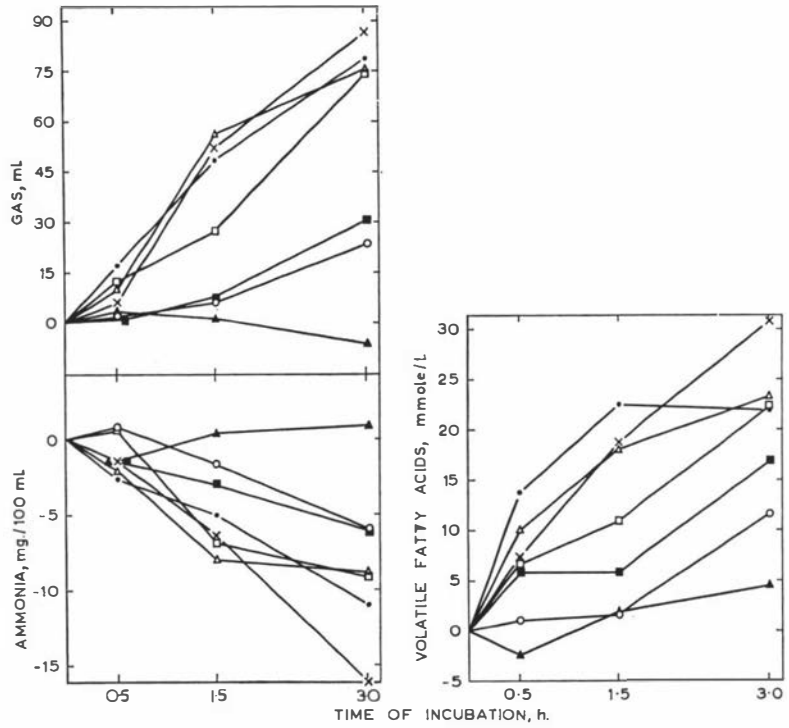

FIG. 3.—*Effect of soluble carbohydrates on gas production and VFA concentrations in rumen liquor* (100 *ml.*) *and ryegrass juice extract* (10 *ml.*) *incubated at* 39°

(Rates with rumen liquor and ryegrass extract = 0)

× galactose; △ sucrose; □ lactose; ● glucose; ■ galacturonic acid; ○ xylose; ▲ D-arabinose (1·0 g. of sugars; 0·5 g. of galacturonic acid)

in the untreated pasture (approximately 2·2% of dry matter) increased protein synthesis sufficiently to utilise the additional nitrogen ingested.

The effect of starch in lowering ammonia concentrations in the rumen of pasture-fed animals is in agreement with observations of Annison[20] and of Lewis & McDonald[1] after the dosage of animals on hay and concentrate diets with starch. In so far as ammonia levels in the portal blood are related to ammonia levels in the rumen,[22] starch supplementation would seem to improve the utilisation of dietary nitrogen in ruminants grazing on pasture high in protein.

The lack of any consistent difference in the levels of total VFA as a result of either the difference in the level of nitrogen in the feed or the addition of starch was unexpected in view of the findings of Lewis & McDonald[1] that casein added to the rumen of sheep at the same time as starch resulted in higher concentrations of total VFA. In addition, the present *in vitro* experiments show that there is a direct relationship between the effect of added sugar on ammonia utilisation and the formation of VFA. Reduction in ammonia concentration, without an accompanying increase in VFA concentration when starch is added, may well indicate that starch was being utilised as an energy source in protein synthesis by rumen bacteria.

With the exception of ribose, the sugars present in ryegrass juice extract are among those commonly found in grasses.[3] The absence of sucrose in the extract was likely to be due to enzymic hydrolysis during extraction and subsequent storage. Similarly, arabinose and galactose are usually present in polymeric form in the hemicellulose and pectic fractions respectively[23] and their occurrence as free sugars was probably due to enzymic degradation.[24]

The reduction in ammonia concentration in the rumen liquor when certain soluble sugars were added to the incubation mixture agrees with observations made previously by Pearson

& Smith[25] and McNaught[26] when studying the *in vitro* conversion of non-protein to protein. The rapid fermentation of galacturonic acid in the present work indicates that the presence of a primary alcohol group is not essential to the utilisation of a carbohydrate as was suggested by McNaught. Lewis & McDonald[1] found that the greatest increases in protein utilisation occurred when the food material acting as an energy source was attacked at a rate comparable to the rate at which NPN becomes available in the rumen. In the present work, the very high rate of ammonia formation during the first half-hour of incubation indicated that the nitrogen in the grass juice extract was readily metabolised, and in these circumstances readily metabolised sugars should be effective in facilitating the incorporation of ammonia into bacterial protein.

The considerable reduction in ammonia formation brought about by supplementation of soluble sugars in ryegrass extract with galactose, glucose, sucrose and lactose stresses the importance of the concentrations of readily fermented carbohydrate in pasture grasses and clovers. This will apply to sucrose and mannose in particular and to a lesser extent ribose. Hemicelluloses and pectic substances probably would also be broken down sufficiently quickly to enhance ammonia utilisation. Thus, provided the amount of protein being ingested by an animal is not in excess of the protein requirements of that animal, the availability of soluble sugars and other readily hydrolysed carbohydrates is likely to be the main factor limiting the utilisation of pasture protein when carbohydrate/protein ratios are low, as occurs in grass and clover pastures in early spring and late autumn. The chemical composition of pasture as affected by season requires further investigation before the most efficient utilisation of grassland by ruminants is likely to be achieved.

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References

[1] Lewis, D., & McDonald, I. W., *J. agric. Sci.*, 1958, **51,** 108
[2] Barnett, A. J. G., & Reid, R. L., ' Reactions in the Rumen ', 1961, p. 110 (London: Arnold).
[3] MacKenzie, D. J., & Wylam, C. B., *J. Sci. Fd Agric.*, 1957, **8,** 38
[4] Bailey, R. W., *J. Sci. Fd Agric.*, 1958, **9,** 748
[5] Hirst, E. L., MacKenzie, D. J., & Wylam, C. B., *J. Sci. Fd Agric.*, 1959, **10,** 19
[6] Johns, A. T., *N.Z. J. Sci. Technol.*, 1955, **37A,** 301
[7] Belasco, I. J., *J. Animal Sci.*, 1956, **15,** 496
[8] Robertson, J. A., & Hawke, J. C., *J. Sci. Fd Agric.*, 1964, **15,** 274
[9] Bathurst, N. O., & Allison, R. M., *N.Z. J. Sci. Technol.*, 1949, **31B,** 1
[10] ' Official Methods of Analysis ', 9th Edn, 1960, p. 284 (Washington, D.C.: Ass. off. agric. Chem.)
[11] Hough, L., Jones, J. K. N., & Wadman, W. H., *J. chem. Soc.*, 1950, p. 1702
[12] Wise, C. S., Dimler, R. J., Davis, H. A., & Rist, C. E., *Analyt. Chem.*, 1955, **27,** 33
[13] Pridham, J. B., *Analyt. Chem.*, 1956, **28,** 1967
[14] Bailey, R. W., personal communication
[15] Richards, E. L., *J. Dairy Res.*, 1963, **30,** 223
[16] Bell, D. J., in ' Modern Methods of Plant Analysis ' (Eds. K. Paech and M. V. Tracey), 1955, Vol. 2, p. 1 (Berlin: Springer-Verlag)
[17] Bath, I. H., *Analyst*, 1958, **83,** 451
[18] McDonald, I. W., *Biochem. J.*, 1952, **51,** 86
[19] Annison, E. F., Chalmers, M. I., Marshall, S. B. M., & Synge, R. L. M., *J. agric. Sci.*, 1954, **44,** 270
[20] Annison, E. F., *Biochem. J.*, 1956, **64,** 705
[21] Ferguson, W. S., *J. agric. Sci.*, 1948, **38,** 33
[22] Lewis, D., Hill, K. J., & Annison, E. F., *Biochem. J.*, 1957, **66,** 587
[23] Bailey, R. W., *Proc. N.Z. Soc. Animal Prodn*, 1962, **22,** 99
[24] Wylam, C. B., *J. Sci. Fd Agric.*, 1953, **4,** 527
[25] Pearson, R. M., & Smith, J. A. B., *Biochem. J.*, 1943, **37,** 153
[26] McNaught, M. L., *Biochem. J.*, 1951, **49,** 325