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THE EFFECT OF LIPID ON THE DIGESTION OF CELLULOSE BY THE RUMINANT.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Agricultural Science in Animal Science at Massey University.

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REVIEW OF LITERATURE

1. Metabolism of Lipids by Ruminants

Many workers have added fats, oils and fatty acids to the rations of sheep, beef cattle and dairy cows to investigate the effects of lipid on either one or more of the following; intake, digestibility and energy utilisation of rations, live weight gains, methane production, ammonia production, N retention, milk production, milk constituents, total VFA production and VFA molar proportions or the value of lipid as a source of energy. These factors will be discussed in subsequent sections of this review.

Lipids which have been fed or infused into ruminants include coconut, cod liver, corn, cottonseed, linseed, palm kernel, peanut, soyabean, tung and whale oils, animal fats (lard, tallow and poultry fat) and long chain fatty acids (palmitic, stearic, oleic, linoleic and linolenic).

Since the amount of lipid ingested by adult ruminants is substantial, the investigations into the effects of lipids are warranted. A cow consuming 45Kg. of pasture daily will ingest about 500g. of lipids and, under conditions of stall-feeding, pregnant and lactating animals may receive rations which provide up to 1.0Kg. of lipids daily (Garton, 1967).

Lipolysis

When lipids are ingested by ruminants extensive lipolysis occurs in the rumen. Garton, Hobson & Lough (1958) incubated linseed oil and tung oil with rumen contents (1.0g./100ml.) and more than 75% of the total lipid was recovered at the end of the incubation time in the form of free higher fatty acids. When they slaughtered sheep 7 hr. after feeding, 80-90% of the lipid in the rumen, abomasum and upper part of the small intestine was in the form of higher free fatty acids.

Glycerol Fermentation

The fermentation of the glycerol moiety of lipid triglycerides has also been studied. Johns (1953); Garton, Lough & Vioque (1959); and Hobson & Mann (1961) found that the main product of glycerol fermentation was propionate. Johns (1953) showed that the fermentation of free glycerol by rumen liquor from sheep in-vitro and in-vivo led to the formation of propionate, though no more than 50% of the glycerol which was metabolised could be accounted for on a 'Carbon balance' basis, while the fate of the remainder is unknown. Hobson & Mann (1961) have shown that 50-60% of the glycerol carbon was accounted for as VFA's (largely propionate) when glycerol-fermenting organisms from the rumen of sheep were grown in media containing glycerol. In a report from the Rowett Research Institute (1962) it was noted that the most important members of the glycerol fermenting flora in the sheep rumen are strict anaerobes of the group Selenomonas ruminantium var. lactilyticas. The main product of fermentation of glycerol by the selenomonads and some lipolytic bacteria was propionate, but other acids were also formed. Garton, Lough & Vioque (1959) detected no glycerol in the rumen after hydrolyses of linseed oil, but 4 hr. after glycerol was infused into the rumen only 50% was present, and after 24hr. no glycerol was detectable. VFA's did not account for more than 50% of the glycerol metabolised and the predominant acid was propionate. Recently Wright (1969) found that rumen micro-organisms, obtained from cattle fed clover-ryegrass pasture, can metabolise (U-^{14}C)glycerol with CO_2(17%), acetate(31.4%) and propionate(20.6%) as the major end products. Butyrate(7.6%), lactate(3.9%) and the micro-organisms(6.5%) were also recipients of the ^{14}C. Only 13% of the radioactivity was unaccounted for after 12hr. incubations, although 9% could possibly be contributed to CH_4 which was not analysed.

It was also found that bacteria were much more active as glycerol fermentors than protozoa.

Hydrogenation

Hydrogenation is another modification of the dietary lipid in the rumen. Shortland *et al.*(1955) noticed that the main dietary fatty constituent of pasture-fed animals, linolenic acid, appeared only in traces in the depot fats of ruminants. They showed that linolenic acid was not only hydrogenated to linolenic but also to the monene or to the stearic acid. The ingestion of soyabean oil by cows resulted in an increase in stearic acid of both milk fat and adipose tissue (Tove & Mochrie, 1963), demonstrating the efficiency and completeness of hydrogenation within the rumen. Wood *et al.*(1963) introduced $(1-^{14}C)$linolenic acid into the rumen of a sheep, and with the rumen-omasal orifice ligated, only 0.6-1.0% of the radioactivity appeared as steam-volatile breakdown products (none of which contained less than five carbons), the rest that could be identified remained as various C_{18} long chain fatty acids.

Although lipolysis and hydrogenation are known to occur in the rumen, lipids are involved in many interactions with feed substrates that are not fully understood.

The Effect of Lipid on Feed Intake, Digestibility, and Live Weight Gains

Feed intake may be lowered by adding lipid to the ration. When 500-700g. of linseed oil or whale oil was infused daily into the rumen of cows by Robertson & Hawke (1964), a decrease in intake occurred. Molasses has been added to rations to increase the palatability and therefore the intake of fats (Vanschoubroek, 1966).

In most experiments the digestibility of rations has been significantly

decreased by the addition of fat (Brooks _et al._ 1954; Ervin _et al._ 1956; Summers _et al._ 1957; Ward _et al._1957; Brethour _et al._ 1958; White _et al._ 1958; Warner, 1960; Davison & Woods, 1961; Grainger _et al._ 1961; Nicholson _et al._ 1962; Buysse, 1963; Davison & Woods, 1963a; Mohammed _et al._ 1964; Robertson & Hawke, 1964; and Vanschoubroek, 1966). Vanschoubroek (1966) questioned whether the value of fat added to the ration was similar to that of fat present in the ration in a natural condition. Added fat apparently decreased food utilisation more than an equal quantity of fat originally present in the feed (Brooks _et al._ 1954). Robertson & Hawke (1964) showed from _in-vitro_ and _in-vivo_ investigations that lipid depressed digestion of fibrous carbohydrates. Ervin _et al._ (1956) found that the addition of tallow tended to reduce crude fibre breakdown, the live weight gains of steers being reduced when fed wheat straw which had a high crude fibre content. On account of the effect of added lipid on the digestibility of the ration, Vanschoubroek (1966) suggested that it is advisable not to exceed 9% fat addition to fattening cattle rations which already contain 2-3% crude fat while the maximum amount of fat advisable for a high yielding dairy cow is 700g./day.

In a few cases adding fat did not depress the utilisation of the ration (Swift _et al._ 1948; Esplin _et al._ 1963; and Orth _et al._ 1966). 4% tallow was added to a mixed ration by Esplin _et al._ (1963) and no effect upon ration components occurred, whereas when 0.5-1.0kg. of beef tallow was added, digestion in the rumen of six cows increased (Orth _et al._ 1966). Swift _et al._ (1948) added corn oil (total E.E. = 6.4%) to sheep rations and an increase in digestibility of all constituents of the mixed ration occurred however, when the amount of corn oil was doubled (E.E. = 9.7%) the effect was just the reverse. Rations differing by only 1% of fat produced no significant

differences in digestibility but when differences of 3% in fat content of the ration were compared, digestibility differences were significant($P < 0.05$).

Besides generally decreasing the digestibility of rations, the effect of fat also decreases weight gains of ruminants (Ward et al. 1957) and Brown et al. 1962). However, when Willey et al. (1952) fed some steer calves cottonseed oil, the fat fed animals consumed less but had higher feed efficiencies per 100lb. gain, and when Broster et al. (1965) added 130ml. of cod liver oil to dairy heifers the rate of live-weight gain improved in all experiments. Broster et al. (1965) could reach no conclusion on whether the cod liver oil made any contribution to the energy metabolism of the animal other than that due directly to its calorific value. Vanschoubroek (1966) noticed that from feeding trials it appeared that the net energy value of added fat was not as high as from calorimetry experiments, due possibly to the depressive action of fat on the other nutrients of the ration.

Other variables which can influence the effect of fat were described by McDowall et al. (1957). They considered that the amount and type of roughage accompanying the fat, and the physical condition of the oil at time of feeding were important. Fats and oils distributed throughout the ration appeared to have less effect than fats given in concentrated forms.

The Effects of Lucerne Ash and Calcium on Rations containing Supplementary Fat

Many experiments have been undertaken to examine the effect of lucerne ash, and later to ascertain the active component(s) in lucerne ash that can reverse the depression in digestibilities observed with rations containing supplementary fat (Brooks et al. 1954; Summers et al. 1957; Ward et al. 1957; White et al. 1958; Davison & Woods 1961; Grainger et al. 1961; and Davison & Woods 1963a). White et al. (1958) found that the effect of lucerne ash

in restoring the digestibility of cellulose in a ration that contained 5% corn oil was produced by 4.4g.Ca or 4.4g.Ca + 0.86g.P. Phosphorous alone or a trace mineral mixture had no effect. Further work by Grainger et al. (1961), when Ca,Fe,K,Zn, and Mg were added to the daily ration containing 7% corn oil, discovered that Ca and Fe alleviated or partially alleviated the effect of corn oil on cellulose digestion. K further increased the corn oil effect, and Mg and Zn caused the wethers to stop eating after 7 days. The form of calcium made no difference, $CaCO_3$ and $CaCl_2$ being equally effective in increasing the digestion of organic matter and cellulose in a ration containing corn oil (Davison & Woods, 1963a).

Davison & Woods (1963b) found that $CaCO_3$ additions did not increase the performance of lambs fed a concentrate ration with or without added white grease and Brethour et al. (1958) found that the addition of lucerne ash to the fat ration (15% animal fat) for sheep did not improve weight gains. This latter result could possibly be due to the high fat level because when Ward et al. (1957) added lucerne ash it counteracted the decreased weight gains and low feeding efficiency of the 2.6% level of corn oil, but only partially the effect of 10% corn oil.

The Effect of Lipid on Nitrogen Retention, CH_4 and NH_3 Production

Although corn oil depressed nitrogen retention in lambs, sheep and cattle, it was significantly increased by the addition of lucerne ash (Summers et al. 1957; Ward et al. 1957; and Davison & Woods, 1961).

When linseed oil was added to both high roughage and high concentrate rations for sheep, CH_4 production was depressed (Czerkawski et al. 1966). Blaxter & Czerkawski (1966) considered that much energy of the diet was inevitably loss to the ruminant as CH_4, so control of CH_4 production clearly

has an economic bearing. Palmitic, stearic, oleic, linoleic and linolenic acids reduced CH_4 production, the magnitude of the reduction increasing slightly with increasing unsaturation of the fatty acids and the reduction could be due to a selective toxicity of the fatty compounds in which methanogenic organisms were more affected than cellulolytic ones (Czerkawski *et al.* 1966).

The effect of lipid on NH_3 production is variable. Jayasinghe (1961) added 10-15% peanut oil to a protein supplement for sheep causing less NH_3 to be produced in the rumen. There was a significant reduction in both protozoal concentrations and NH_3 levels when 60g. of linseed oil was fed to sheep (Purser & Moir, 1966). In *in-vitro* experiments (Robertson & Hawke, 1964) linseed oil decreased NH_3 production when incubated with ryegrass fibre, but increased NH_3 production when it was incubated with soluble ryegrass constituents. When 500-700g. of linseed or whale oil was infused into cows daily, levels of NH_3 rose appreciably.

Effect of Lipid on Milk Production and Milk Constituents

Storry & Rook, (1966) have shown that the secretion of the major fatty acids in the milk were reduced when feeding a low-hay, high-concentrate diet to lactating cows causing a fall in the milk fat content. There were also changes in the rumen VFA proportions, with an increase in propionate and valerate and a decrease in acetate. Approximately 60% of the variation in milk fat content was associated with the increase in propionate. On the return of the animals to high-hay diets, recovery of the initial VFA proportions occurred within 4 days but the recovery in milk fat content was not complete until 2-3 weeks had elapsed. Davis (1967) considered that the time lag in recovery of the milk fat suggests that factors other than the amount of acetate or the acetate:propionate ratio are involved.

Different lipids have also had different effects on milk fat production in dairy cows (Van Soest, 1963; and Vanschoubroek, 1966), these differences being contributed to by their degree of saturation or unsaturation. Garner & Sanders (1938) increased butter fat yield by increasing butterfat % from adding palm oil, butter, or lard and considered the beneficial oils are those containing large proportions of saturated fatty acids. Lipids containing a large proportion of unsaturated fatty acids have generally had the effect of depressing milk fat (Shaw & Ensor, 1959).

Many workers (Garner & Sanders, 1938; Brown et al. 1962; Moody et al. 1964; and Beitz & Davis, 1964) have shown that the addition of lipids to rations of lactating cows, although not altering milk production, did lower the milk fat content while in other cases only individual fatty acids were affected. Tove & Mochrie (1963) noticed that soyabean oil increased the stearic acid content and cottonseed oil increased the linoleic acid content of milk fat. Mohammed et al. (1964) found evidence that an increasing amount of C_{12} was present in the milk when coconut oil was fed.

Although milk composition was not altered when high levels(600ml./day) of various oils and fats were given to lactating cows by McDowall et al. (1957), the milk, cream, and butter were tainted to different degrees and there were immediate effects on the properties of butterfat including increases in iodine value and refractive index, and changes in the softening point, saponification value and Reichert value. Beitz & Davis (1964) believed that the mechanism whereby cod liver oil caused a decrease in milk fat synthesis was by its effect on milk fat production somewhere beyond the rumen, probably at the udder. The postulate by Shaw & Ensor (1959) that cod liver oil affected milk fat synthesis by altering the rumen VFA, the same as high grain diets, was not confirmed in the study by Beitz & Davis (1964).

Effect of Lipid on Total VFA Concentrations

Total VFA concentrations have been increased (Shaw & Ensor, 1959), decreased (Robertson & Hawke, 1964; and Orth *et al.* 1966) or have remained unaltered (Elam & Davis, 1962; and Esplin *et al.* 1963) when lipids were fed to cattle. When cod liver oil, oleic and linoleic acid were administered to cows on normal diets they increased the total VFA concentrations in the rumen fluid, linoleic acid having the greatest effect. Linoleic acid also increased total VFA the most when given to cows on diets which had already decreased milk fat content markedly (Shaw & Ensor, 1950). When oleic acid was fed to dairy cows, total VFA's fell slightly (Orth *et al.* 1966), and although total VFA's did not decrease when 500g. of linseed oil was given to cows by Robertson & Hawke (1964), at the higher lipid feeding (700g./day) a decrease in total VFA was paralleled by a tendency for intake to fall on successive days of treatment. They also conducted two experiments *in-vitro*, one incubating linseed oil with ryegrass juice, the other incubating linseed oil with ryegrass fibre. In the former experiment total VFA was higher than controls after 1 hr., but no difference was apparent after 3 hr. In the latter experiment, the total VFA's produced were lower after 48hr. than the controls, although slightly higher after 12 and 24hr.

Drenching steers with either 8% soyabean oil (Elam & Davis, 1962) or 4% tallow and 4% hydrolysed vegetable oil (Esplin *et al.* 1963) did not alter the total VFA produced.

Effect of Lipid on VFA Molar Proportions

Shaw & Ensor (1959) administered cod liver oil, oleic and linoleic acid to cows on a normal diet and found that cod liver oil especially exerted a remarkable effect by decreasing the molar proportions of rumen acetate and

increasing the molar proportions of propionate while the effects on rumen butyrate were variable. Elam & Davis, (1962); Nicholson _et al_. (1963); Nottle & Rook (1963); and Robertson & Hawke (1964) subsequently confirmed these effects of added lipid.

The addition of cod liver oil by Nicholson _et al_. (1963) to rations containing a buffer resulted in a higher molar percentage of propionate and lower acetate and butyrate percentages, than when the basal ration was fed alone. The buffer had already had similar effects on acetate and propionate and these effects were greatly accentuated by the addition of cod liver oil. Nottle & Rook (1963) found that cod liver oil caused a pronounced fall in the proportion of rumen acetate and a complementary increase in propionate when added to a basal ration for cattle.

Although soyabean oil, when given as a drench to steers, increased the proportion of propionate in ruminal fluid, there was no effect on the proportion of acetate (Elam & Davis, 1962).

Robertson & Hawke (1964) infused 500g. of linseed oil into cows daily, causing an immediate decrease in acetate and considerable increases in the concentrations of propionate and butyrate. Two _in-vitro_ experiments were also conducted by Robertson & Hawke. Linseed oil was incubated for 3 hr. with ryegrass juice causing only small and variable effects on acetate and butyrate concentrations but slightly higher propionate concentrations than those found in control incubations. When linseed oil was incubated with ryegrass fibre for 48hr., the concentration of acetate was decreased while propionate concentration increased. The concentration of butyrate was affected only slightly. Robertson & Hawke considered that the increase in propionate must have been due to an effect of the lipid on carbohydrate

TABLE 1. The proportions of individual VFA's in the rumen liquor of cattle fed rations containing added lipid.

Reference	Ration Fed and Lipid Added	Acetate	Molar Percentage Propionate	Butyrate	Valerate
Shaw & Ensor, 1959.	Basal Diet	67.4	15.4	11.8	5.3
J. Dairy Sci., 42, 1238.	" + 300ml. oleic acid	61.4	21.5	14.3	2.7
J. Dairy Sci., 42, 1238.	"	78.9	9.9	7.7	3.4
	" + 300ml. linoleic acid	52.2	29.9	14.0	3.8
	"	71.1	14.1	12.5	1.9
	" + 300ml. cod liver oil	56.1	28.2	9.1	5.2
Elam & Davis, 1962.	Basal Diet	54.7	26.3	19.1	-
J. Anim. Sci., 21, 568.	" + 8% crude soyabean oil	51.3	33.4	15.4	-
Nicholson et. al. (1963).	Basal Diet	60.5	21.8	14.7	3.0
Can. J. Anim. Sci., 43, 309.	" + $NaHCO_3$	55.3	26.0	15.4	3.3
	" + " + 60ml. cod liver oil	48.1	39.2	9.0	3.6
	Cow A				
Nottle & Rook, 1963.	Basal Diet	67.2	17.4	12.4	3.1
Proc. Nutr. Soc., 22, vii.	" + 270g. linseed oil	66.6	18.4	12.0	3.0
	" + 270g. cod liver oil	64.5	20.8	11.4	3.4
	" + 270g. beef tallow	69.7	16.4	11.4	2.6
	Cow B				
	Basal Diet	66.6	18.1	12.3	3.0
	" + 270g. linseed oil	67.0	18.5	11.1	3.4
	" + 270g. cod liver oil	60.3	23.8	12.2	3.7
	" + 270g. beef tallow	68.4	17.0	11.8	3.0

digestion. Nottle & Rook (1963) found that the proportions of acetate and propionate were not affected when linseed oil was added to a basal ration for cattle.

When tallow was fed with dairy cow rations by Orth et al. (1966), total acetate initially increased and then tended to decline. Tallow added to the rations of cattle by Nottle & Rook (1963) increased the rumen VFA proportions of acetate with a complementary decrease in propionate.

When linoleic acid was incubated in-vitro for 8 hr. with lucerne meal by Satter et al. (1967), the reduction in the acetate:propionate ratio which occurred was related to the amount of linoleic acid dispersed in the incubating medium. Four different mixtures of fatty acids containing increasing levels of unsaturated fatty acids were fed by Clarke & Roberts (1967) to fistulated sheep. There was little difference in acetate levels but propionate gradually increased and butyrate gradually increased as dietary unsaturated fatty acids increased.

Brown et al.(1962) found that although dietary fat had no effect on rumen acetate or propionate it increased valerate and higher acids. When coconut oil was added to concentrate mixtures by Mohammed et al. (1964) the only effect was a depression in the total butyrate and valerate contents of the bovine rumen.

When tallow and hydrolysed vegetable and animal fat (Esplin et al. 1963) and cod liver oil (Beitz & Davis, 1964; and Broster et al. 1965) were fed, the proportions of the VFA's in the rumen were unaltered.

A comparison of the rumen VFA proportions in the rumen liquor of cattle fed rations containing various lipids is shown in Table 1.

2. Interconversion of Individual Volatile Fatty Acids

The interconversion of VFA's was suspected by James *et al.* (1956) who perfused (1-^{14}C)propionate into a cow and isolated ^{14}C - acetate, n-butyrate and n-caproate from the udder and milk. Although Flavin *et al.* (1956) stated that (1-^{14}C)propionate could not give rise to ^{14}C-acetate, James *et al.* (1956) knew that (1-^{14}C) acetate could give rise to ^{14}C-formate in the rabbit, and so postulated that a similar loss of a methyl carbon from (1-^{14}C)propionate could produce (1-^{14}C) acetate. Leng & Brett (1966) using a constant infusion technique with labelled VFA's, measured simultaneously the production rates of acetate, propionate and butyrate in the rumen of sheep. Interconversions of acetate or butyrate with propionate or vice versa were small in extent, but conversion of butyrate to acetate accounted for 6-13% of the acetate produced, while the conversion of acetate to butyrate accounted for 40-50% of the butyrate produced. This work also confirmed that there was a correlation between production rates and concentrations of the individual acids in the rumen.

3. Propionate Metabolism

The two pathways of propionate formation are the dicarboxylic acid or randomised pathway and the direct, reductive, non-randomised or acrylate pathway, and are readily differentiated on the basis of differences in the distribution of ^{14}C in the propionate formed from position-labelled substrates (Baldwin, 1963). When ^{14}C-glucose was incubated to determine the contribution of the two pathways to propionate, 70-100% was labelled as though propionate was formed via the randomising pathway and 0-30% via the non-randomising pathway. The contribution of the acrylate pathway increased with higher carbohydrate availability of the diet (Baldwin *et al.* 1963).

Although in the past it was believed that insufficient propionate was produced in the rumen to meet the demands for gluconeogenesis, Leng _et al._ (1967) found that when using ^{14}C-propionates, 45-62% of the glucose carbon arose from propionate carbon and only 32% of the propionate produced in the rumen was actually converted into glucose. Therefore, considerably more propionate was produced in the rumen than required to synthetise all the glucose entering the body pool. When Bergman _et al._ (1966) infused ^{14}C-propionate into the veins of sheep, about 50% of the propionate was converted to glucose, and 40% synthetised to other compounds. They concluded that protein probably accounts for much of the glucose requirements of the adult ruminants and that, depending on the diet, propionate probably forms only 20-40% of the glucose.

4. Cellulose Digestion

The digestion of cellulose has been used as a criterion for comparing _in-vitro_ fermentation systems; as one of many criteria for comparing the digestibility of pastures; and cellulose has been used to compare the importance of individual micro-organisms in the rumen. LeFevre & Kamstra (1960) found that sheep and cattle rumen fluid, as inoculum in _in-vitro_ systems, gave similar cellulose digestion coefficients and 48hr. _in-vitro_ fermentations yielded cellulose digestion coefficients similar to the value obtained _in-vivo_. Cellulose digestion has also been measured _in-vivo_ (Davison & Woods, 1960; LeFevre & Kamstra, 1960; and El-Shazly _et al._ 1961).

Forage Composition in Relation to Digestion in the Rumen

The amount of forage cellulose digested by rumen bacteria decreases with increasing maturity and lignification of the plant (Dehority & Johnson, 1961), caused by a physical barrier of lignin between the bacteria and the cellulose. They also found that there appeared to be a basic difference

between grasses and legumes in regard to the amount of cellulose which could be digested per given amount of lignin in the plant. Of the seven cellulolytic strains of bacteria used by Dehority & Scott (1967), only three were able to digest forage hemicelluloses. Two of the four strains of _Ruminococcus flavefaciens_ had a markedly reduced ability to digest cellulose from lucerne.

Parks _et al._ (1964) considered that the chemical components of ryegrass which appeared to be most clearly related to the intraruminal acid ratios were soluble sugars, lignin and protein. As the level of soluble sugar and protein in the ryegrass decreased and the level of lignin increased, the molar percent of propionate in the rumen contents decreased and that of acetate increased. The proportion of acetate in the rumen of cows given cut grass indoors (Bath & Rook, 1961) was inversely related to the water soluble carbohydrate content of the cut grass.

The Effect of Various Feeds accompanying Cellulose

> 'When cellulose is incubated with rumen liquor or a suspension of micro-organisms, relatively more propionate than acetate is formed. In contrast, feeds containing large amounts of cellulose, such as, dried grass or wheaten hay, yield predominantly acetate as an end product'. (Barnett & Reid, 1961).

Satter _et al._ (1966) realised that the observations by Barnett & Reid would lend support to the suggestion that the nature of end products formed from cellulose is partially dependent upon the nature of the accompanying feed components. When they incubated ^{14}C-cellulose and ^{14}C-hemicellulose for 4 hr. in either the presence of lucerne hay alone (H) or lucerne + grain(1:2.5)(G), they obtained the following results:-

		Cellulose		Hemicellulose	
		H	G	H	G
Molar %	Acetate	67.1	62.9	69.3	64.5
	Propionate	18.6	30.1	23.3	27.5
	Butyrate	13.1	5.7	6.9	7.4
	Valerate	1.1	0.6	0.5	0.8
	Acetate:Propionate	3.6	2.1	3.0	2.4

Acetate and propionate accounted for over 90% of the VFA's produced from ^{14}C-cellulose or hemicellulose when fermented in-vitro in the presence of dried grass + hay. Acetate:Propionate ratios from cellulose and hemicellulose were 1.7:1 and 2.4:1 respectively, considerably higher than those obtained when cellulose or hemicellulose were fermented as a sole substrate (Bath & Head, 1961). ^{14}C-cotton cellulose within a nylon bag was incubated in the rumen of a hay-fed cow for 6 hr. followed by 4 more hours in incubation vessels. The molar proportions of labelled VFA's formed were:-

	Distribution of total ^{14}C-VFA		
Substrate	Acetate(%)	Propionate(%)	Butyrate(%)
(1-^{14}C)cellulose	32.6	27.3	40.1
(2-^{14}C)cellulose	36.6	28.9	34.5
(6-^{14}C)cellulose	52.1	27.0	20.9

The results were different when the ^{14}C-cotton was incubated in the rumen of a high-concentrate-fed cow.

Studies by El-Shazly et al. (1961) indicated that the depression of cellulose digestion by starch was due primarily to competition between the cellulolytic and amylolytic groups of bacteria for nutrients, especially N_2.

When starch was added to an *in-vitro* rumen liquor medium from sheep, by Ichhponani & Sidhu (1966), there was a reduction of digestibility of cellulose linearly related to the amount of starch added (0.25- 1.5g./100ml.). An increase in urea progressively increased the digestion of cellulose. Cellulose digestion was similarly inhibited when sheep were fed an increasing proportion of corn in hay and corn rations. Partial or complete alleviation of the inhibition of cellulose digestion could be obtained by adding urea to the rations except when large amounts of corn were fed (El-Shazly *et al.* 1961).

5. Techniques of Sampling from the Rumen

Bath & Head (1961) considered that absorption of the VFA's takes place through the rumen wall but the relative rates of absorption of the different VFA's appears to be dependent upon the conditions within the rumen. They reasoned that a knowledge of the proportions of the VFA's in the rumen contents at any given time does not necessarily indicate the proportions in which they are formed. However, more recently, the infusion of labelled VFA's into the rumen of sheep by Bergman *et al.* (1965), and Weller *et al.* (1967) showed that the mixture of acids finally produced in the rumen had a composition very similar to that of the mixture present in the rumen fluid.

An attempt was made by Bryant (1964) to obtain a clearer understanding of the nature and magnitude of any stratification in the rumen of the pasture-fed animal, and to select the most suitable site for obtaining a representative rumen liquor sample. This was found to be either from the centre of the ingesta or a composite from the dorsal, centre and ventral regions. This was confirmed by Davey (1965). Lane *et al.* (1968) had a

system whereby the entire ruminal contents were removed and after being mixed, the contents were sampled by an automatic sampling device. Sutherland et al. (1962) used a pump that could circulate the rumen contents of sheep, with representative samples being easily withdrawn from the pump circuit. One drawback of this method was the interference with the NH_3 concentration of the rumen liquor.

VFA's of the Rumen Liquor: VFA concentration was higher in the dorsal region of the rumen in cattle than in the ventral region (Bryant, 1964; Davey, 1965; Lampila, 1965; and Lane et al. 1968), and the relative proportions of individual acids did not vary with sampling position (Bryant, 1964; and Lane et al. 1968). When using radioactive VFA's, Leng & Leonard (1965) had difficulty in sampling because of a variation in specific activity between various sites in the rumen, even after time had elapsed to allow complete mixing. The magnitude of the differences in VFA concentration between positions within the rumen was dependent on the sampling time during the day and on the day itself. A diurnal variation in ruminal VFA concentrations occurs in grazing sheep (Ulyatt & Leonard, 1965). Total VFA concentration was highest in the evening and lowest in the morning and although changes in molar proportions were not as marked, the acetate proportion decreased and those of propionate and butyrate increased with an increase in VFA concentration. Davey (1965) noticed that VFA concentration increased to a peak 3-6 hr. after the start of feeding, this peak being associated with the lowest acetate:propionate ratio.

Another problem in sampling was the differences in the activity of rumen liquor samples collected. Although collected on different days, they were collected at the same time of day and from the same animal on the same dietary regime (Lampila, 1965).

<u>pH of the Rumen Liquor</u>: Ingesta in the dorsal region of a rumen has a lower pH than the ingesta in the ventral region (Bryant, 1964; Davey, 1965; and Lane *et al.* 1968) Inconsistent pH levels at the ventral and reticulum positions noticed by Bryant (1964) were considered to be a result of rumination and consequent entry of increasing volumes of saliva into the rumen. The pH of the rumen contents was usually between 5.5 and 7.3 (Kay, 1963). Within the normal range, the pH depended principally on the balance achieved between production and absorption of VFA's and the buffering powers of the rumen contents. Fatty acid concentration and buffering power varied independently so that a clear correlation between pH and the concentration of VFA's was seldom seen. Short term fermentations with whole rumen contents from cows, when on different diets, showed that although VFA formation was most rapid at pH 6.5 and microbial protein formation greatest at pH 6.3, it varied with carbohydrate and ammonia levels (Lampila, 1965). pH remained between 6-7 with only small variations in different parts of the rumen when cows were fed hay or mainly hay diets. When more carbohydrate was fed the average pH fell and variations within the rumen increased.

6. In-vitro Experimentation

Criteria for In-vitro Experiments

The main advantage of *in-vitro* apparatus is the ability to use it to study activity of micro-organisms away from the control and influence imposed by the host animal. Several criteria of normal rumen functions which can be applied to *in-vitro* studies with the whole rumen microbial population include; the maintenance of numbers and normal appearance of the bacteria, selenomonads and protozoa of the rumen; the maintenance of normal rates of digestion of cellulose, starch and protein and of normal interactions between these; and

the ability to predict quantitative results in-vivo (Warner, 1956; and Bryant, 1964). Johnson (1966) considered it desirable to satisfy as many criteria of validity as possible, and any criteria sacrificed must be kept in mind when interpreting the results.

For the microbial population to remain normal in numbers and activity in-vitro, it was shown to be necessary to use as test substrates only substances similar to the diet fed to the animal from which the rumen liquor inoculum was taken (Warner, 1956). Motility of micro-organisms appeared to be a sensitive criterion of normal rumen function, but care was exercised to ensure maintenance of temperature and anaerobiosis of the specimen during examination.

The rate of digestion of the cellulosic contents of the rumen as ordinarily measured might be very different from the rate of digestion of cellulose newly introduced into an artificial system. Since it is practically essential to strain the rumen liquor for use in-vitro, some diminution of the rate of digestion of cellulose occurs, since many of the cellulolytic micro-organisms would remain attached to the large plant particles removed in straining (Lane et. al. 1968).

Comparison of In-vitro Systems

No major differences were found by Johnson (1966) between the closed and continuous flow systems of in-vitro apparatus when cellulose digestion, total VFA and NH_3 production were used as criteria of microbial activity. The rate of flow from the continuous flow apparatus had no marked effect on cellulose digestion and microscopic examination of the bacteria showed little difference between the systems after 30hr. Disadvantages of the continuous flow system was its complexity and the inability to perform large numbers of

experiments with it while the all-glass system was advantageous because of its simplicity and ease of duplication (El-Shazly *et. al.* 1960; and Johnson, 1966). The closed systems are subject to criticism as to whether the micro-organisms being propagated were truly typical of rumen populations in the intact animal. The organisms proliferating *in-vitro* could be transient contaminants from the soil and feed, or may be one normally occurring in the rumen in small numbers which proliferates preferentially in the *in-vitro* medium (Dehority, 1963). The invariable elimination of protozoa from the population is another criticism of closed systems.

Lowe *et al.* (1949) compared the all-glass and semi-permeable bag systems. The differences in cellulose digestion in favour of the semi-permeable bag were thought to be due only to an inhibitory influence of fission products accumulating in the glass bottles. Acetate and propionate were found in the same proportions irrespective of which apparatus the fermentation was carried out in. Less VFA/g. of cellulose digested was formed in the semi-permeable bag than in the glass bottle.

Pre-Incubation Gassing of In-vitro Bottles

Normally *in-vitro* fermentations are initially gassed with CO_2 and N_2 to procure an anaerobic environment. Boda & Johns (1962), however, carried out fermentations in the presence of O_2 without materially affecting results.

Period of In-vitro Fermentation

The time period of fermentation for *in-vitro* rumen studies is entirely the function of the study itself. Cellulose digestion is not only initiated after a longer period of incubation, but usually at a slower rate than digestion of more soluble carbohydrates while digestion of native forage celluloses is initiated at a much earlier time (Johnson, 1966). In experiments using a cellulose dialysis sac (El-Shazly *et al.* 1960), the sac could not be

used for periods longer than 30hr. since it weakened around the rubber stopper, due to bacterial attack. Marston (1948) fermented cellulose *in-vitro* for either 24, 26, 36 or 48hr. and found that maximum VFA production had occurred by 36hr. Cellulose digestion in twenty-four fermentations was below that obtained *in-vivo* after a 24hr. incubation, but as mentioned previously LeFevre & Kamstra (1960) noticed that the 48hr. fermentations yielded cellulose digestion coefficients similar to values obtained *in-vivo*. The object of work by Karn *et al.* (1967) was to determine if the rate of cellulose digestion between two fermentation time intervals was a better predication of forage nutritive value than the cellulose digestion value obtained after a specific fermentation time. Results indicated that rates of cellulose digestion were not more highly correlated with *in-vivo* data than a single cellulose digestion figure obtained at a given fermentation time.

Optimum pH for In-vitro Fermentations

Most of the rumen microbial activity occurs at an optimum rate between a pH range of 6.5 - 7.0. Rumen bacteria seem to be somewhat more sensitive to pH levels above than below 7.0. The necessity for adjusting pH in *in-vitro* experiments depends largely upon the rate, extent and type of fermentation, i.e., cellulose digestion acid production occurs at a much slower rate than during starch digestion (Johnson, 1966).

Optimum Temperature for In-vitro Fermentations

Temperature should be standardised, since differences of as little as 0.5°C have invalidated comparisons between individual fermentations. Care should be taken to prevent the temperature from rising over 40°C during all phases of fermentation (Johnson, 1966).

Termination of In-vitro Fermentations

In some cases it suffices merely to remove the *in-vitro* bottles from a waterbath and refrigerate them. For more exact timing of the termination of fermentations, acids, alcohols, and mecuric chloride have been added. Acids have the disadvantage of releasing CO_2, which may create sufficient foaming to coat the sides of vessels with portions of the substrate material (Johnson, 1966).

OBJECT OF THE PRESENT WORK

The present work was undertaken to investigate the effect of additional lipid on the fermentation of grass fibre by rumen micro-organisms. To enable a series of experiments to be conducted under similar conditions, in-vitro equipment was used.

Preliminary experiments were conducted to confirm that the effect of lipid on VFA's was to decrease acetate and increase propionate concentrations, as found by Robertson & Hawke (1964) when they incubated linseed oil with grass fibre in rumen liquor. Subsequent investigations extended the preliminary experiments with a wide range of lipid levels (0.2 - 3.0g./100ml. rumen liquor) and a number of different lipids (peanut, cod liver and linseed oils). These experiments involved the use of impermeable apparatus and enabled the effect of lipid on total and individual VFA's to be further documented.

In-vitro experiments, using micro-artificial rumens containing rumen liquor and grass fibre and diffused with continuous flows of clarified rumen liquor(dialysate), were conducted in an attempt to measure and compare the rates of total VFA, acetate, propionate and butyrate production in the presence and absence of added lipid. ^{14}C-cellulose was used to extend these investigations.

^{14}C-cellulose was also used in an attempt to establish the source of the increased propionate production which occurred when lipid was added to rumen liquor containing grass fibre.

Since almost all the work reported on the fermentation of glycerol has shown that the prodominant VFA formed is propionate, $(2-^{14}C)$glycerol was incubated in rumen liquor to substantiate this former work and determine the importance of the contribution of the glycerol moiety of triglycerides to the propionate formed, under the conditions of the present experiment.

The effect of lipid on the concentrations of ammonia in rumen liquor was also investigated, ammonia being another end product of rumen fermentations affected by the addition of lipid.

MATERIALS AND METHODS

Treatment of Animals

Non-lactating, monozygous twin cows (AA x Jersey; and later Jersey) each with a rumen fistula were the main source of rumen liquor. They were fed twice daily (7.00 a.m. and 3.00 p.m.) with 10lb. of good meadow hay either on a concrete pad or in a holding yard with no access to fresh pasture. Water was available *ad libitum*. Feeding of this diet started at least two weeks before initial sampling.

Rumen liquor samples were extracted, after withholding food overnight, from the mid-region of the rumen as suggested by Bryant, 1964; and Davey 1965, and strained through several layers of muslin into a thermos flask previously warmed to 40°C. Since rumen liquor from cattle and sheep is interchangeable if similar rations are fed (LeFevre & Kamstra, 1960), rumen liquor was also obtained from hay-fed fistulated Romney wethers using the same feeding regime and sampling technique as for the cows.

Clarified Rumen Liquor (Dialysate)

Large quantities (10-20 litres) of rumen liquor were collected from both cows and strained through muslin. Fibrous material was returned to the rumen. The strained rumen liquor was centrifuged in an Alfa-Laval Cream Separator (Model 105) operating at approximately 8200 r.p.m. The supernatant was recentrifuged twice and this removed the heavier particular material from the rumen liquor. The rumen liquor obtained from the above treatment was siphoned into a Sharple's Super Centrifuge and centrifuged at 57,000-75,000*g*. using a Type 8R 34 standard bowl. This was repeated on the supernatant using a Type 24B (Virus) bowl.

The fluids collected, from both the main outlet tap and the overflow tap, were equally clarified and were clearer than samples obtained by using the method of Dawson, Ward & Scott, 1964. The clarified rumen liquor was stored in a refrigerator ($9^{o}C$) until used.

Grass Fibre

3" - 6" Ariki ryegrass (17% dry matter) was collected in the autumn and passed through a Bentall 'Protess' extraction plant. The resultant grass fibre was washed with hot tap water and left to soak for 2 hr. This washing procedure was repeated before squeezing the fibre dry between layers of muslin and drying in a freeze-drier for 24hr. 380g. of freeze-dried fibre was obtained from 1150g. of fresh grass. The freeze-dried fibre, enclosed in a plastic bag, was frozen ($-9^{o}C$) until required. The same fibre (24.9% dry matter) was used for all the experiments. Dry matter was determined by drying a grass fibre sample at $100^{o}C$ for 24hr. in a forced-draught oven.

Lipid Substrates

Raw linseed oil, cod liver oil and peanut oil were used as substrates in the in-vitro incubations. The oils were added as oil/water emulsions (50:50 w/v) with 1% Lissapol N.X. (Imperial Chemical Industries Ltd.) as emulsifying agent. The emulsions were prepared by shaking and were stable.

Artifical Saliva

Artificial saliva (30ml./100ml. rumen liquor) was used in all the in-vitro experiments using the impermeable apparatus. The artificial saliva (McDougall, 1948) was a bicarbonate-phosphate buffer of pH 7.0. The buffer was made by dissolving the $NaCl$, KCl, $CaCl_2$ (anhydrous), $MgCl_2$ (anhydrous), and $NaHCO_3$ in an appropriate volume of distilled water and by adding a solution of $NaH_2PO_4.2H_2O$ until pH 7.0 was obtained using a

Radiometer 25 pH meter. The phosphate buffer was stored in a refrigerator (9°C).

Radioactive Materials

Radioactive compounds used were ^{14}C-tobacco leaf residues (^{14}C-cellulose ...not uniformly labelled) (Specific activity = 28.0 μc./mg.) and (2-^{14}C)glycerol (Specific activity = 8.0mc./m-mole), both obtained from The Radiochemical Centre, Amersham, England.

Preparation: The ^{14}C-cellulose was in a powder form and when required was weighed in small (1cm.) glass or polypropylene vials. The (2-^{14}C)glycerol was dissolved into 10ml. of distilled water (50 μc./10ml.). Of this stock solution 1ml. containing 5 μc. was diluted by another 10ml. of distilled water (0.$\overline{45}$ μc./ml.). Both compounds were stored in a refrigerator (-2°C) until required.

Impermeable Apparatus

When gas production was measured (preliminary experiment) one pint glass bottles fitted with rubber stoppers were used as incubation vessels. Pressure hosing connected the six manometers to one of the leads through the stoppers, while the second lead was fitted with a valve allowing the addition of oil emulsion, and acidification of the contents. All the bottles were held in a shaker rack immersed in a waterbath which was maintained at a temperature of 39°C ± 1°C.

When gas production was not measured 130ml. glass bottles, with rubber stoppers and bunsen valves attached, were used. In experiments containing many treatments a five litre glass flask was used to enable quick preparation of the incubation vessels. N_2 was blown into the stoppered flask and through the rumen liquor/buffer mixture (10.3) it contained, to enable thorough mixing, to create anaerobic conditions, and

by pressurising the flask, filling incubation vessels rapidly with the required volume of solution.

A modification of this experimental procedure was made in experiments containing ^{14}C-cellulose to permit collection of $^{14}CO_2$ in 'Hyamine' hydroxide (British Drug House, Poole, England). Incubation vessels were gassed with N_2 prior to the experiment to ensure anaerobic conditions and at the completion of the experiment to expel all the $^{14}CO_2$ into the 'Hyamine' hydroxide.

Semi-permeable Apparatus

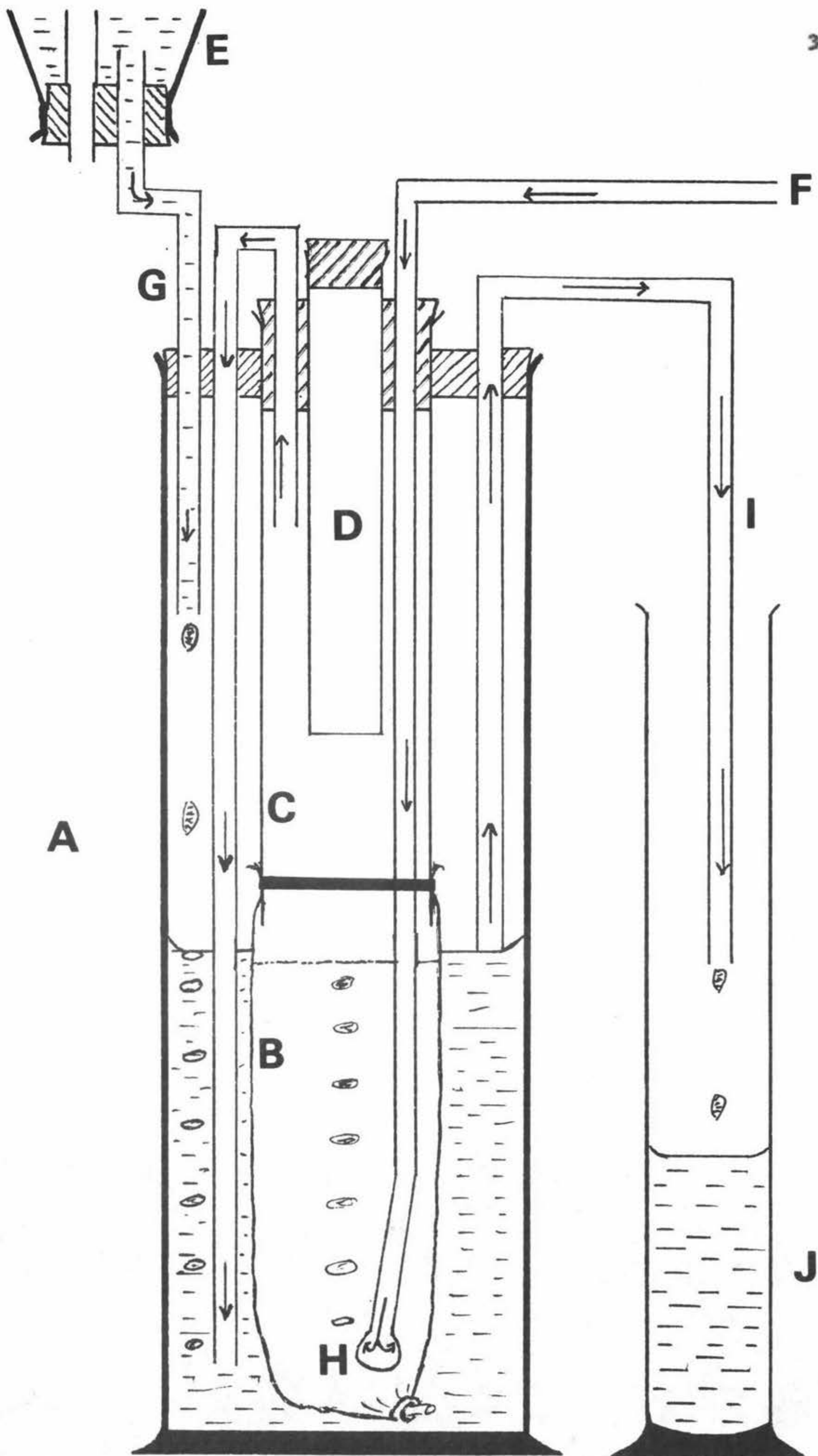
The three micro-artificial rumens used were based on the apparatus described by Dawson, Ward & Scott, 1964 (Fig. 1.). The apparatus was immersed in a thermostatically controlled waterbath (39°C) throughout the entire incubation period.

The clarified rumen liquor was gravity-fed from a reservoir (an inverted 2 litre conical flask), the rate of infusion being adjusted to about 40ml./hr. The flow of N_2 through the apparatus is shown in Fig. 1.

Problems with the semi-permeable apparatus were experienced. Frothing of rumen liquor invariably occurred once gassing commenced causing rumen liquor to spill over into the dialysate and tended to lift the grass fibre and lipid to the top of the rumen liquor. Low gas flow rates reduced frothing and further improvement was obtained by attaching a gas distribution bulb, containing four small holes, to the end of the glass tubing in the dialysis sac which diminished the size of the gas bubbles and the amount of frothing. Nevertheless, the problem was never completely solved in the artificial rumens, with frothing liable to occur up to 12hr. from the start of incubation.

FIG. 1. THE MICRO-ARTIFICIAL RUMEN. (Dawson, Ward & Scott, 1964). A. Large glass jar. B. Tied-off dialysis sac connected to a glass tube (C) by a rubber band. D. Glass tube through which substrate was introduced into the dialysis sac. E. Reservoir containing clarified rumen liquor (Dialysate). F. Entrance tube for N_2 gas. G. Entrance tube for dialysate. H. Gas distribution bulb. I. Exit tube for N_2 gas and dialysate. J. 100ml. measuring cylinder containing collected dialysate.

Arrows denote the flow of N_2 gas and/or dialysate through the apparatus.


E
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Due to microbial attack (Warner, 1956; El-Shazly *et al.*, 1960), the cellulosic membrane of the dialysis sacs partially disintegrated when incubated for 48hr. and even after 24hr. extreme care had to be taken when handling them.

In the initial experiments 50ml. of rumen liquor was added to the dialysis sac (36mm. diameter), but the rumen liquor volume was increased to 100ml. (dialysis sac 65mm. diameter) in latter experiments to enable larger amounts of VFA's to be produced and therefore more readily detected.

Analytical Methods

Gas Production

Gas production was measured by displacement of water from large manometers (260ml.) which were attached to the *in-vitro* bottles by rubber pressure hosing. If gas production exceeded the capacity of the manometers, gas could be released via a valve after readings had been recorded. Acidification of rumen liquor enabled more exact timing of the period of fermentation (Johnson, 1966).

Ammonia Determination

Ammonia in rumen samples was determined by the method of Conway & O'Malley (1942). 1ml. of boric acid (0.5% in 20% alcohol, containing a mixed indicator of bromocresol green 0.33% and methyl red 0.066% in alcohol) was pipetted into the centre chamber of a Conway dish. In the outer chamber 1ml. of saturated potassium carbonate was pipetted to one side while 1ml. of rumen liquor was accurately pipetted on the other side of the dish. A glass cover-slip was placed upon each Conway dish and sealed with gum acacia fixative. The dish was slowly swirled to completely mix the rumen liquor and potassium carbonate. Triplicate rumen samples were analysed for NH_3

concentrations by titrating the boric acid mixture with 0.05N HCl following an incubation of 2 hr. at room temperature.

Isolation and Determination of the VFA's in Rumen Fluid

Total volatile fatty acids (VFA's) were isolated from rumen fluids by steam distillation in a manner similar to Johns(1955), but with some modifications. Total VFA's were determined in 5ml. of rumen contents by having 1 ml. of $10N.H_2SO_4$, saturated with $MgSO_4$, added to it followed by steam distillation in the Markham still for not less than 12min. Quantitative recovery of the acids was obtained by collecting 70ml. distillates in 20ml. of CO_2-free distilled water. Collection of a further 70ml. distillate to serve as a blank was initially only collected at regular intervals during a series of distillations, but later on they were collected for each rumen sample. The distillate was titrated with standardised NaOH solution, in the vicinity of 0.05N., with phenolphthalein as indicator. After quantitative analysis, an excess of 0.05N.NaOH was added to each sample before evaporating to dryness in either a forced-draught oven (80-100°C), on a boiling waterbath, or in a rotary evaporator.

NaOH Standardisation: The NaOH used for titration was standardised each day using approximately 0.05g. of potassium hydrogen phthalate dissolved in 90ml. of CO_2-free distilled water with phenolphthalein as indicator.

Distillation and Titration Techniques: Difficulties with reproducing titration results led to a small investigation into the best method of distillation and titration (see Table 2). The figures in Table 2 are the mean values of three determinations.

TABLE. 2. A comparison between the importance of placing ice around a conical flask during the collection of the distillate; collecting the distillate in 20ml. of CO_2-free distilled water; and bubbling CO_2-free air through the distillate prior to titration.

Method	Titration (ml. NaOH)
Ice, H_2O	10.94
Ice, H_2O and Bubbling	10.15
H_2O, Bubbling	10.11
Bubbling	9.86

Besides the normal procedure of bubbling CO_2-free air through the distillate prior to titration, it was found advisable to collect the distillate in at least 20ml. of CO_2-free distilled water. The presence of ice appeared to be of no importance. This technique of using CO_2-free distilled water eliminated variation within-stills but large between-still variations persisted and were especially large when distilling clarified rumen liquor. In latter experiments only one Markham still was used.

<u>Individual Volatile Fatty Acids</u>

<u>Preparation and Storage</u>: Two separate distillates from 5 ml. samples of rumen liquor were combined and evaporated to dryness. A small volume of distilled water was used to transfer sodium salts of the VFA's into a small vial and the sample was evaporated to dryness. The stoppered vials were stored in a refrigerator until required.

In preparation for separation into individual VFA's, 0.2ml. of distilled water was added to each vial and thoroughly mixed with an Adams

Cyclo-Mixer (Clay-Adams Inc. N.Y.).

Gas-Liquid Chromatography using the Titrimetric Method (James and Martin)

The proportions of acetic, propionic and butyric acids in the sodium salts were determined, after acidification, by gas-liquid chromatography (James & Martin, 1952). n- & iso-valeric acid was not determined.

The column was packed with 15% polythylene glycol adipate (PEGA) (Applied Science Laboratories Inc., State College, Pa.) liquid phase on Celite 545 (Koch-Light Laboratories Ltd., Bucks, England) as solid support and held in a vapour jacket at 137°C in boiling methyl cellosolve (Shell Methyl Oxitol).

Column Preparation: Initially the packed columns did not resolve the VFA's satisfactorily, but suitable columns were obtained by combining the techniques of James & Martin (1952), Youssef & Allan (1966), and Storry & Millard (1965). Celite 545 was initially size-graded (James & Martin) and then heated at 400°C for 6 hr., allowed to cool, then washed with conc. HCl for 15min. to remove Fe and other basic impurities. After repeated washing with boiling and then cold distilled water to remove HCl, the Celite was finally rinsed in 0.75% v/v orthophosphoric acid and dried overnight at 105°C.

The Celite was thoroughly mixed with PEGA (dissolved in $CHCl_3$) (85:15 w/w), and the $CHCl_3$ was then evaporated slowly accompanied by continuous stirring until visibly dry and the mixture completely dried at 100°C in an oven overnight. The column material was packed into the 4' x ⅛" glass column using a Vibro-tool (Burgess Products Co. Ltd., England) to assist consolidation. N_2 was passed through the column a little above the normal running pressure, and the column vibrated again or tapped lightly on the floor. Finally a 1" long plug of fibre glass yarn was placed in the

column. This plug was changed approximately every 50 analyses. The packed column was conditioned at 137°C for 60hr. and operated at 137°C with the nitrogen pressure between 9-12lb./in.^{2}

Column Operation: The VFA salts were transferred to the chromatography column by taking them up into a hypodermic syringe (5-10ml.). Either one or two drops (0.01-0.03ml.) of the aqueous solution were added to a Teflon boat containing the acidification material, a mixture of Celite and $KHSO_4$ (50:50 w/w). The Teflon boat was rapidly pushed into the column and the N_2 released. The titration cell was filled with phenol red indicator (0.005% w/v in distilled water). Titration was with 0.02-0.05N. NaOH, the former generally being more applicable for VFA's from rumen liquor. Complete separations of acetate, propionate and butyrate usually took 40-50min. Normally, duplicates of each sample were analysed but if duplicates were omitted a standard VFA sample was used after every sixth separation to check the efficiency of the column.

Varian Aerographs

In addition to using a gas-liquid chromatograph fitted with an automatic titrator, the proportions of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric were determined using two models of Varian Aerograph (1520 and 1200). The former was equipped with both a flame ionisation detector (F.I.D.) and a thermal conductivity detector (T.C.D.). The latter had only a F.I.D. but, unlike the former, had no stream splitter. N_2 or H_2 were used as carrier gases.

Column Preparation

The funnel coating method (McNair & Bonelli, 1966) to prepare the various columns was used. 5g. of support was sufficient for any one column and was added to 25ml. of the liquid phase solution in a Buchner flask.

The pressure in the flask was reduced for a few minutes with a water aspirator. The pressure was then released and the flask allowed to stand for 15min. The material was then put into a sintered glass funnel and allowed to drain freely until it settled. A vacuum was applied for 5min. before the coated support was spread on filter paper to dry. After air drying, the coated support was dried in a forced-draught oven (80-100°C) overnight. When coating with phosphoric acid was required the coated support was mixed with the appropriate phosphoric acid/aqueous solution and the water slowly removed with a rotary evaporator. The support was then dried in a forced-draught oven overnight.

The columns were packed by plugging one end with glass fibre, inserting the same end on to a water aspirator and with the aid of a funnel attached to the other end, the coated support was sucked into the 8' x ⅛" coiled stainless steel columns or alternatively 7' x ⅛" coiled glass columns, using an electric vibrator (Vibro-tool) to ensure firm, even packing. Finally glass wool was used to plug the other end also. Columns were also packed under positive gas pressures of about 30-40lb./in.^{2} Most columns were conditioned for 12-24hr. at 100°C higher than at which the columns were used. A low carrier gas flow was streamed through the columns during conditioning.

Materials used: PEGA (Applied Science Laboratories Inc., State College, Pa.); and Anakrom 50/60-AS (Analabs Incorporated).

Columns Prepared: 7% PEGA-Anakrom 50/60; and 7% PEGA + 1% H_3PO_4-Anakrom 50/60.

Separation of ^{14}C-VFA's using the Titrimetric Method (James & Martin)

Separation of ^{14}C-VFA's with the titrimetric apparatus required a few modifications from that of non-radioactive separations. The contents of

the titration cell were removed after each fatty acid had emerged, to give samples of Na salts of the acid contaminated with phenol red indicator. Gas flow was decreased to give greater retention times and thus allow more time for removal of acids, replenishment of indicator solution, and permit adequate washing. The apparatus used to remove the acids from the titration cell consisted of a Buchner flask fitted with a bung through which passed two leads, one fitted with a Pasteur pipette, the other with a Griffin pipette filler. The titration cell contents were mixed with excess NaOH when taken up into a Buchner flask, transferred into beakers and evaporated to dryness. Separate apparatus was used for each of the three VFA's collected (acetate, propionate and butyrate), the apparatus being well cleaned between samples.

After evaporation of the salts of each acid to dryness, 6ml. of distilled water was added and a 5ml. sample taken for steam distillation in order to remove the phenol red colouration. The distillates were collected in five times the NaOH required to neutralise the VFA's present. The colourless distillates were evaporated to dryness, and the salts taken up in an appropriate volume of distilled water for determination of radioactivity.

Separation of ^{14}C-VFA's using a Varian Aerograph 1520

A 10 μl. micro-syringe (Hamilton Co. Ltd., Whittier, California) was used to inject aliquots of the VFA samples on to the column.

The stream splitter on the 1520 Varian Aerograph allowed collection of individual free volatile fatty acids in collection tubes packed with siliconised glass fibre which was moistened with toluene scintillation fluid. After each acid had passed through the F.I.D. or T.C.D. the collection tube was replaced by another collection tube in readiness for the next acid.

The collection tube was then eluted with 10ml. of the toluene scintillation fluid directly into counting vials.

Measurement of Radioactivity

All radioactive samples were counted using a Packhard Tri-Carb liquid scintillation counter. Counting efficiency was determined using ^{14}C-hexadecane and ^{14}C-hexadecanewas used as an internal standard when quenching was suspected.

10ml. of Bray's scintillation solution (60g. naphthalene, 4g. 2,5 diphenyloxazole (PPO), 0.2g. 1,4-bis-2-(5-phenyloxazoyl)benzene (POPOP), 100ml. methanol, 20ml. ethylene glycol made up to one litre with dioxane) was added to counting vials containing sodium salts of VFA's. 10ml. of toluene scintillation solution (0.6% PPO and 0.05% POPOP) was used when determining the radioactivity of free VFA's and CO_2-absorbing 'Hyamine' hydroxide.

pH Readings

a pH meter (Radiometer, Copenhagen) was used to determine the pH of rumen liquor samples.

Statistical Methods

A number of separate experiments were carried out in this study. The experiments designed for statistical analysis were of a factorial nature, carried out in duplicate and initially analysed by an analysis of variance (Snedecor & Cochran, 1967). The significance of differences between duplicate experiments, lipid treatments, times of incubation, grass fibre levels and the various interactions, for rumen liquor, NH_3 and total and individual VFA concentrations were determined. The difference between pairs of means required (D value required) for 5% significance was calculated

from tables provided by Joyce M. May (1952) of the Studentized Range ($Q = \bar{X}max. - \bar{X}min.)/S\bar{x}$) by the formula $D = Q_{0.05} \cdot S\bar{x}$(Snedecor & Cochran, 1967). The Studentized Range test is a more conservative test for significance than the least significant difference (LSD) test, requiring a larger difference for significance. The LSD test was used however to detect significance at the 10% level.

Since percentages are not normally distributed, the analysis of the proportional distribution of ^{14}C among the individual VFA's in one of the experiments using ^{14}C-cellulose, was done by transforming the proportions into angles (angle = arcsin. $\sqrt{\text{percentage}}$). (Bliss, 1937, as reproduced in Snedecor & Cochran, 1967).

EXPERIMENTAL AND RESULTS

1. Preliminary experiments

Two experiments (denoted (i) and(ii) were conducted to re-investigate the experiment by Robertson & Hawke (1964); they studied the fermentation products formed when grass fibre was incubated with rumen liquor in the presence or absence of added lipid. The design of the two experiments is shown in Table 3. Manometers were connected

TABLE 3. Design of the preliminary experiments to investigate the effect of lipid on the VFA and ammonia production of rumen liquor containing grass fibre within impermeable apparatus.

Sample	Rumen Liquor(ml.)	Phosphate Buffer(ml.)	Grass Fibre (g.)	Linseed oil Emulsion (ml.)	Distilled H_2O (ml.)	Incubation Time(hr.)
Blank	100	30	-	-	2	0
	"	"	-	-	2	48
Control	"	"	1	-	2	12
	"	"	1	-	2	24
	"	"	1	-	2	48
Experimental	"	"	1	4	-	12
	"	"	1	4	-	24
	"	"	1	4	-	48

to all bottles except in the case of the two 24hr. samples. The fermentations were stopped by the addition of 4ml. of 10N. H_2SO_4 and analyses of gas, ammonia, and total and individual volatile fatty acid (VFA) formation were carried out on each sample.

1.1 Gas production

Gas production from rumen liquor containing grass fibre was twice that of endogenous fermentation (Table 4). Rates of gas production appeared to be faster in the presence of lipid at 12hr., although at 48hr. the total gas production was only slightly higher for the samples containing lipid.

TABLE 4. Gas, ammonia, and total VFA production from rumen liquor(100ml.) incubated with linseed oil and grass fibre.

	Incubation Time(hr.)	Experiment (i)			Experiment (ii)		
		B*	C**	E***	B	C	E
Gas Production(ml.)	0	32	32	32	112	112	112
	12	-	175	191	-	188	219
	24	-	-	-	-	-	-
	48	160	275	287	126	254	256
Ammonia Production(mg.NH_3/100ml.)	0	7.82	7.82	7.82	9.10	9.10	9.10
	12	-	18.96	19.64	-	17.85	21.59
	24	-	27.46	26.16	-	25.76	24.75
	48	29.24	41.65	38.85	23.12	33.32	28.39
Total Volatile Fatty Acid Production(m-mole/l.)	0	84.6	84.6	84.6	98.4	98.4	98.4
	12	-	118.2	123.5	-	139.5	132.8
	24	-	141.3	143.1	-	151.6	161.8
	48	112.1	160.8	170.3	110.6	190.0	190.4

B* Blank; rumen liquor only.

C** Control; rumen liquor + grass fibre(1.0g.).

E*** Experimental; rumen liquor + grass fibre(1.0g.) + linseed oil(2.0g.).

Gas production figures underlined denote some losses.

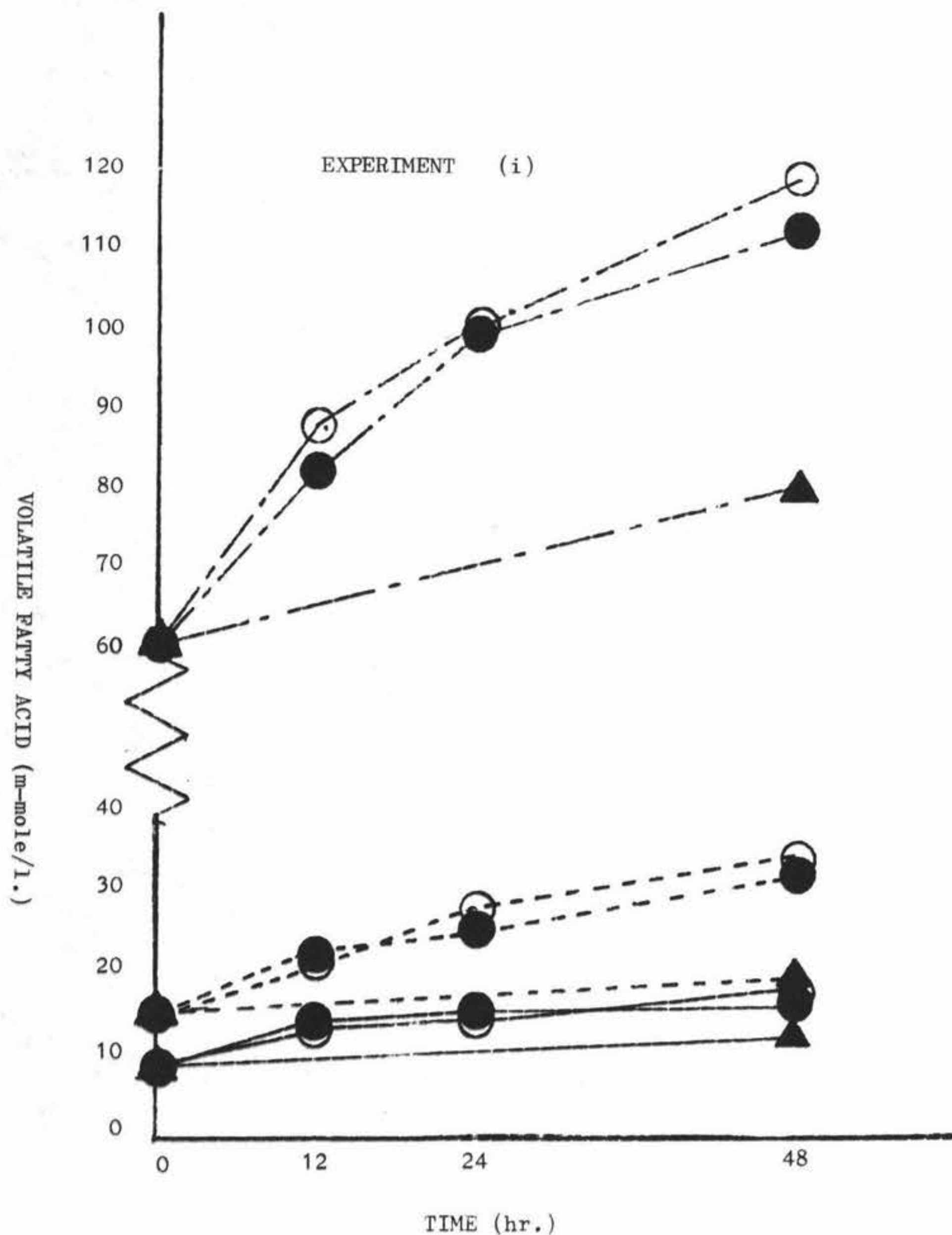
1.2 Ammonia production

In both experiments ammonia production was highest in the presence of lipid after 12hr. but lower after 24 and 48hr. (Table 4). Endogenous ammonia production after 48hr. was two-thirds of that produced in samples containing lipid and grass fibre.

1.3 Total and individual VFA production

In experiment (i) total VFA production was consistently higher in the presence of lipid whereas, in experiment (ii), the control sample had produced more VFA by 12hr. (Table 4). This position had reversed by 24hr., although by 48hr. VFA production was approximately the same for both control and added lipid samples in experiment (ii). In experiment (i), 40% of the VFA production could be attributed to endogenous fermentation in the control and added lipid samples after 48hr. This contribution was only 12% in experiment (ii).

In experiment (i), at 48hr. acetate production was higher in the presence of lipid than when fibre was incubated alone. However, this was reversed in experiment (ii) (Table 5). In both experiments propionate and butyrate production was highest after 48hr. in the presence of lipid, with the increase in propionate and butyrate in experiment (ii) being comparable to the decrease in acetate production. Experimental error most likely accounts for the low propionate production at 12hr. in experiment (ii). Variations in the concentrations of the individual VFA's (acetate, propionate and butyrate) under the different treatments and times of incubation are shown graphically in Fig. 2.



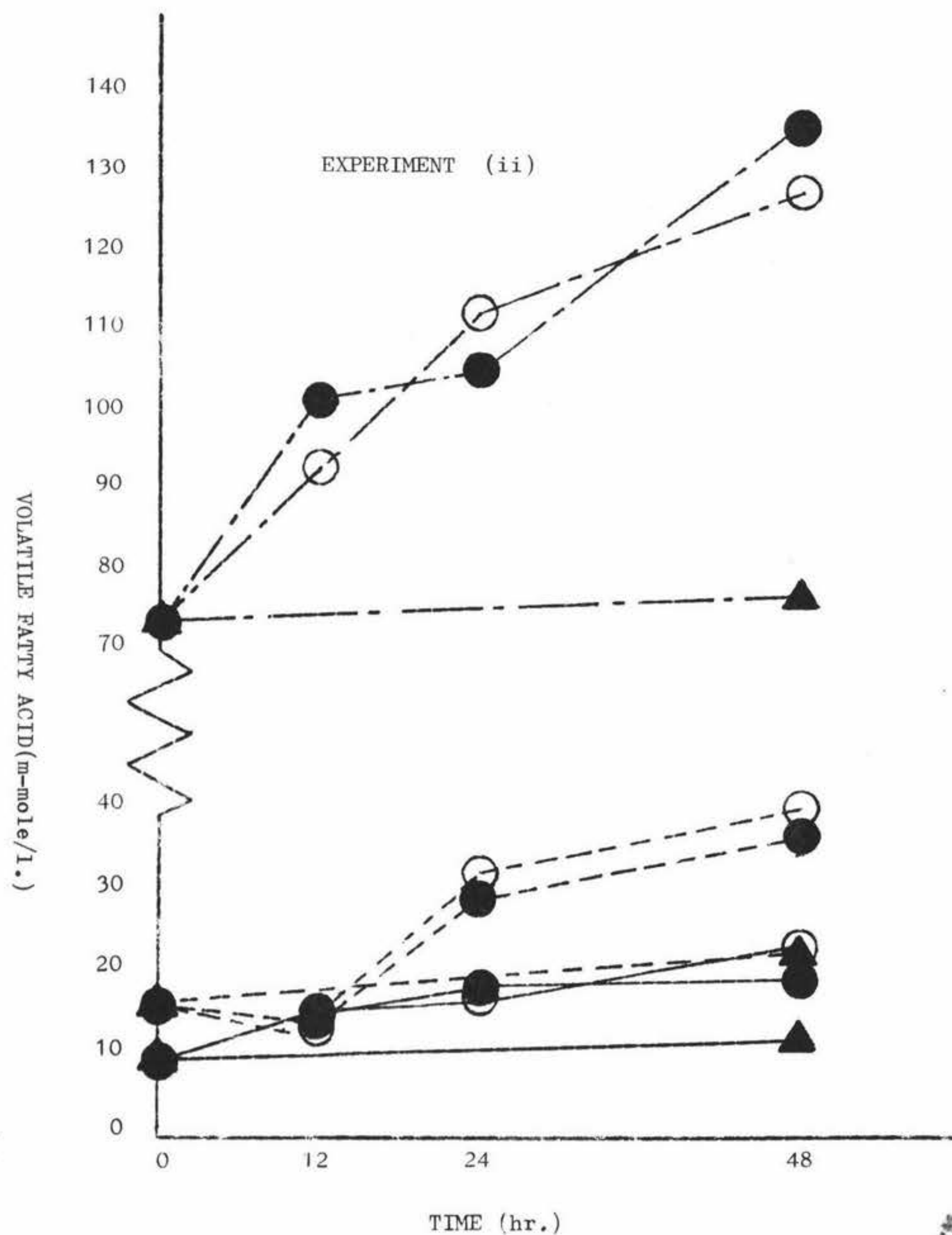


FIG. 2. The effect of linseed oil(2.0g.) on individual VFA concentrations (m-mole/l.) when 0.5g. of grass fibre was incubated in rumen liquor (50ml.) for 48hr. (a) 0.5g. of grass fibre + 2.0g. of linseed oil ○ ; (b) 0.5g. of grass fibre ● ; (c) no substrate added. ▲ .
—·—·—Acetate – – – – –Propionate ———— Butyrate

TABLE 5. Concentrations of acetic, propionic and butyric acids(m-mole/l.) formed on incubation of rumen liquor(100ml.) with linseed oil and grass fibre.

	Incubation Time(hr.)	Experiment (i) B*	Experiment (i) C**	Experiment (i) E***	Experiment (ii) B	Experiment (ii) C	Experiment (ii) E
Acetate Production	0	61.39	61.39	61.39	73.40	73.40	73.40
	12	-	83.14	88.57	-	101.84	93.28
	24	-	101.10	101.17	-	105.30	112.18
	48	80.38	112.24	118.95	76.71	135.45	127.28
Propionate Production	0	14.76	14.76	14.76	15.98	15.98	15.98
	12	-	21.76	21.65	-	13.95	13.28
	24	-	25.90	28.00	-	29.05	32.59
	48	19.61	31.74	33.84	22.09	35.28	40.48
Butyrate Production	0	8.45	8.45	8.45	9.02	9.02	9.02
	12	-	13.30	13.28	-	15.29	15.29
	24	-	14.30	13.92	-	17.07	17.04
	48	12.12	16.82	17.51	11.81	19.27	22.64

B* Blank; rumen liquor only.

C** Control; rumen liquor + grass fibre(1.0g.).

E*** Experimental; rumen liquor + grass fibre(1.0g.) + linseed oil(2.0g.).

2. Effect of different amounts of lipid substrate on total and individual VFA production and ammonia production from grass fibre incubated in rumen liquor for 24, 36, 48 or 72hr.

The design of the experiment is shown in Table 6. After incubation

TABLE 6. Design of the experiment to determine the effect of different levels of lipid substrate on VFA and ammonia production from grass fibre incubated in rumen liquor for 24, 36, 48 or 72hr.

Sample		Rumen Liquor (ml.)	Phosphate Buffer (ml.)	Grass Fibre (g.)	Linseed Oil Emulsion (ml.)	Incubation Time(hr.)	Distilled H_2O(ml.)
Blank	1;22.	50	15	-	-	0	1.5
	2;23	"	"	-	-	24	"
	3;24	"	"	-	-	36	"
	4;25	"	"	-	-	48	"
	5;26	"	"	-	-	72	"
Control	6;27	"	"	0.5	-	24	1.0
	7;28	"	"	"	-	36	"
	8;29	"	"	"	-	48	"
	9;30	"	"	"	-	72	"
Experimental (1g.lipid/100ml.)	10;31	"	"	"	1.0	24	0.5
	11;32	"	"	"	"	36	"
	12;33	"	"	"	"	48	"
	13;34	"	"	"	"	72	"
Experimental (2g.lipid/100ml.)	14;35	"	"	"	2.0	24	-
	15;36	"	"	"	"	36	-
	16;37	"	"	"	"	48	-
	17;38	"	"	"	"	72	-
Experimental (3g.lipid/100ml.)	18.39	"	"	"	3.0	24	1.5
	19;40	"	"	"	"	36	"
	20;41	"	"	"	"	48	"
	21;42	"	"	"	"	72	"

each sample was centrifuged for 10min. at 1000-2000r.p.m. to precipitate the undigested fibre. The total VFA, individual VFA, and ammonia concentrations were measured in the supernatant.

2.1 Total VFA production

The overall effect of lipid was to decrease the production of total VFA ($P<0.05$). When the means for each lipid level were compared only the difference between no lipid and 1.0g. of lipid was significant (Table 7, Appendix 1a).

TABLE 7. Effect of lipid and time of incubation on the formation of total VFA(m-mole/l.) in rumen liquor(50ml.) incubated with grass fibre(0.5g). (Mean values).

Lipid Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Lipid Means	Signif. of diffs. ($P<0.05$)
0	90.05	100.15	104.90	109.65	101.19	
1	86.30	96.90	101.90	105.55	97.66	1<0
2	88.00	96.85	101.30	111.40	99.39	
3	87.80	95.95	105.35	108.60	99.43	
Incubation Time Means	88.04	97.46	103.36	108.80	$\overline{X}$ = 99.42	
Significance of diffs. ($P<0.05$)	24<36, 48 & 72.	36<48, & 72.	48<72.			

S.E. per fermentation bottles mean = ± 2.19 m-mole/l.

Total VFA production increased with increasing time of incubation, irrespective of lipid treatment, with total VFA production at all incubation times differing significantly ($P<0.01$). There were no significant interactions between lipid levels and incubation times.

The addition of 0.5g. of grass fibre to incubations increased total VFA production ($P<0.01$) (Appendix 1b & 6a).

2.2 Acetate production

Lipid depressed the production of acetate, the differences in acetate production between all lipid levels being significant ($P<0.01$) (Table 8, Appendix 2a).

Acetate production also increased with increasing time of incubation ($P<0.01$) although when the means for each incubation were compared, the differences in acetate production between the 36 and 48hr. incubations, and between the 48 and 72hr. incubations were not statistically significant. There were no significant interactions between incubation times and lipid levels.

TABLE 8. Effect of lipid and time of incubation on the formation of acetate(m-mole/l.) in rumen liquor(50ml.) incubated with grass fibre(0.5g.). (Mean values).

Lipid Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Lipid Means	Signif. of diffs. ($P<0.05$)
0	62.87	69.63	72.02	74.55	69.77	0>1,2,3.
1	58.49	63.93	65.63	67.33	63.84	1>2,3.
2	57.01	58.42	59.51	64.27	59.80	2>3.
3	53.12	54.74	59.16	60.12	56.77	
Incubation Time Means	57.83	61.68	64.15	66.55	$\bar{X}$ = 62.54	
Significance of diffs. ($P<0.05$)	24<36, 36<72. 48 & 72.					

S.E. per fermentation bottles mean = ± 1.84 m-mole/l.

The addition of 0.5g. of grass fibre to the rumen liquor incubations increased acetate production ($P<0.05$) (Appendix 2b & 6b).

2.3 Propionate production

Lipid increased the production of propionate, the production for all lipid levels differing significantly ($P<0.01$) (Table 9, Appendix 3a). Propionate production increased with increasing time of incubation irrespective of lipid treatment, the production for all incubation times also differing significantly ($P<0.01$).

TABLE 9. Effect of lipid and time of incubation on the formation of propionate(m-mole/l.) in rumen liquor(50ml.) incubated with grass fibre(0.5g.). (Mean values).

Lipid Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Lipid Means	Signif. of diffs. ($P<0.05$)
0	18.36	20.71	23.05	15.29	21.83	0<1,2,3.
1	18.74	22.99	25.82	27.06	23.65	1<2,3.
2	21.28	27.42	31.22	35.23	28.79	2<3.
3	24.04	30.98	35.40	36.42	31.71	
Incubation Time Means	20.60	25.52	28.87	30.97	$\bar{X}$ = 26.49	
Significance of diffs. ($P<0.05$)	24<36, 48 & 72.	36<48, & 72.	48<72.			

S.E. per fermentation bottles mean = $\pm$ 1.12 m-mole/l.

The significant ($P<0.01$) interaction between lipid levels and incubation times can be attributed to the large increases in propionate production when 2.0 or 3.0g. of lipid were incubated with grass fibre in rumen liquor for more than 24hr.

The addition of 0.5g. of grass fibre brought about a significant increase in propionate production ($P<0.01$) (Appendix 3b & 6c).

2.4 Butyrate production

The overall effect of lipid was to increase the production of butyrate ($P<0.01$). When the means for each lipid level were compared only the differences between no lipid and 2.0 or 3.0g. of lipid were significant (Table 10, Appendix 4a).

Butyrate production increased with increasing time of incubation ($P<0.01$) although the only significant difference was between the 24 and 72hr. incubations. The interactions between lipid levels and incubation times were not significant.

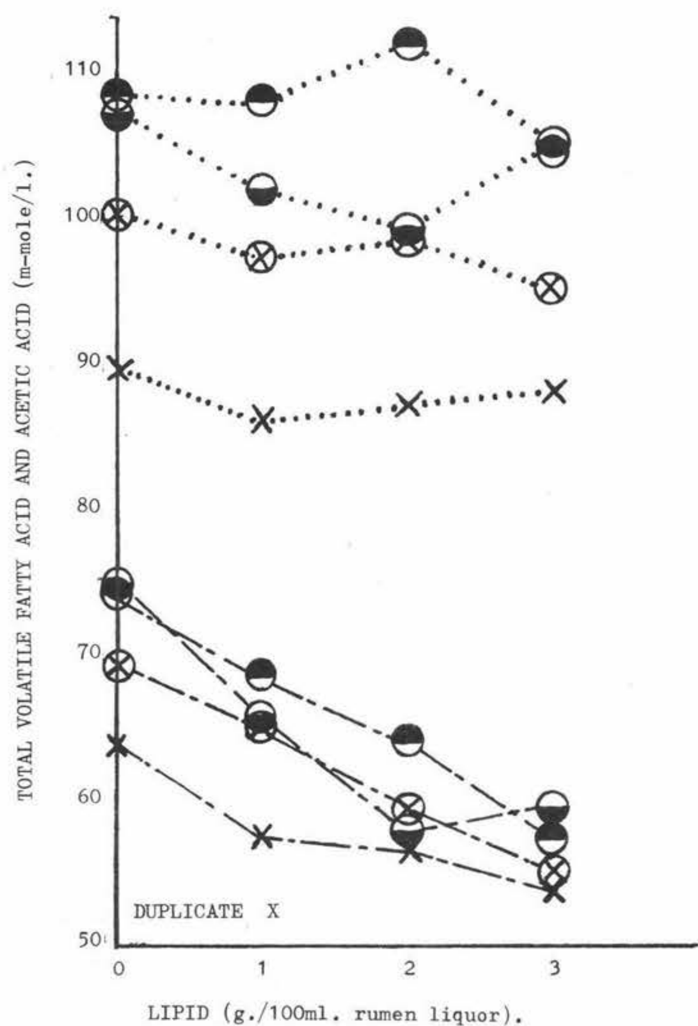
TABLE 10. Effect of lipid and time of incubation on the formation of butyrate(m-mole/l.) in rumen liquor(50ml.) incubated with grass fibre(0.5g.). (Mean values).

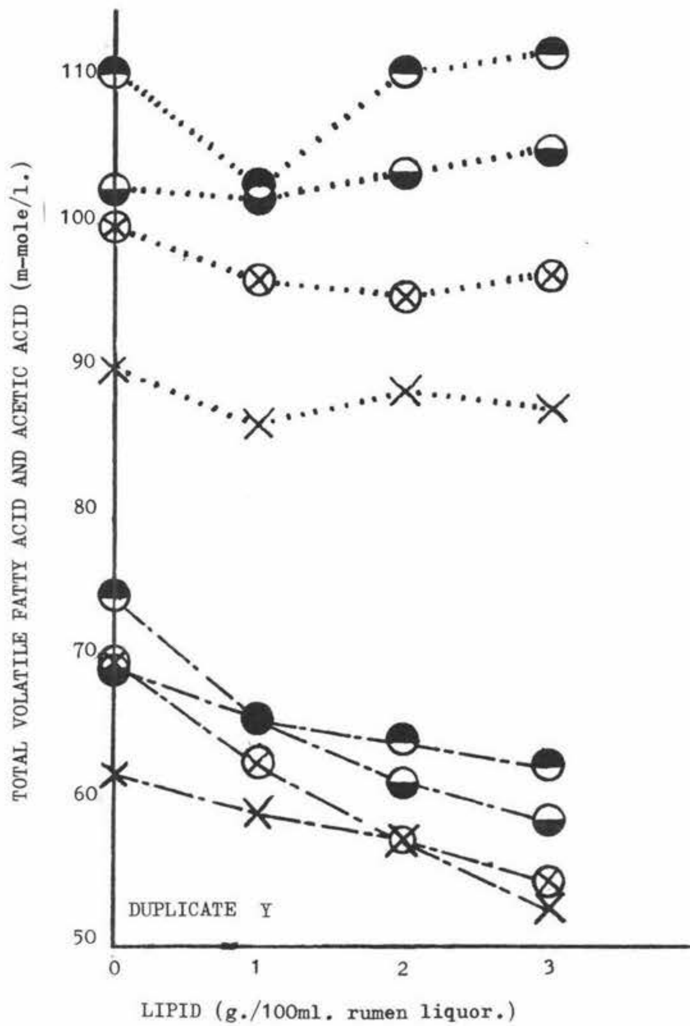
Lipid Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Lipid Means	Signif. of diffs. ($P<0.05$)
0	8.83	9.82	9.84	9.91	9.60	0<2,3.
1	9.08	9.99	10.46	11.17	10.18	
2	9.72	11.02	10.58	11.91	10.81	
3	10.65	10.23	10.79	12.13	10.95	
Incubation Time Means	9.57	10.27	10.42	11.28	$\bar{X}$ = 10.38	
Significance of diffs. ($P<0.05$)	24<72.					

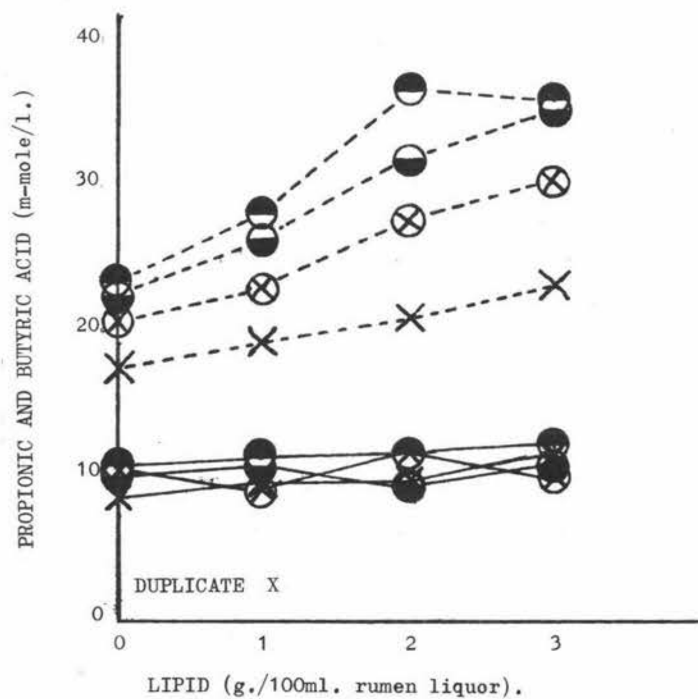
S.E. per fermentation bottles mean = ± 0.72 m-mole/l.

The addition of 0.5g. of grass fibre increased butyrate production ($P<0.01$) (Appendix 4b & 6d).

The effect of lipid on total VFA, acetate, propionate and butyrate production for each incubation time is shown graphically in Fig. 3.







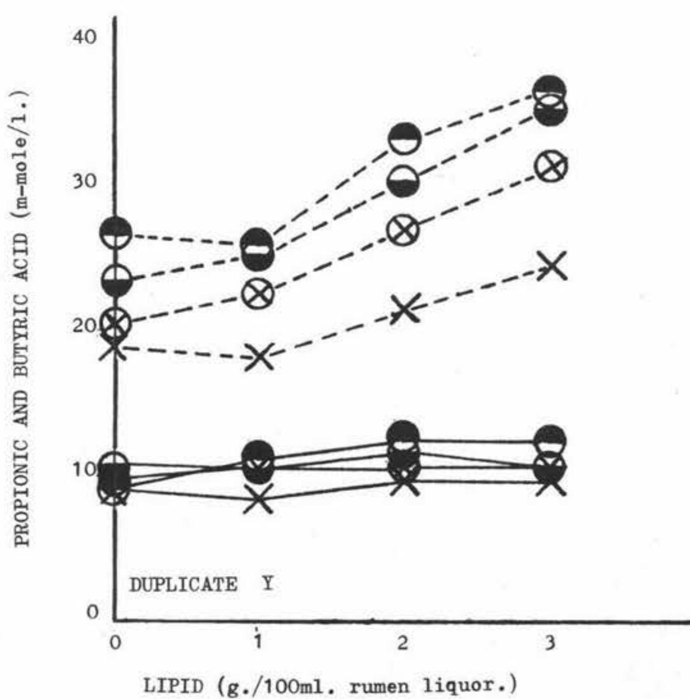


FIG. 3. The effect of linseed oil (0,1,2 & 3g./100ml. rumen liquor) on total and individual VFA concentrations (m-mole/1.) when 0.5g. of grass fibre was incubated in rumen liquor (50ml.) for 24hr. ✕ ; 36hr. ⊗ ; 48hr. ⊖ ; or 72hr. ◓ .

.... total VFA —.—.— Acetate — — — — Propionate ———— Butyrate

2.5 Ammonia production

The overall effect of lipid was to decrease the production of ammonia($P < 0.01$). When the means for each lipid level were compared only the differences between no lipid and 2.0 or 3.0g. of lipid were significant. The difference between no lipid and 1.0g. of lipid was, however, close to being significant at the 5% level (Table 11, Appendix 5a).

Ammonia production increased with increasing time of incubation ($P < 0.01$) although the only significant difference was between the 24 and 72hr. incubations.

The addition of 0.5g. of grass fibre increased ammonia production ($P < 0.05$) (Appendix 5b & 6e). However, the differences in ammonia production appeared to be primarily affected by the depressive effect of lipid rather than by the increase in production from grass fibre or from increasing the time of incubation.

TABLE 11. Effect of lipid and time of incubation on the formation of ammonia(mg.NH_3/100ml.) in rumen liquor(50ml.) incubated with grass fibre(0.5g.). (Mean values).

Lipid Added(g./100ml.)	TIME OF INCUBATION(hr.)				Added Lipid Means	Signif. of diffs. ($P < 0.05$)
	24	36	48	72		
0	26.19	31.62	37.02	41.02	33.96	$0 > 2,3$.
1	23.69	25.63	27.50	32.56	27.34	
2	22.19	25.08	25.42	30.65	25.84	
3	23.29	23.34	27.63	30.01	26.07	
Incubation Time Means	23.84	26.42	29.40	33.56	$\bar{X} = 28.30$	
Significance of diffs. ($P < 0.05$)	$24 < 72$.					

S.E. per fermentation bottles mean = ± 5.29mg.NH_3/100ml.

The effect of incubation time on ammonia production when various levels of lipid were added is shown graphically in Fig. 4. The ammonia production from samples containing only rumen liquor was nearly always greater than samples containing grass fibre and lipid. The samples containing grass fibre as the only substrate produced the greatest amounts of ammonia. In Fig. 5 ammonia concentrations are plotted against lipid levels to illustrate that the greatest difference in ammonia production is between the presence and absence of lipid, rather than between lipid levels.

2.6 pH determinations of rumen liquor incubations containing grass fibre and linseed oil within impermeable apparatus

One of the criticisms of the use of impermeable apparatus (glass bottles) for _in-vitro_ experiments is that end products are not removed but are believed to accumulate to such an extent that rumen liquor fermentations are inhibited. With the knowledge that rumen micro-organisms can be inhibited outside a pH range of 6.0-7.0 (Barnett & Reid, 1961), and rather than interfere with the thermal and anaerobic environments of the incubations in the previous experiment, a second experiment, in which the pH of six rumen liquor incubations were recorded regularly throughout a 72hr. incubation, was conducted to determine the validity of the previous experiment where grass fibre and linseed oil were added to rumen liquor incubations within impermeable apparatus. Two 130ml. fermentation bottles, both containing 50ml. of rumen liquor, had 0.5g. of grass fibre added; two had 0.5g. of grass fibre + 1.5g. of linseed oil added, while the remaining two had no additional substrates added to the rumen liquor. pH readings were taken at 12, 24, 36, 48 and 72hr.

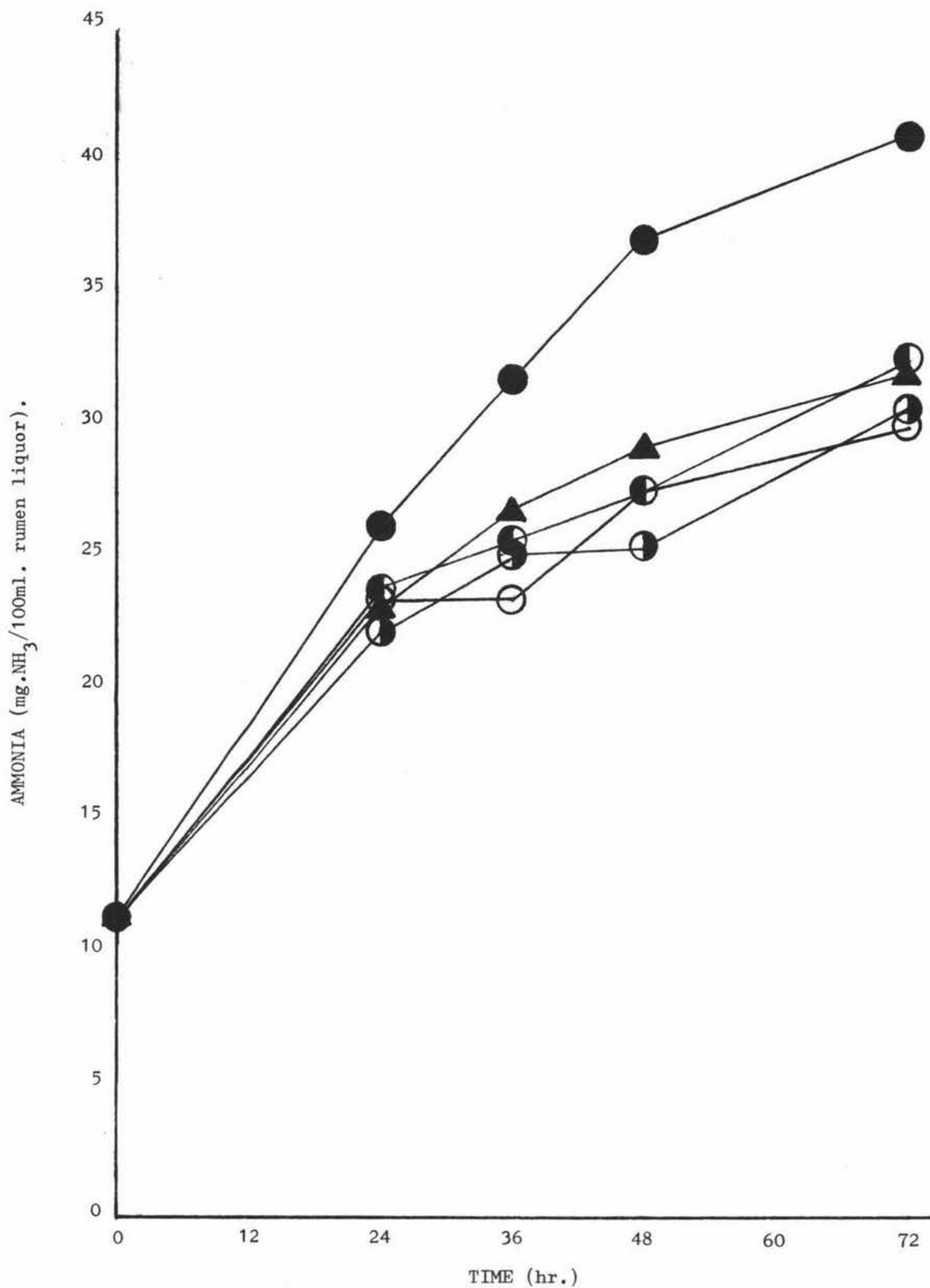


FIG. 4. The effect of time of incubation on the ammonia concentration ($mg.NH_3$/100ml.) of rumen liquor (50ml.) containing 0.5g. of grass fibre and linseed oil (0,1,2 & 3g./100ml.).

▲ rumen liquor

● " " +0.5g. of grass fibre.

◐ " " + " " " " +0.5g. of linseed oil.

◑ " " + " " " " +1.0g. " "

○ " " + " " " " +1.5g. " "

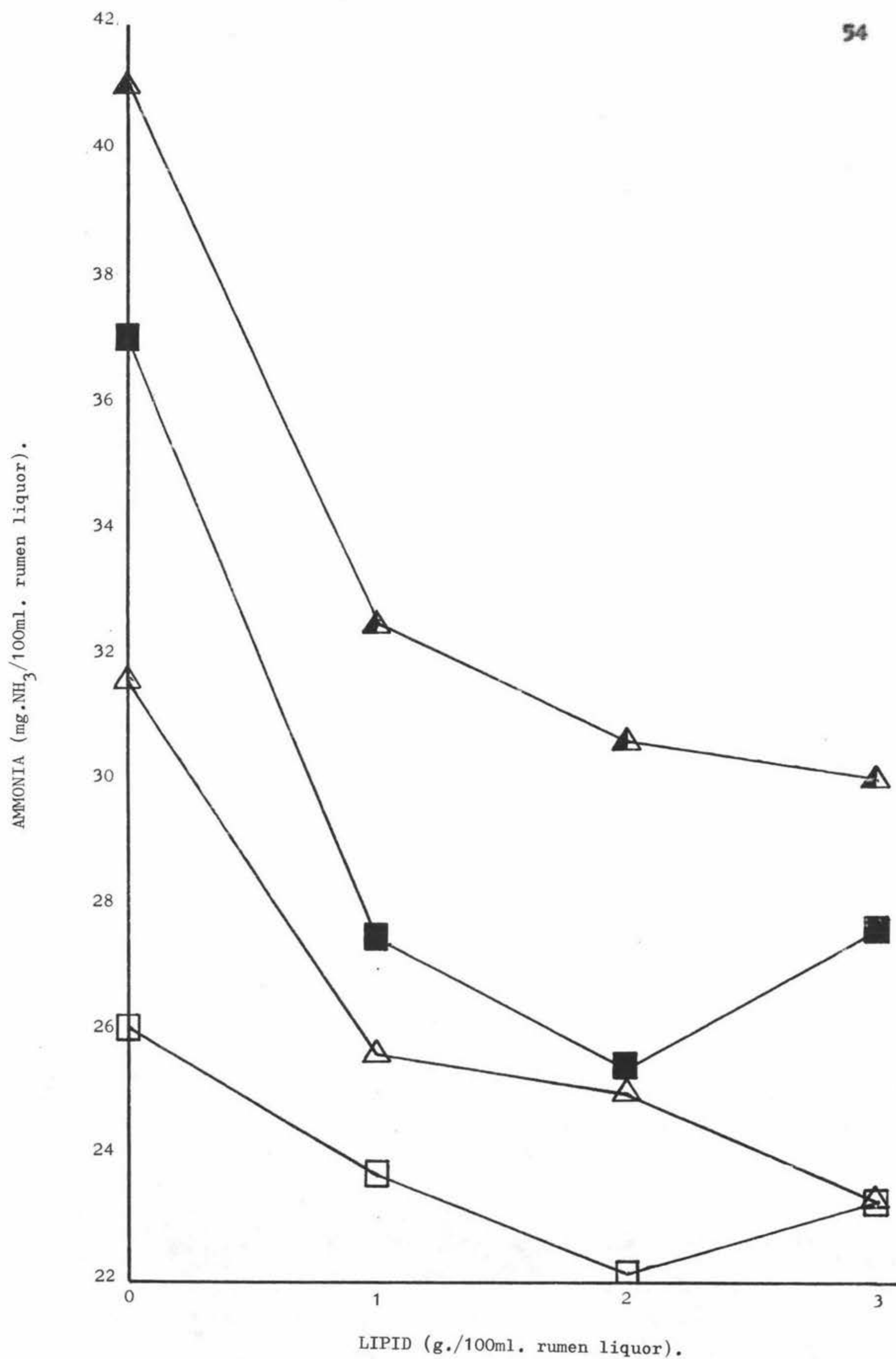


FIG. 5. The effect of linseed oil (0,1,2 & 3g./100ml. rumen liquor) on ammonia concentrations (mg.NH_3/100ml.) when 0.5g. of grass fibre was incubated in rumen liquor (50ml.) for 24hr. □ ; 36hr. △ ; 48hr. ■ ; or 72hr. ▲ .

TABLE 12. The pH rumen of liquor(50ml.) incubated with 0.5g. of grass fibre, with grass fibre and 1.5g. of linseed oil, or without any additional substrate.

Sample	Duplicate	12	24	36	48	72
		TIME OF INCUBATION(hr.)				
Blank:	A	6.81	6.81	6.94	7.12	7.40
Rumen liquor.	B	6.75	6.85	7.09	7.22	7.35
Control:	A	6.32	6.30	6.40	6.50	6.60
Rumen liquor + grass fibre.	B	6.42	6.39	6.51	6.62	6.80
Experimental:	A	6.35	6.03	6.03	6.04	6.10
Rumen liquor + grass fibre + linseed oil.	B	6.34	6.06	6.00	6.12	6.20

Rumen liquor pH = 6.80 Rumen liquor + buffer pH = 6.96

⟶ Increasing pH.

The pH of rumen liquor was depressed to the greatest extent in the incubations containing linseed oil (Table 12). Rumen liquor incubations with no additional substrates added reached their lowest pH value at or before 12hr. When grass fibre was added the pH did not reach a minimum until 24hr. whereas linseed oil and grass fibre caused the pH to fall for 36hr. before slowly increasing. Incubations containing only rumen liquor increased their pH values above the initial rumen liquor + buffer pH value of 6.96. By 72hr., the pH value had risen the least in incubations containing linseed oil and the most in the incubations containing no additional substrates.

3. The effect of various lipids on volatile fatty acid production

This experiment was conducted to compare cod liver oil and peanut oil with linseed oil so as to determine which oil was the most effective in increasing propionate production when incubated in rumen liquor with grass fibre (Table 13).

TABLE 13. Design of the experiment comparing the effect of linseed oil, cod liver oil and peanut oil on the production of VFA's in rumen liquor(50ml.) incubated for 48hr. with or without grass fibre.

Sample		Phosphate Buffer (ml.)	Lipid Emulsion (1.0ml.)	Grass Fibre (g.)	Incubation Time(hr.)	Distilled H_2O(ml.)
Zero Blank	1;10	15	-	-	0	0.5
Blank	2;11	"	-	-	48	"
Experimental	3;12	"	Linseed oil	-	"	-
"	4;13	"	Cod liver oil	-	"	-
"	5;14	"	Peanut oil	-	"	-
Control	6;15	"	-	0.5	"	0.5
Experimental	7;16	"	Linseed oil	"	"	-
"	8;17	"	Cod liver oil	"	"	-
"	9;18	"	Peanut oil	"	"	-

3.1 Total and individual VFA production from rumen liquor containing grass fibre

Linseed oil, cod liver oil, and peanut oil all depressed total VFA production significantly ($P < 0.1$) when incubated with grass fibre in rumen liquor (Table 14, Appendix 7i).

TABLE 14. The effect of various lipids on total VFA concentrations (m-mole/l.) of rumen liquor incubated for 48hr. with 0.5g. of grass fibre.

LIPIDS(1g./100ml.)	Duplicates Z	Duplicates W	Lipid Means	Signif. of diffs. (P<0.1)
No lipid (0)	104.6	105.4	105.00	
Linseed oil (L)	103.2	103.2	103.20	L<0.
Cod liver oil (C)	104.3	103.9	104.10	C<0.
Peanut oil (P)	103.6	102.8	103.20	P<0.
Duplicate Means	103.9	103.8	$\bar{X}$ = 103.85	

S.E. per fermentation bottle = ± 0.48 m-mole/l.

Cod liver oil and peanut oil depressed acetate production significantly ($P<0.05$) but linseed oil did not. Cod liver oil also produced significantly less acetate than linseed oil or peanut oil ($P<0.05$) when incubated with grass fibre in rumen liquor (Table 15, Appendix 7ii).

TABLE 15. The effect of various lipids on acetate concentration(m-mole/l.) of rumen liquor incubated for 48hr. with 0.5g. of grass fibre.

LIPIDS(1g./100ml.)	Duplicates Z	Duplicates W	Lipid Means	Signif. of diffs. (P<0.05)
No lipid (0)	70.00	69.09	69.55	
Linseed oil (L)	68.80	67.38	68.09	
Cod liver oil (C)	62.96	63.16	63.06	C<0,L,P.
Peanut oil (P)	65.77	67.39	66.58	P<0.
Duplicate Means	66.88	66.76	$\bar{X}$ = 66.82	

S.E. per fermentation bottle = ± 0.95 m-mole/l.

Peanut oil slightly increased while linseed oil decreased propionate production, but only cod liver oil increased propionate production significantly ($P<0.05$) when incubated with grass fibre in rumen liquor. Cod liver oil produced significantly more propionate than linseed oil or peanut oil ($P<0.05$) (Table 16, Appendix 7iii).

TABLE 16. The effect of various lipids on propionate concentration (m-mole/l.) of rumen liquor incubated for 48hr. with 0.5g. of grass fibre.

LIPIDS(1g./100ml.)	Duplicates		Lipid Means	Signif. of diffs. ($P<0.05$)
	Z	W		
No Lipid (O)	23.56	24.87	24.22	
Linseed oil (L)	22.80	23.96	23.38	
Cod Liver Oil (C)	27.16	26.73	26.95	C>O,L,P.
Peanut oil (P)	24.83	24.36	24.60	
Duplicate Means	24.59	24.98	$\bar{X}$ = 24.79	

S.E. per fermentation bottle = ± 0.69 m-mole/l.

Although linseed oil and peanut oil increased butyrate production when incubated with grass fibre in rumen liquor, only cod liver oil increased butyrate production significantly ($P<0.1$). Cod liver oil produced significantly more butyrate than linseed oil and peanut oil ($P<0.1$) (Table 17, Appendix 7iv).

TABLE 17. The effect of various lipids on butyrate concentration(m-mole/l.) of rumen liquor incubated for 48hr. with 0.5g. of grass fibre.

LIPIDS(1g./100ml.)	Duplicates		Lipid Means	Signif. of diffs. ($P<0.1$)
	Z	W		
No Lipid (O)	11.04	11.44	11.24	
Linseed oil (L)	11.60	11.86	11.73	
Cod liver oil (C)	14.18	14.01	14.10	C>O,L,P.
Peanut oil (P)	13.00	11.05	12.03	
Duplicate Means	12.45	12.09	$\bar{X}$ = 12.27	

S.E. per fermentation bottle = ± 0.77 m-mole/l.

The differences between duplicate analysis for total VFA, acetate, propionate and butyrate production were not significant ($P > 0.05$) (Appendix 7).

Cod liver oil, when incubated with grass fibre in rumen liquor, produced more propionate and butyrate and less acetate than when the other lipids were added, so cod liver oil was used as lipid substrate in all subsequent experiments.

3.2 Comparison of the effect of lipid upon the production of total and individual VFA's of rumen liquor incubated with or without grass fibre

In the absence of added lipids, 0.5g. of grass fibre increased the production of total and individual VFA's (Table 18). However, the influence of the added fibre also depended on the type of lipid present.

TABLE 18. The effect of various lipids on total VFA, acetate, propionate and butyrate concentrations(m-mole/l.) of rumen liquor(50ml.) incubated for 48hr. with or without grass fibre. (Mean values).

	Added fibre (g./100ml.)	No lipid	Lipid(1g./100ml.)		
			Linseed oil	Cod liver oil	Peanut oil
Total VFA	0	65.45	69.55	71.15	67.75
	1	105.00	103.20	104.10	103.20
Acetate	0	43.53	46.12	44.94	43.97
	1	69.55	68.09	63.06	66.58
Propionate	0	13.62	15.83	18.41	15.12
	1	24.22	23.38	26.95	24.60
Butyrate	0	8.30	7.61	7.80	8.67
	1	11.24	11.73	14.10	12.03

When lipids were added to rumen liquor containing 0.5g. of grass fibre, total VFA and acetate productions were decreased, while butyrate and propionate were increased, except for linseed oil which depressed propionate production.

When lipids were incubated in rumen liquor alone, total VFA, acetate and propionate productions were increased whereas butyrate was decreased, with the exception of peanut oil which increased butyrate production. These effects on total VFA, acetate and butyrate were the opposite to those which occurred in the presence of fibre.

4. <u>The use of ^{14}C-cellulose in rumen fermentations of lipid and grass fibre within impermeable apparatus</u>

Initially, an exploratory experiment of three incubations was carried out (Table 19) to determine total VFA production, percentage incorporation of ^{14}C into CO_2 and VFA, and the relative proportions of ^{14}C in acetic, propionic and butyric acids. The use of ^{14}C-cellulose as a marker was an attempt to establish the source of VFA's formed in rumen fermentations of grass fibre and lipid. At the conclusion of the incubation period the samples were frozen (-4°C) until analysed.

TABLE 19. Design of exploratory experiment when ^{14}C-cellulose(28.0 μc./mg.) was incubated in rumen liquor(50ml.) for 48hr. with grass fibre(0.5g.) and with or without cod liver oil(1.5g.).

Sample	Phosphate Buffer (ml.)	Grass Fibre (g.)	Cod Liver Oil Emulsion (ml.)	^{14}C-cellulose (mg.)	Distilled H_2O(ml.)
Control (1)	15	0.5	-	1.95	1.5
Control (2)	"	"	-	2.13	1.5
Experimental (3)	"	"	3.0	2.15	-

4.1 <u>Total and individual VFA production</u>

Although the lowest total VFA production occurred in the presence of lipid, the greatest amount of propionate and the least acetate was produced in the presence of lipid (Table 20).

TABLE 20. Total and individual VFA concentrations of rumen liquor incubated for 48hr. with grass fibre, ^{14}C-cellulose, and with or without cod liver oil.

Sample	m-mole/100ml.			
	Acetate	Propionate	Butyrate	TOTAL
Control (1)	10.31	2.73	1.46	14.50
Control (2)	11.20	2.63	1.30	15.13
Experimental (3)	9.32	3.03	1.35	13.70

4.2 Incorporation of ^{14}C into VFA and CO_2

The incorporation of ^{14}C into VFA did not appear to be dependent upon treatment (Table 21a). An overall average of 62.63% was found in this experiment.

TABLE 21. Distribution of ^{14}C into VFA (a) and CO_2 (b) from ^{14}C-cellulose incubated in rumen liquor for 48hr. with grass fibre and with of without cod liver oil.

(a)

Sample	Total disintegrations/min. in Sample	Total disintegrations/min. in VFA	% incorporation
Control (1)	121.212×10^6	79,367,870	65.48
Control (2)	132.401×10^6	79,952,030	60.39
Experimental (3)	133.644×10^6	82,050,140	61.39

(b)

Sample	Total disintegrations/min. in Sample	Total disintegrations/min. in CO_2	% incorporation
Control (2)	132.401×10^6	538,692	0.41
Experimental (3)	133.644×10^6	1,427,534	1.07

The incorporation of ^{14}C into CO_2 was only a very small proportion of the total radioactivity (Table 21b).

4.3 Distribution of ^{14}C among individual VFA's

When lipid was added there was a decrease in ^{14}C-acetate which was approximately balanced by increases in ^{14}C-propionate and ^{14}C-butyrate production (Table 22). Whereas 68% and 64% of the total ^{14}C was incorporated into acetate in the samples containing grass fibre, only 53% was incorporated into acetate when lipid was added. Conversely, although 34% of the ^{14}C was

incorporated into propionate when lipid was present, when it was absent only 25–26% was incorporated. Of the total ^{14}C-VFA, approximately 74% was incorporated into ketogenic compounds in the absence of lipid, and only 66% when lipid was added.

TABLE 22. The distribution of the total ^{14}C-VFA, formed from ^{14}C-cellulose, in acetate, propionate and butyrate.

Sample	% distribution of the total ^{14}C			
	Acetate	Propionate	Butyrate	Acetate & Butyrate
Control (1)	68.14	25.44	6.42	74.56
Control (2)	63.79	26.33	9.88	73.67
Experimental (3)	53.43	33.68	12.89	66.32

A second experiment (Table 23) was conducted to extend the exploratory experiment using ^{14}C-cellulose. Only samples 1 – 9 were connected to test-tubes containing the CO_2-collecting 'Hyamine' hydroxide.

TABLE 23. Design of the experiment when ^{14}C-cellulose (28 µc./mg.) was incubated in rumen liquor(50ml.) for 48hr. with grass fibre (0.5g.) and 0.1 – 1.0g. of cod liver oil.

Sample	Phosphate Buffer (ml.)	Grass Fibre (g.)	Cod Liver Oil Emulsion (ml.)	^{14}C-cellulose (mg.)		Distilled H_2O(ml.)
1;10.	15	-	-	1.09	0.94	1.00
2;11.	"	0.5	-	1.00	0.96	1.00
3;12.	"	"	0.2	1.00	1.09	0.90
4;13.	"	"	0.3	1.00	1.00	0.85
5;14.	"	"	0.4	1.00	1.09	0.80
6;15.	"	"	0.5	1.06	1.07	0.75
7;16.	"	"	1.0	1.00	0.91	0.50
8;17.	"	"	1.5	1.09	1.08	0.25
9;18.	"	"	2.0	1.14	1.00	-

4.4 Net production of total VFA, acetic, propionic and butyric acids

Net VFA production figures were determined by subtracting the zero hour VFA concentration(m-mole/l.) from the VFA concentrations of all the 48hr. incubations.

Although all levels of lipid increased the net production of total VFA, the difference between the means for different lipid levels were not significant ($P>0.05$) (Appendix 8a).

Most, but not all levels of lipid, decreased the net production of acetate. However, no significant differences were found between the means for any of the levels of lipid ($P>0.05$) (Appendix 8b).

All lipid levels increased the net production of propionate although only the difference between the means for no lipid and 2.0g. of lipid was significant ($P<0.05$) (Table 24, Appendix 8c).

TABLE 24. Net production of propionate(m-mole) from grass fibre(0.5g.), and ^{14}C-cellulose incubated in rumen liquor for 48hr. with 0.1 - 1.0g. of cod liver oil.

Lipid Added(g./100ml.)	Duplicates D_1	D_2	Lipid	Signif. of diffs. ($P<0.05$)
0	11.79	10.50	11.15	
0.2	15.43	14.14	14.79	
0.3	13.35	14.48	13.92	
0.4	15.53	16.30	15.92	
0.5	15.66	12.09	13.88	
1.0	16.84	16.47	16.66	
1.5	17.80	15.95	16.88	
2.0	19.22	18.45	18.84	2.0>0
Duplicate Means	15.70	14.79	$\overline{X} = 15.25$	

S.E. per fermentation bottle = ± 1.43 m-moles.

All levels of lipid increased the net production of butyrate but only the difference between the means for no lipid and 2.0g. of lipid was significant ($P<0.05$) (Table 25, Appendix 8d).

TABLE 25. Net production of butyrate(m-mole) from grass fibre(0.5g.), and ^{14}C-cellulose incubated in rumen liquor for 48hr. with 0.1-1.0g. of cod liver oil.

Lipid Added(g./100ml.)	Duplicates D_1	D_2	Added Lipid Means	Signif. of diffs. $(P<0.05)$
0	5.00	5.10	5.05	
0.2	5.58	8.51	7.04	
0.3	7.25	6.84	7.04	
0.4	8.92	9.09	9.01	
0.5	8.04	7.40	7.72	
1.0	9.56	9.46	9.51	
1.5	8.35	8.79	8.57	
2.0	11.86	8.99	10.43	2.0>0
Duplicate Means	8.07	8.02	$\overline{X}$ = 8.05	

S.E. per fermentation bottle = ± 1.12 m-moles

4.5 Incorporation of ^{14}C into VFA and CO_2

The percentage of ^{14}C incorporated into VFA ranged from 37.76% to 63.09% with an overall average incorporation of 46.28%. No trends in incorporation were apparent with the presence or absence of grass fibre and lipid or with lipid level.

As found in the exploratory experiment, the incorporation of ^{14}C into CO_2 was very low ranging from 0 - 1.8%. It was highest with the 0.3g. of lipid while no incorporation was found with 0.5 - 2.0g. levels of lipid.

4.6 Distribution of ^{14}C among individual VFA's

The figures used in these analyses were the percentages transformed to angles; the percentages representing the distribution of total ^{14}C-VFA in either acetic, propionic or butyric acids.

Lipid decreased the production of ^{14}C-acetate (Table 26, Appendix 9a) and increased the production of ^{14}C-propionate (Table 27, Appendix 9b) and ^{14}C-butyrate (Appendix 9c).

TABLE 26. ^{14}C-acetate production from ^{14}C-cellulose incubated in rumen liquor with 0.5g. of grass fibre and 0.1 - 1.0g. of cod liver oil. (Percentages transformed to angles).

Lipid Levels(g./100ml.)

Duplicate	0	0.2	0.3	0.4	0.5	1.0	1.5	2.0	Duplicate Means
D_1	54.45	53.73	51.59	50.42	51.47	48.85	50.01	48.73	51.16
D_2	53.19	52.53	49.54	51.41	51.30	52.12	50.36	49.43	51.24
Added Lipid Means	53.82	53.13	50.57	50.92	51.39	50.49	50.19	49.08	$\bar{X}$ = 51.20
Significance of diffs. ($P<0.1$)			0.3<0.	0.4<0.	0.5<0.	1.0<0.	1.5<0.	2.0<0.	

TABLE 27. ^{14}C-propionate production from ^{14}C-cellulose incubated in rumen liquor with 0.5g. of grass fibre and 0.1 - 1.0g. of cod liver oil. (Percentages transformed to angles).

Lipid Added(g./100ml.)

Duplicate	0	0.2	0.3	0.4	0.5	1.0	1.5	2.0	Duplicate Means
D_1	27.63	27.28	28.59	29.47	28.93	30.00	29.53	30.13	28.95
D_2	27.69	27.42	31.24	28.66	29.33	28.86	30.00	30.66	29.23
Added Lipid Means	27.66	27.35	29.92	29.07	29.13	29.43	29.77	30.40	$\bar{X}$ = 29.09
Significance of diffs. ($P<0.1$)			0.3>0, & 0.2.	0.4>0.2.	0.5>0.2.	1.0>0, & 0.2.	1.5>0, & 0.2.	2.0>0, & 0.2.	

The differences in ^{14}C-acetate production between no lipid and all the other lipid levels, except the 0.2g. lipid level, were significant ($P < 0.1$).

Differences in ^{14}C-propionate production were significant between no lipid and all the other lipid levels except for the 0.2, 0.4 & 0.5g. lipid levels ($P < 0.1$). There were also significant differences between the lower ^{14}C-propionate production of the 0.2g. lipid level and all the other levels of lipid.

There were no significant differences in ^{14}C-butyrate production between lipid levels ($P > 0.05$).

There were also no significant differences between duplicates in either ^{14}C-acetate, ^{14}C-propionate or ^{14}C-butyrate production ($P > 0.05$) (Appendix 9).

5. The incubation of (2-^{14}C)glycerol (0.45 μc./ml.) in rumen liquor with grass fibre and cod liver oil

The extent of the contribution of the glycerol moiety of triglycerides to VFA, or more specifically, the extent of the contribution of glycerol to propionate production both in the presence and absence of grass fibre and lipid was investigated by adding (2-^{14}C)glycerol to rumen fermentations within impermeable apparatus (Table 28).

TABLE 28. Design of the experiment when (2-^{14}C)glycerol (0.45 μc./ml.) was incubated in rumen liquor(50ml.) for 48hr. with grass fibre(0.5g). and 0.15 - 1.5g. of cod liver oil.

Sample		Phosphate Buffer (ml.)	Grass Fibre (g.)	Cod Liver Oil Emulsion (ml.)	(2-^{14}C) glycerol (ml.)	Distilled H_2O(ml.)
Blank	1;6.	15	-	-	1	1.50
Control	2;7.	"	0.5	-	"	1.50
Experimental	3;8.	"	"	0.3	"	1.35
"	4;9.	"	"	1.0	"	1.00
"	5;10.	"	"	3.0	"	-

5.1 Net production of total VFA, acetic, propionic and butyric acids

Net VFA production figures were determined by subtracting the zero hour VFA concentration (m-mole/l.) from the VFA concentrations of all the 48hr. incubations.

The effect of lipid on the net production of total VFA was variable, 0.3g. and 3.0g. of lipid increased while the 1.0g. of lipid decreased total VFA production. There were, however, no significant differences between the lipid levels ($P > 0.05$) (Appendix 10a).

Lipid decreased the net production of acetate and increased that of butyrate. No significant differences between lipid levels were found for either acid ($P > 0.05$) (Appendix 10b & 10d).

Lipid increased the net production of propionate. However, only the differences between the means for no lipid and the 0.3, 1.0 & 3.0g. levels of lipid were significant ($P < 0.01$) (Table 29, Appendix 10c). Although not confirmed by the Studentized Range test, a significant difference ($P < 0.05$) in the net production of propionate between the lipid levels (0.3, 1.0 & 3.0g.) was detected by the analysis of variance F test; this probably being the increase in the net production of propionate between the low(0.3 & 1.0g.) and the high(3.0g.) levels of lipid.

TABLE 29. Net production of propionate(m-mole) from grass fibre(0.5g.) and (2-^{14}C)glycerol incubated in rumen liquor for 48hr. with 0.15- 1.5g. of cod liver oil.

Lipid Added(g./100ml.)	Duplicates D_1	Duplicates D_2	Added Lipid Means	Signif. of diffs. ($P < 0.01$)	Signif. of diffs. ($P < 0.05$)
0	7.73	6.22	6.98		
0.3	10.23	8.94	9.59	0.3 > 0.	
1.0	9.75	8.95	9.35	1.0 > 0.	
3.0	11.49	9.75	10.62	3.0 > 0.	3.0 > 0.3, & 1.0.
Duplicate Means	9.80	8.47	$\bar{X} = 9.13$		

S.E. per fermentation bottle = ± 0.28 m-mole/l.

There was a significant difference between duplicates in the net production of propionate ($P < 0.01$).

5.2 The percentage incorporation of ^{14}C from (2-^{14}C)glycerol into total and individual VFA's

The percentage of the ^{14}C from (2-^{14}C)glycerol incorporated into VFA, in the various treatments, ranged from 51.33% to 61.24%, with an overall average of 54.95% (Table 30). No trends in total incorporation were present in these data.

TABLE 30. Percentage incorporation of ^{14}C from (2-^{14}C)glycerol into total and individual VFA's, and the total activity of each (disintegrations/min. x 10^3).

Sample	VFA	Duplicate X		Duplicate Y	
		Total Activity	% incorporation	Total Activity	% incorporation
Blank	Acetate	471	46.67	507	50.24
	Propionate	22	2.18	34	3.37
	Butyrate	25	2.48	37	3.67
	TOTAL	518	51.33	578	57.28
Control	Acetate	479	47.47	457	45.29
	Propionate	60	5.94	52	5.15
	Butyrate	79	7.83	37	3.67
	TOTAL	618	61.24	546	54.11
0.3g. Lipid	Acetate	408	40.43	424	42.02
	Propionate	67	6.64	87	8.62
	Butyrate	73	7.24	48	4.76
	TOTAL	548	54.31	559	55.40
1.0g. Lipid	Acetate	419	40.53	374	37.06
	Propionate	76	7.53	69	6.84
	Butyrate	87	8.62	75	7.43
	TOTAL	582	57.68	518	51.33
3.0g. Lipid	Acetate	397	39.34	376	37.26
	Propionate	80	7.93	67	6.64
	Butyrate	75	7.43	83	8.23
	TOTAL	552	54.70	526	52.13

The majority of the ^{14}C was incorporated into acetate with only a small portion being incorporated into propionate and butyrate. In all samples the combined incorporation of ^{14}C into propionate and butyrate rarely exceeded one/third of the ^{14}C incorporated into acetate. The incorporation of ^{14}C into propionate and butyrate was least when grass fibre and lipid were not added to the rumen liquor. The effect of grass fibre and increasing levels of lipid was to decrease the incorporation of ^{14}C into acetate and increase the incorporation of ^{14}C into propionate and butyrate.

6. In-vitro experiments using the micro-artificial rumen apparatus

A series of experiments were undertaken to determine the production of total and individual VFA's in rumen liquor incubated for 24hr. with grass fibre and with or without cod liver oil within a dialysis sac from which end products of fermentation were removed by a continuous flow (40ml./hr.) of clarified rumen liquor(dialysate). In all experiments the three micro-artifical rumen units, described earlier, were arranged experimentally as in Table 31. In the first two experiments (denoted a and b) the dialysate from each artificial rumen was collected as one sample,

TABLE 31. Experiments using the micro-artificial rumen apparatus. Design of experiments to determine the VFA production from 50ml. of rumen liquor incubated for 24hr. with either 0.5g. of grass fibre; grass fibre and 1.5g. of cod liver oil; or without additional substrates.

UNIT	Grass Fibre(g.)	Cod Liver Oil Emulsion(ml.)	Incubation Time(hr.)	Distilled H_2O(ml.)
Blank	-	-	24	1.5
Control	0.5	-	"	1.5
Experimental	0.5	3.0	"	-

its pH adjusted to 9.0 - 9.5 with N/5 NaOH and evaporated to approximately 100 - 200ml. in a rotary evaporator, before being analysed. In a further two experiments (denoted c and d) the dialysate was collected in 80ml. portions every 2 hr. for analysis.

Since only one cow was available during experiments a and b, rumen liquor was obtained from a hay-fed sheep. Bovine rumen liquor was used in experiments c and d.

6.1 Experiments a and b

Table 32a shows the VFA concentrations within each apparatus of both

TABLE 32a. The total VFA concentrations, in experiments a and b, of the rumen liquor(50ml.) incubated for 24 hr. with grass fibre and cod liver oil, and of the clarified rumen liquor(dialysate) collected from each artificial-rumen unit.

Sample	Sampling Time (hr.)	Experiment a		Experiment b	
		m-mole/l.	m-mole/50ml.	m-mole/l.	m-mole/50ml.
Rumen Liquor:	0	59.1	2.96	58.0	2.90
Blank	24	55.1	2.76	53.1	2.66
Control	24	61.4	3.07	58.1	2.91
Experimental	24	56.7	2.84	57.7	2.89
Clarified Rumen Liquor:	0	53.20	-	53.20	-
Blank	24	57.29	-	54.93	-
Control	24	60.63	-	55.53	-
Experimental	24	57.38	-	57.59	-

TABLE 32b. The total and individual VFA production(m-mole/50ml.) in experiments a and b, from rumen liquor(50ml.) incubated for 24hr. with grass fibre and cod liver oil.

Sample	Experiment a				Experiment b			
	A*	P**	B***	Total	A	P	B	Total
Blank	3.36	-	0.91	3.89	0.90	0.38	0.21	1.49
Control	5.18	1.02	1.34	7.54	1.21	1.29	-	2.34
Experimental	3.76	-	0.54	4.06	1.63	1.73	1.02	4.38

A* Acetate P** Propionate B*** Butyrate

Blank; rumen liquor only.

Control; rumen liquor + grass fibre(0.5g.).

Experimental; rumen liquor + grass fibre(0.5g.) + cod liver oil(1.5g.).

experiments. After 24hr., in both experiments a and b, the VFA concentration within the dialysis sacs containing added lipid and grass fibre were lower than at the beginning of the experiment. The same was true for the dialysis sacs containing only rumen liquor, whereas an increase in VFA concentration occurred in the apparatus with grass fibre alone as substrate.

There were increases in the VFA concentrations of the dialysate, the lowest increase in VFA concentration occurring in the dialysate collected from the apparatus containing rumen liquor alone. Although in experiment a the highest VFA concentration was in the dialysate collected from the apparatus containing grass fibre, in experiment b the highest was in the dialysate collected from the apparatus containing both grass fibre and cod liver oil.

The production of total and individual VFA's is shown in Table 32b. The results of experiments a and b were variable even although both experiments were run under similar conditions. In experiment a the addition of grass fibre to rumen liquor produced the largest amounts of acetate, propionate and butyrate, whereas in experiment b the addition of grass fibre and cod liver oil to rumen liquor produced the most propionate and butyrate. The most acetate produced in experiment b was in the rumen liquor containing only grass fibre.

The amounts of the individual VFA's produced were obtained by determining the proportions of the fatty acids in the rumen sample and the dialysate before and after incubations. Since the production of total VFA was often small, in some cases negative values for

individual fatty acids could result from experimental errors when determining proportions of the acids from chromatographic data. Differences between the totals and the sums of individual VFA's have occurred because of these negative values (Table 32b).

6.2 Experiments c and d

The results of experiments c and d are shown in Tables 33 and 34. The total VFA concentration of each 80ml. portion was determined but the individual VFA's were only determined in alternate portions. The amounts of each fatty acid in the portions not analysed were estimated by averaging the proportions of each acid in the preceding and succeeding portions. The results of Table 33 are given in terms of both (m-mole/50ml.) and (m-mole/l.), the latter being used for conformity and to allow comparison with previous

TABLE 33. The VFA production, in experiments c and d, from rumen liquor(50ml.) incubated for 24hr. with grass fibre and cod liver oil. (0-24hr. samples).

Expt.	Sample	VFA Production(m-mole/50ml.)				VFA production(m-mole/l.)			
		A*	P**	B***	Total	A	P	B	Total
c	Blank	1.88	0.38	0.85	3.11	37.6	7.6	17.0	62.2
	Control	2.25	0.62	1.06	3.93	45.0	12.4	21.2	78.6
	Experimental	2.70	1.00	1.04	4.74	54.0	20.0	20.8	94.8
d	Blank	1.08	0.53	0.30	1.91	21.6	10.6	6.0	38.2
	Control	1.85	0.33	0.48	2.66	37.0	6.6	9.6	53.2
	Experimental	2.40	0.23	0.64	3.27	48.0	4.6	12.8	65.4

A* Acetate P** Propionate B*** Butyrate

Blank; rumen liquor only.

Control; rumen liquor + grass fibre(0.5g.).

Experimental; rumen liquor + grass fibre(0.5g.) + cod liver oil(1.5g.).

experiments. Total VFA production was lower in experiment d than in experiment c. In both experiments the highest total VFA formation occurred when lipid and grass fibre were substrates (see Figs. 6 & 7).

In experiment c more acetate and propionate but less butyrate was formed when lipid was incubated in rumen liquor than when only grass fibre was incubated. However, in experiment d more acetate and butyrate but less propionate was formed in the presence of lipid than when only grass fibre was added.

The results from experiments c and d have been recalculated (see Table 34) to allow for the decreases in VFA concentration within

TABLE 34. The corrected total VFA production(m-mole/50ml.), in experiments c and d, from rumen liquor(50ml.) incubated for 24hr. with grass fibre and cod liver oil.

	Experiment c			Experiment d		
	B*	C**	E***	B	C	E
Dialysate (0-24hours)	+ 3.11	+ 3.93	+ 4.74	+ 1.91	+ 2.66	+ 3.28
Remaining Clarified Rumen Liquor	+ 0.80	+ 0.38	+ 0.83	-	-	+ 0.11
Decreases in VFA concentration within the dialysis sac	- 1.38	- 1.57	- 1.59	- 0.63	- 0.46	- 0.50
TOTAL	2.53	2.74	3.98	1.28	2.20	2.67

B* Blank; rumen liquor only.

C** Control; rumen liquor + grass fibre(0.5g.).

E*** Experimental; rumen liquor + grass fibre(0.5g.) + cod liver oil(1.5g.).

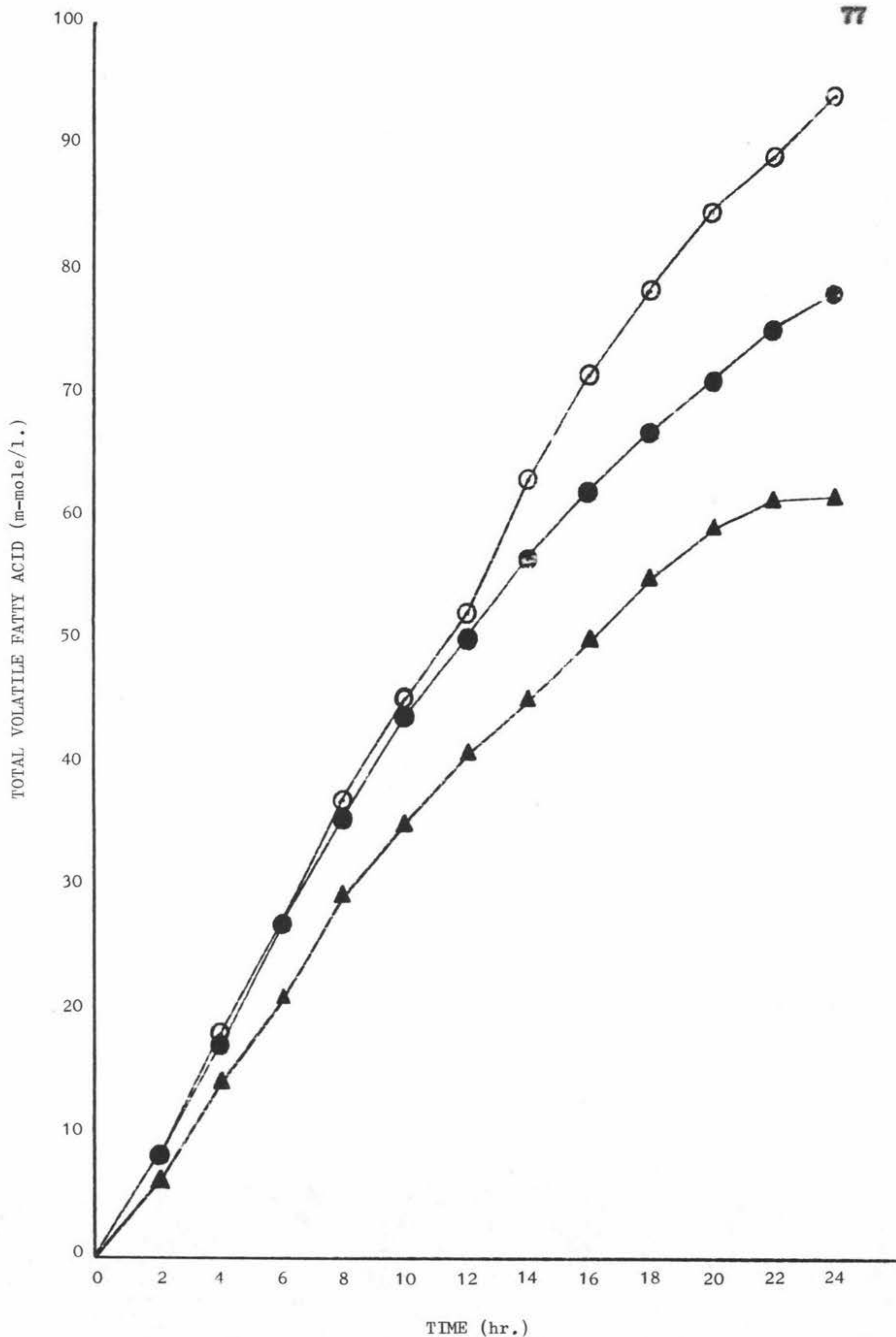


FIG. 6. Total VFA production(m-mole/l.) from rumen liquor(50ml.) incubated with (a) 0.5g. of grass fibre + 1.5g. of cod liver oil ○ ; (b) 0.5g. of grass fibre ● ; (c) or without additional substrates ▲ , for 24hr. within the semi-permeable apparatus. (Experiment c.)

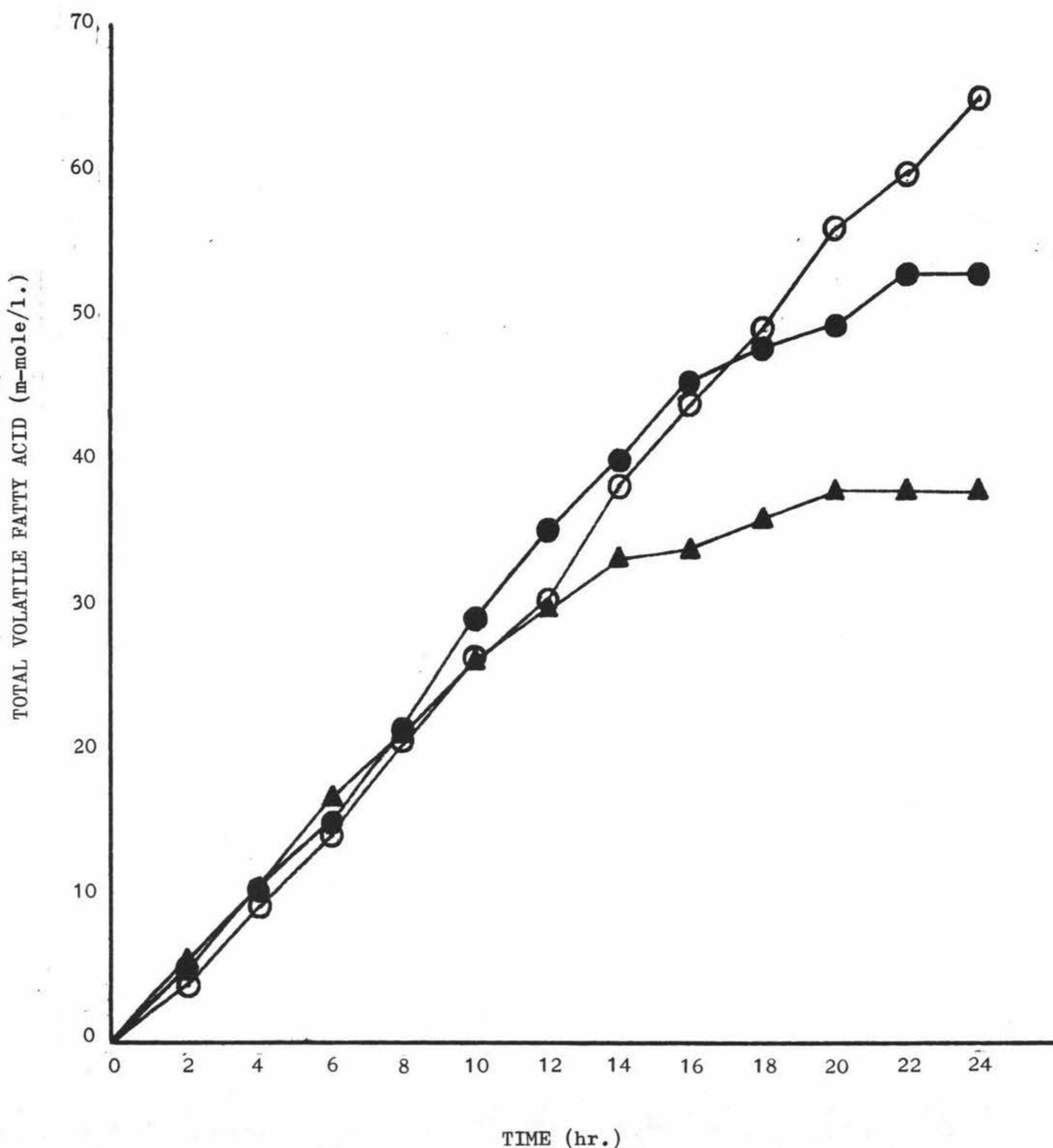


FIG. 7. Total VFA production(m-mole/l.) from rumen liquor(50ml.) incubated with (a) 0.5g. of grass fibre + 1.5g. of cod liver oil ○ ; (b) 0.5g. of grass fibre ● ; (c) or without additional substrates ▲ , for 24hr. within the semi-permeable apparatus. (Experiment d.)

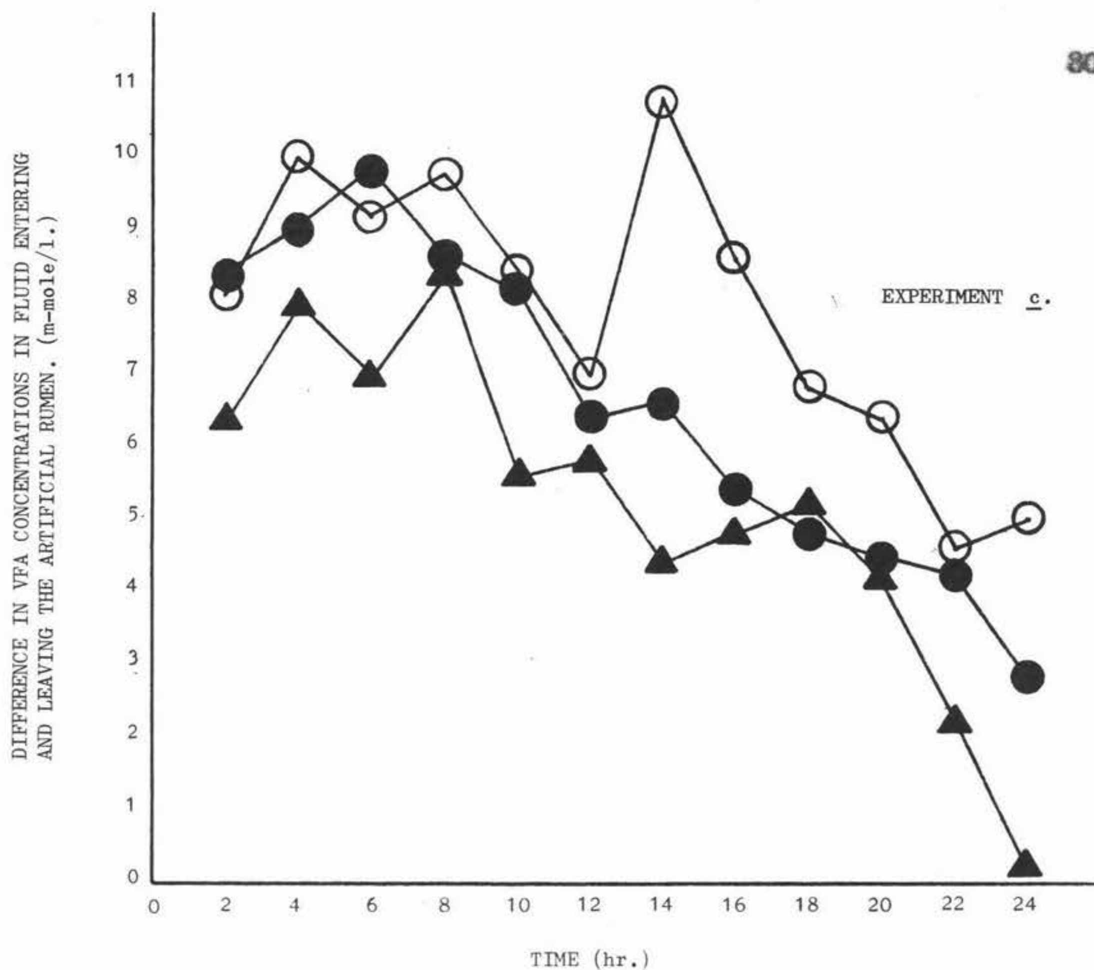
the dialysis sacs, when they occurred, and for the accumulated VFA in the clarified rumen liquor remaining in the apparatus at the conclusion of the incubation period. The corrections required were variable but no overall effect on the production of total VFA occurred, i.e., in both experiments the incubations containing added lipid still had the highest production of total VFA.

The rate of total and individual VFA production in experiments c and d are shown graphically in Figs. 8 and 9.

In experiment c the peak rate of total VFA production for each incubation appeared to be between 4 - 8 hr. (Fig. 8) with the incubation containing lipid continuing with a higher rate of total VFA production from 14 - 24hr. In experiment d the peak rate of total VFA production for the incubation containing only rumen liquor was at 6 hr. while the peak rates of total VFA production for the incubations containing grass fibre or grass fibre and lipid were at 10 and 14hr. respectively. Once again the lipid and grass fibre incubation continued with a higher rate of total VFA production from 14 - 24hr.

In both experiments c and d, the peak rate of acetate production was at 14hr. for the grass fibre and lipid incubations; 10hr. for the grass fibre incubations and 6 hr. for the incubations containing only rumen liquor. In experiment d the peak rate continued from 6-14hr. in the latter incubation (Fig. 9).

In experiments c and d the rate of propionate production appeared to slowly decrease for all the incubations until finally by 14hr. no propionate could be detected (Fig. 9). In experiment c there was a recovery after 14hr. with peak rates of propionate production for all



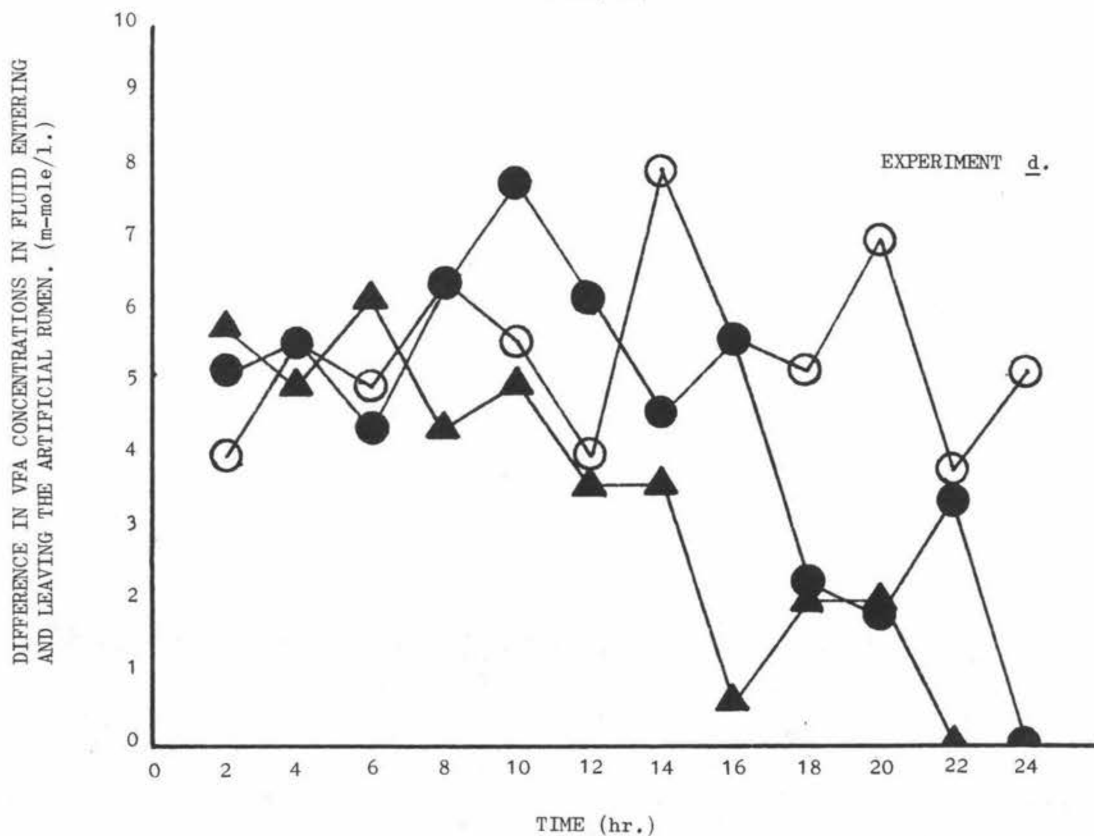


FIG. 8. Rate of total VFA production (m-mole/l./2hr.) within the semi-permeable apparatus containing 50ml. of rumen liquor incubated for 24hr. with (a) 0.5g. grass fibre + 1.5g. of cod liver oil ○ ; (b) 0.5g. of grass fibre ● ; or (c) without additional substrates ▲ .

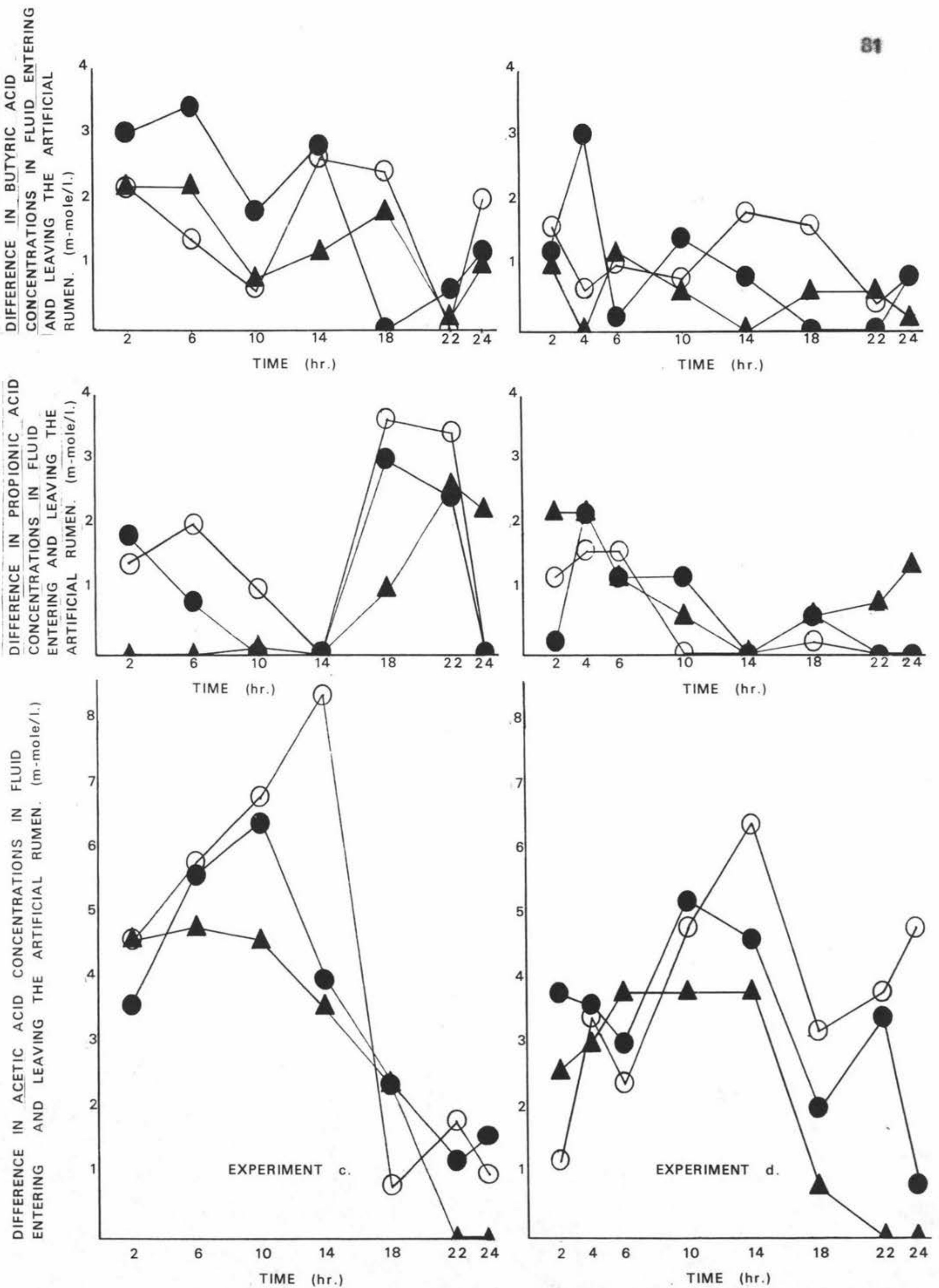


Fig. 9. Rate of production of individual VFA's (m-mole/l./2hr.) within the semi-permeable apparatus containing 50ml. of rumen liquor incubated for 24hr. with (a) 0.5g. of grass fibre + 1.5g. of cod liver oil ○; (b) 0.5g. of grass fibre ●; or (c) without additional substrates ▲.

three incubations occurring at 18 - 22hr. In experiment d, although some recovery in propionate production had occurred after 14hr., the peak rates of propionate production had taken place at 2 hr. for all incubations.

The rate of butyrate production was extremely variable between experiments and between incubations within each experiment (Fig. 9). In both experiments c and d, the incubations containing grass fibre had the highest rates of butyrate production at 6 hr. and 4 hr. respectively, while the peak rates of butyrate production for incubations containing lipid were highest at 14hr. All incubations showed some indication of a gradual decrease in the rates of butyrate production over the 24hr. period.

Without exception, the propionate production of the incubations containing lipid were highest at the same time as the lowest rates of acetate and butyrate production occurred. Conversely, when the rates of acetate and butyrate production were highest the rates of propionate production were among the lowest that occurred during the incubations.

No similar generalisations could be found for the incubations containing grass fibre or rumen liquor alone.

7. Use of ^{14}C-cellulose in rumen fermentations of lipid and grass fibre within the micro-artificial rumens

Grass fibre was supplemented with a small amount of ^{14}C-cellulose as a marker in order to determine accurately when the increased propionate formation, observed when lipid was added to rumen liquor incubations within semi-permeable apparatus (experiment 6), occurred. Approximately 80ml. portions of dialysate were collected every 2 hr. for analysis. The micro-artificial rumens were arranged experimentally as in Table 35.

TABLE 35. Incubation of ^{14}C-cellulose in the semi-permeable apparatus. Design of the experiment in which ^{14}C-cellulose (28 μc./mg.) was incubated in rumen liquor(100ml). for 24hr. with grass fibre(0.5g.) and 1.0 - 3.0g. of cod liver oil.

UNIT	Grass Fibre (g.)	Cod Liver Oil Emulsion (ml.)	^{14}C-cellulose (mg.)	Incubation Time(hr.)	Distilled H_2O(ml.)
Control	1	-	5.02	24	3.0
Experimental 1	1	2.0	5.00	"	2.0
Experimental 2	1	6.0	5.02	"	-

7.1 Total VFA production

No overall formation of VFA was detected when grass fibre, or grass fibre and 3.0g. of cod liver oil were incubated. In fact, decreases in VFA concentration of the rumen liquor within the dialysis sacs containing these substrates exceeded the increases in VFA concentration detected in the dialysate (Table 36). The most likely source of error is in the determinations of total VFA in the dialysate. Difficulties were encountered in obtaining acceptable repeatability when determining the total VFA of these samples. This difficulty was not encountered with whole rumen liquor.

TABLE 36. The production and decreases in total VFA (m-mole) of rumen liquor (100ml.) incubated for 24hr. in a dialysis sac with grass fibre (0.5g.), ^{14}C-cellulose, and cod liver oil (1.0 and 3.0g.).

UNIT	Production	Decreases
Control	4.85	5.60
Experimental 1	5.58	5.42
Experimental 2	5.00	5.70

7.2 Total and individual ^{14}C-VFA production

In this experiment, VFA production did occur because ^{14}C-VFA was formed (Fig. 10). ^{14}C-VFA production was at a peak for all incubation units 6 hr. after the commencement of the experiment. Total ^{14}C-VFA production was greatest with the low lipid level (1.0g.cod liver oil/100ml.) and least with the high lipid level (3.0g.cod liver oil/100ml.). Excluding the amount of ^{14}C-VFA produced at 16hr. (which was accidentally lost), the percentage incorporation of ^{14}C-cellulose into VFA's was 26.14% for the low lipid; 24.12% for the control; and 22.38% for the high lipid incubation. Compared with previous experiments of this study, in which ^{14}C-cellulose was used, these were low percentages of incorporation.

It was intended to determine the distribution of ^{14}C between the acetic, propionic and butyric acids by gas-liquid chromatography, but this was not successful because of extremely poor reproducibility due to adsorption of VFA's on to the columns and ghosting phenomena. An investigation of the extent to which adsorption and carryover of VFA's was occurring on the columns used was undertaken. ^{14}C-acetate of known radioactivity was added to a VFA sample and its gas-liquid chromatographic behaviour investigated.

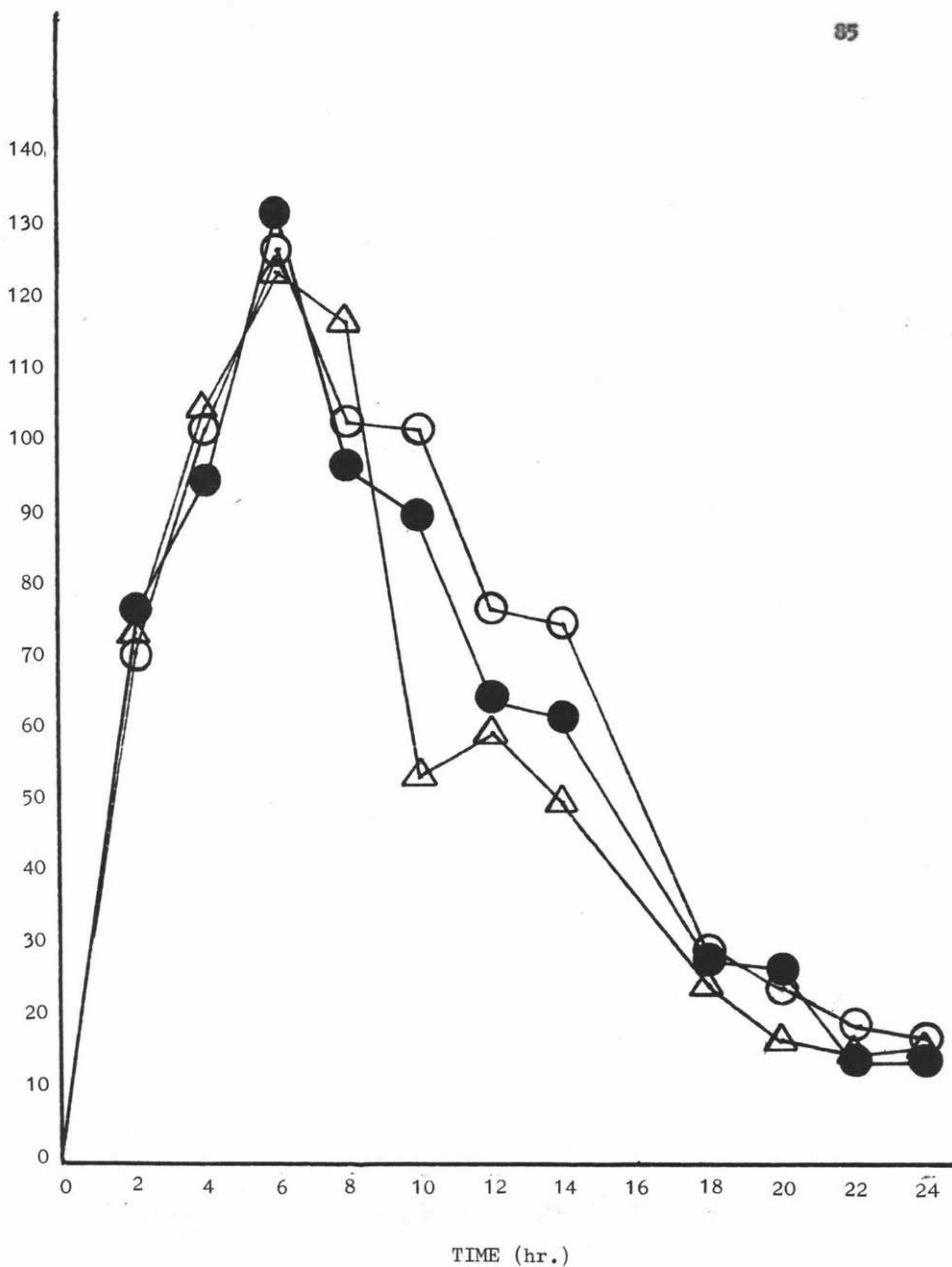


FIG. 10. The production of ^{14}C-VFA from ^{14}C-cellulose incubated with rumen liquor in the presence of (a) 0.5g. of grass fibre ● ; (b) 0.5g. of grass fibre + 1.0g. of cod liver oil ○ ; or (c) 0.5g. of grass fibre + 3.0g. of cod liver oil △ , for 24hr. within the semi-permeable apparatus.

7.3 Investigations into the adsorption and carryover of VFA's on gas-liquid chromatography columns

A 10 µl. aliquot of ^{14}C-VFA was injected and separated on a freshly packed 4' x ½" glass column (15% PEGA - Celite 545) and ^{14}C-free fatty acids collected by the titrimetric method. Two consecutive non-radioactive VFA samples were separated followed by another ^{14}C-VFA sample. Finally, two more non-radioactive samples were separated and collected for analyses. The results are presented in Table 37. Although the ^{14}C-acetate adsorbed on the column was subsequently displaced by successive VFA samples, this carryover of VFA's was low and relatively constant for both ^{14}C-VFA samples.

TABLE 37. Use of radioactive and non-radioactive acetate incorporated in VFA samples and separated on a gas-liquid chromatography column (15% PEGA - Celite 545).

Sample(10 µl.)	disintegrations/min.	% Carryover
Radioactive	13,172	
Non-radioactive	346	2.56
Non-radioactive	46	0.34
	13,564	
Radioactive	16,776	
Non-radioactive	453	2.63
Non-radioactive	104	0.60
	17,333	

A similar column (15% PEGA - Celite 545) which had separated approximately 200 radioactive VFA samples (mainly ^{14}C-acetate) was analysed for adsorbed radioactivity. The column, containing 9-10g. of solid support, was estimated to have adsorbed a total radioactivity of 2080 - 2310 disintegrations/min.

A large loss of the ^{14}C injected on to the column could not be accounted for. Only 44.22% and 56.32% of the ^{14}C was recovered, respectively, from the two ^{14}C-VFA samples separated. Even allowing for losses from acidification in the normal manner, a large amount of ^{14}C was still unaccounted for.

a 7' x ⅛" stainless steel column was freshly prepared (7% PEGA - Anakrom 50/60) and used for the separation of ^{14}C-VFA samples. The free VFA's were detected by a thermal conductivity detector and collected in collection tubes containing siliconised glass fibre moistened with toluene scintillation fluid

Two methods of injecting 1.0 - 2.0 µl. of VFA samples (radioactive and non-radioactive, alternately twice) were tried. One method involved taking up the required aliquot of the non-acidified VFA sample, then a small amount of air, followed by 1.0 µl. of 85% H_3PO_4. In the second method the VFA sample was acidified with HCl and the acidified mixture used for injection upon the column.

The percentage carryover (the proportion of the total radioactivity detected in the non-radioactive separations) is shown in Table 38.

The method of injection may have influenced the effect of carryover which increased from 28 - 37% when acidified VFA samples were used, or this increase may be an accumulative effect of the ^{14}C-samples injected on the column.

The adsorbed radioactivity of the 7% PEGA - Anakrom 50/60 column, used for the previous VFA separations, was 200 disintegrations/min.

TABLE 38. Use of radioactive and non-radioactive acetate incorporated in VFA samples and separated on a gas-liquid chromatography column (7% PEGA - Anakrom 50/60).

(a) Non-acidified VFA aliquots

Sample	disintegrations/min.	% Carryover
2.0 µl. radioactive sample	1,723	
1.0 µl. non-radioactive sample	669	27.97
1.0 µl. radioactive sample	1,409	
1.0 µl. non-radioactive sample	547	27.97

(b) Acidified VFA aliquots

Sample	disintegrations/min.	% Carryover
1.0 µl. radioactive sample	899	
1.5 µl. non-radioactive sample	527	36.96
1.0 µl. radioactive sample	940	
1.5 µl. non-radioactive sample	742	44.11

Only 7530 disintegrations/min. were recovered after an estimated 16,220 disintegrations/min. were injected on to the 7% PEGA - Anakrom 50/60 column. Only 42.55% of the ^{14}C was recovered when injecting the non-acidified VFA aliquots compared with a recovery of 53.1% when injecting the acidified VFA aliquots. However, the initial losses from acidification make both methods comparable. A 13.89% loss of ^{14}C occurred when the VFA sample was acidified before injection on to the column. Over a period of a week this increased to 18.20%.

The percentage carryover was higher on the 7% PEGA - Anakrom 50/60 column than that on the 15% PEGA - Celite 545 column. The low carryover occurring on the 15% PEGA - Celite 545 column was acceptable and the results from this column considered to be valid. The results obtained from the 7% PEGA - Anakrom 50/60 column were not satisfactory.

DISCUSSION

The results of the in-vitro experiments carried out in this study have indicated that the addition of lipid has a distinctive effect upon fermentation of cellulose and probably other insoluble carbohydrates of grass fibre by micro-organisms, an effect capable of influencing the formation of the VFA's and other end products of fermentation.

Effect of Lipid on the Production of Total and Individual VFA's

Preliminary investigations, in which the total and individual VFA productions from rumen liquor incubated with grass fibre were analysed to determine the effect of lipid, can be compared with similar experiments conducted by Robertson & Hawke (1964). The higher total VFA production that occurred at 48hr. in the presence of lipid (Table 4) did not agree with the results of Robertson & Hawke (1964) where lipid depressed total VFA production at 48hr., although at 12 and 24hr. the higher VFA produced agreed with their results. Robertson (1968) believed that the higher VFA production, which occurred when he administered lipid to cows, was probably due to an effect of lipid on carbohydrate digestion. Grass fibre was the major substrate added to the rumen liquor incubations of this present study.

When a number of different lipid levels were used(1,2 & 3.0g./100ml. rumen liquor), the production of total VFA was decreased (Table 7). This was not in agreement with the results of the preliminary experiments but it was interesting to note that the lowest level of lipid added (1.0g./100ml. rumen liquor) depressed the total VFA production to the greatest extent.

Robertson & Hawke (1964) found in both their experiments that when lipid was added there were decreases in acetate which were approximately balanced by increases in propionate. Only one of the preliminary experiments

agreed with the results of Robertson & Hawke while the other had no complementary decrease in acetate even although propionate production had increased. Nevertheless, lipid did increase propionate production substantially from a substrate normally recognised to be fermented to acetate, and in one of the preliminary experiments acetate production was decreased when lipid was added, giving adequate cause for further investigations to be conducted. When analysing the effects of various levels of lipid on the individual VFA's (Tables 8, 9 & 10) it was found that lipid significantly decreased the production of acetate and significantly increased the production of both propionate and butyrate. Increasing amounts of lipid further accentuated these effects. No increase occurred in total production when lipid was added (in fact a decrease occurred), yet a large decrease in acetate production to account for the decrease in total VFA production was considerably offset by increases in propionate and butyrate production.

A similar pattern of total and individual VFA productions emerged for experiments 3, 4, 5 & 6. When lipid was added in experiment 5 (Appendix 10a) total VFA production was variable between lipid levels; it was depressed in experiment 3 (Appendix 7i) and in the initial exploratory incubations of experiment 4 (Table 20); and increased in a second incubation of experiment 4 (Appendix 8a) and in experiment 6 (Table 33) where the increase in production was gained by higher rates of production during the latter stage of the incubations. Not all the changes in acetate, propionate and butyrate concentrations were statistically significant, but when lipid was added to experiments using impermeable apparatus, acetate was decreased and propionate and butyrate productions were increased.

The total and individual VFA productions at various times of incubation when various levels of lipid were added, revealed little more than expected, the rates of production did fall off with time, although the 72hr. incubations contained significantly higher total VFA and propionate productions than the 48hr. incubations.

Use of Micro-Artificial Rumens

Three micro-artificial rumens were used a number of times primarily to determine whether the addition of lipid alters the time at which peak production of total and individual VFA's occurs, compared with when VFA's are formed in incubations containing no lipid. The results (Tables 32 & 33) were extremely variable and inconsistent, the only truly valuable information obtained was that incubations containing lipid had the lowest rates of acetate and butyrate production at the same time as when the highest rates of propionate production occurred.

The total VFA production differed considerably between experiments 6 (a, b, c & d) and this was similar to observations by Lampila (1965) who mentioned the problem of differences in activity of rumen liquor samples collected from the same animal on the same regime but on different days. These differences in total VFA production led to some conflicting results and also led to two experiments being discarded. One experiment was discarded because the rumen liquor, although strained through muslin cloth prior to the incubation, as in former experiments, still contained fine fibrous material which overwhelmingly contributed to total VFA production, obliterating any possibility of detecting significant treatment differences. On the other hand, another experiment was discontinued when no measureable total VFA production could be detected until 12hr. of incubation.

Comparison of the Impermeable Apparatus and the Micro-Artificial Rumens

The use of impermeable apparatus for in-vitro experiments, especially for lengthy incubations (24, 36, 48 & 72hr.), has been criticised on the grounds that end products of fermentation could inhibit the rate of fermentation and influence the pathways of fermentation (Louw et al. 1949; and Dehority, 1963). It could be argued for example that the large amount of propionate formed when lipids were added was an artifact of the in-vitro system. Besides preventing the accumulation of end products, a further advantage of the micro-artificial rumen was that it allowed the determination of the times and rates at which end products were formed. Sampling at regular intervals was the greatest advantage of the micro-artificial rumen enabling the rate of production of any end product to be determined, but this advantage was offset by the difficulty in detecting the small increments in production. In an attempt to increase the amount of VFA to be detected, the rumen liquor was increased to 100ml., but this failed to procure the increases expected. The dialysis of VFA from the whole rumen liquor in the dialysis sac to the dialysate further confused the situation and correction had to be made for these losses (Table 34). Difficulty was encountered in determining the VFA of dialysate samples and this problem was further aggravated by the increased numbers of VFA determinations required when micro-artificial rumens were used.

Total VFA production was not always higher during a 24hr. period in the micro-artificial rumen than in the impermeable apparatus and the pH results (Table 12) suggest that microbial activity probably would not be inhibited to any great extent. A disadvantage of the micro-artificial rumen was its shorter period of incubation (24hr.) prescribed by the serious deterioration of the cellulosic membrane of the dialysis sac due to bacterial attack. The maximum VFA production, in-vitro, from cellulose does not occur until 36hr.

according to Marston (1948), and in this study substantial VFA production from grass fibre still occurred between 48 - 72hr. There was difficulty in completely preventing frothing and hence carryover of rumen fluid into the dialysate. This problem may have been due to the nature of the rumen contents used.

In future, provided frothing does not occur, the micro-artificial rumen possibly could be consistently successful if at least 200ml. of rumen liquor was incubated within the dialysis sac so that greater amounts of VFA are formed. The increases in the VFA concentrations of the dialysate must be substantial and easily detected if the results are to withstand the inherent experimental errors of the procedure of separating individual VFA's, and the subtraction of the individual VFA zero hour concentration from the VFA concentrations of each succeeding portion of dialysate. Possibly another way of solving this problem would be to decrease the flow rate of the dialysate. As experimental equipment the major disadvantage of the micro-artificial rumen was its complexity and the inability to perform large numbers of experiments with it, a criticism also mentioned by Johnson (1966).

The impermeable apparatus used was advantageous because of its simplicity and ease of duplication. Large numbers of incubations could be conducted at the same time, allowing duplication of all experiments.

Use of ^{14}C-cellulose in the Impermeable Apparatus

^{14}C-cellulose was used as a marker to establish the source of VFA's in rumen fermentations of lipid and grass fibre, and especially to discover if the increased propionate produced in the presence of lipid was being formed from the digestion of carbohydrate as suggested by Robertson (1968).

The addition of lipid did not significantly affect the incorporation of ^{14}C into total VFA (Table 21a). In so far as individual VFA's were concerned, ^{14}C-acetate production was significantly decreased and ^{14}C-propionate production was significantly increased when lipid was added (Tables 26 & 27). The results of this experiment clearly showed that since lipid had an effect on the individual VFA's formed from ^{14}C-cellulose, it most likely had similar effects on the cellulose and the other insoluble carbohydrates of the grass fibre. The choice and use of tobacco ^{14}C-cellulose as a marker appears to have been quite valid since lipid had a similar effect on the formation of individual VFA's from the ^{14}C-cellulose, as it did on the VFA's formed from grass fibre.

When Satter et al. (1964) added (U-^{14}C)cellulose and (U-^{14}C)hemicellulose to rumen ingesta from hay-fed and grain-hay mixture fed cows, the molar proportions of the ^{14}C-VFA's produced were calculated by dividing the total activity (disintegrations/min.) of each fraction collected from the liquid-liquid chromatography columns, by the number of carbons in the molecules. The improbability of all the carbons in butyrate and valerate being labelled was acknowledged. The divisor for propionate ranged from 2.54 - 2.62 depending upon the extent of propionate formation by way of the dicarboxylic acid pathway or the acrylate pathway. Only acetate, propionate and butyrate were determined when ^{14}C-cellulose was incubated in impermeable apparatus, and since the ^{14}C-cellulose was not uniformly labelled, the molar proportions of the ^{14}C-VFA's produced from ^{14}C-cellulose were derived directly from the total activity (disintegrations/min.).

Use of ^{14}C-cellulose in Micro-Artificial Rumens

A reliable way of determining the rates of absolute VFA production

and to eliminate the need for making corrections for the VFA which has dialysed from the dialysis sacs, was to use ^{14}C-cellulose as a marker. Measurements of the rates of production of the individual ^{14}C-VFA's would exclude any VFA from being measured other than that being produced. Unfortunately this experiment was not successfully completed because of the lack of a satisfactory technique to separate the individual VFA's. The titrimetric method of separation (James & Martin) as used in the other experiment in which ^{14}C-cellulose was added, would not be suitable when determining as many as 340 acid samples for each experiment containing three artificial rumen units. Because of the experimental error inherent and the experimental error possible during such a long undertaking, attention was given to separating ^{14}C-VFA's on gas-liquid chromatography columns (Varian Aerograph), and collecting free ^{14}C-VFA's in collection tubes packed with siliconised glass fibre moistened with toluene scintillation fluid. As mentioned in the results, this proved to be unsuccessful due to the carryover of ^{14}C-VFA's in succeeding separations. Many different liquid phases and various solid supports were prepared and used without success, but the two columns used to separate VFA samples containing ^{14}C-acetate (Tables 37 & 38), although being of different length and containing different solid supports, may have pointed out that adsorption was less on the columns coated with the most liquid phase. Wallnofer _et. al._ (1966) separated ^{14}C-VFA's successfully on a column containing Anachrome A 60/80 coated with 20% terephthalic acid carbowax 20M. This is a large percentage of liquid phase compared with recent recommendations of 5 - 7% (Hrionak & Pale, 1967). Youssef and Allen (1966) mentioned that a decrease in percentage of liquid phase (20 - 15%) eliminated column bleeding without affecting the resolution of the acids. Even if higher percentages of liquid phase decrease the amount

of adsorption, the effect of carryover must be completely eliminated when separating ^{14}C-VFA's. The faster separations and better resolutions obtainable from columns with 5 - 7% liquid phase will have to be compromised when wishing to decrease adsorption, hence carryover of free ^{14}C-VFA's.

Effect of Different Lipids on VFA Production

Robertson (1968) used linseed oil as a supplementary source of lipid in several of his experiments because of its similarity in fatty acid composition to that of the lipid of grasses and clovers. The effects of various lipids upon the rumen VFA proportions have been variable. Robertson & Hawke (1964) found that linseed oil added to in-vitro fermentations of rumen liquor and grass fibre increased propionate and decreased acetate production, whereas Nottle & Rook (1963) found linseed oil had no effect on the individual VFA proportions. When cod liver oil was added to rumen liquor it was found to decrease acetate and increase propionate production by Shaw & Ensor (1959); Nicholson et. al. (1963); and Nottle & Rook (1963), but Beitz and Davis, (1964) and Broster et. al. (1965) found that the proportions of the VFA's in the rumen were unaltered when cod liver oil was fed. Shaw & Ensor (1959) noted that when oils of differing fatty acid composition were added to rations for ruminants, alterations in VFA proportions could be expected to occur to a greater or lesser degree depending on the amount and degree of unsaturation of the lipid added. They found that the more highly unsaturated lipids had a greater effect on decreasing the molar proportions of rumen acetate and increasing the molar proportions of propionate. Table 39 contains the fatty acid composition (% weight) of the different oils used in this study

TABLE 39. Composition of Peanut, Linseed and Cod Liver Oil (% weight) (Hilditch, 1956).

		Peanut Oil	Linseed Oil	Cod Liver Oil
Saturated fatty acids	:	15 - 24	6 - 16	15
Unsaturated fatty acids	: Total	85 - 76	94 - 84	85
	Oleic	66 - 39	13 - 36	20
	Linoleic	19 - 37	10 - 25	29
	Linolenic	-	60 - 30	26
	$> C_{18}$	-	-	10

When linseed, peanut and cod liver oils were used, it was found that cod liver oil, in the presence of grass fibre as substrate, had by far the greatest effect of increasing propionate (Table 16) and butyrate (Table 17) production and decreasing acetate production (Table 15). Peanut oil also produced more butyrate and propionate and less acetate than linseed oil. This would seem to be at variance with the hypothesis of Shaw & Ensor (1959), since according to the figures given by Hilditch (1956) in Table 39, linseed oil (being the lipid with the greatest amount and degree of unsaturated fatty acids) would be expected to increase propionate and decrease acetate the most. Linseed oil actually depressed propionate production (Table 16).

When the three different lipids were added to rumen liquor, total VFA increased in the absence and decreased in the presence of grass fibre, yet propionate production was increased in both environments (Table 18). It is difficult to explain the increase in acetate and decrease in butyrate production when lipids were added to rumen liquor in the absence of grass fibre unless the interconversion of acetate to butyrate (Long & Brett, 1966) was inhibited.

Effect of Lipid on Ammonia Concentration of Rumen Liquor

A similarity between the results shown in Table 4 and those obtained by Robertson & Hawke (1964) was the effect of lipid in decreasing the NH_3 production at 48hr., although higher NH_3 production at 12hr. occurred in the presence of lipid in the preliminary experiments of this study.

Irrespective of the different amounts of lipid added, lipid significantly decreased NH_3 production (Table 11). This effect of lipid on NH_3 production was consistent with the results of the preliminary experiments, although, since there was a decrease in total VFA (Table 7) and therefore fermentation, the decrease in NH_3 production could possibly be expected. 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. It is possible that the grass fibre used in this study contained 3% or less nitrogen. However, this was not determined.

Endogenous fermentation accounted for a large proportion of the NH_3 produced in all the incubations. In fact, in the experiment containing various levels of lipid, the NH_3 production was generally higher from endogenous fermentation than in the incubations containing both lipid and grass fibre (Fig. 4). NH_3 production was fairly constant throughout the 72hr. incubations, the rate of NH_3 production decreasing only slowly with time.

Effect of Lipid on the pH of Rumen Liquor

The rumen liquor in the micro-artificial rumens did not always produce greater amounts of VFA over a 24hr. period than the incubations with the impermeable apparatus, and despite the accumulation of VFA's in the latter

apparatus the pH did not fall below 6.0, but instead, after initial falls, rose again. Although the primary purpose of determining the pH of the rumen liquor was to check the validity of the incubations in impermeable apparatus, the results obtained raised some other interesting points, including the increase in pH after the initial decrease in pH. The incubations containing only rumen liquor rose to a pH of 7.40 which probably can be accounted for by endogenous protein fermentation and possible degradation of microbial protein. The effect of lipid was to decrease the pH of the rumen liquor (Table 12) to a greater extent than incubations containing grass fibre alone, and to maintain this low pH for a longer period of time. Although less total VFA was produced, incubations containing lipid had a pH as low as 6.0, which increased only slightly after 36hr. This can be attributed to the depression in NH_3 production caused by lipid.

Use of (2-^{14}C)glycerol

Robertson (1968) suggested that some of the increased propionate formed, when lipid was added, might be derived from the glycerol moiety of the triglycerides since earlier work by Johns (1953); Hobson & Mann, (1961); and Garton, Lough & Vioque (1959) showed that propionate was the main VFA formed when glycerol was incubated in rumen liquor. However, Robertson calculated that 2.0g. of lipid would only account for an increase in the concentration of propionic acid of slightly less than 0.03m-mole/l., whereas the increase in propionic acid effected by lipid (8.5 and 11.3m-mole/l.) far exceeded this calculated value. Wright (1969), using (U-^{14}C)glycerol, found that only 20.6% of the ^{14}C was detected in propionate after a 12hr. incubation. Acetate and CO_2 accounted for 31.4% and 17% respectively. Butyrate, lactate and the micro-organisms themselves were other recipients

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of the ^{14}C, with only 13% of the ^{14}C unaccounted for.

When (2-^{14}C) glycerol was added as substrate in rumen liquor containing both grass fibre and added lipid, approximately 55% of the glycerol was incorporated into VFA's (Table 30), which is similar to that found by Garton, Lough & Vioque; Hobson & Mann; Johns; and Wright. The glycerol could have possibly been fermented to other end products (lactate, citric acid cycle compounds or be present in micro-organisms) but in this study these were not investigated.

Instead of propionate being the major fermentation product from (2-^{14}C)glycerol, most of the ^{14}C was incorporated into acetate with only small portions being incorporated into propionate and butyrate. There would appear to be no reason why glycerol should form propionate predominantly since it would initially form pyruvate via the glycolytic pathway before being converted into VFA's. The products of glycerol fermentations could vary with the nature of the microbial population and it is likely therefore that the dietary regime of the ruminant could have an important bearing on the amounts of each VFA formed. In work done by Johns (1953); Hobson & Mann (1961); and work performed at the Rowett Research Institute (1962), the glycerol was fermented _in-vitro_, while Garton, Lough & Vioque (1959) added glycerol to cows fed hay and concentrate rations, commonly a propionate producing environment. Wright (1969) used rumen liquor from ryegrass-clover fed cows to ferment (U-^{14}C)glycerol to acetate, CO_2, propionate, butyrate and lactate. The rumen liquor used in this present study was obtained from cows feeding on hay, and contained a high proportion of acetate (70%), hence acetate producing micro-organisms. Proportionally higher incorporations of (2-^{14}C)glycerol into acetate occurred in the incubations in which glycerol was the only substrate (Table 30). The addition of grass fibre led to a

greater incorporation of (2-^{14}C)glycerol into propionate and butyrate, while the addition of lipid increased even further the incorporation of (2-^{14}C)glycerol into propionate and butyrate and decreased acetate incorporation. Since these changes in the proportions of ^{14}C-VFA's parallel the changes in the proportions of VFA's brought about by grass fibre and lipid, it would appear that the incorporation of (2-^{14}C)glycerol into VFA's depends on the nature of the microbial population.

Conclusion

Results from this work have shown that the effect of lipid on total VFA production has been variable, although in most cases the change in total VFA production has not been very great. One consistent effect of lipid within the impermeable apparatus has been the increase in propionate and butyrate production and the decrease in acetate. Although acetate can be interconverted to butyrate, the large increases in propionate and the decreases in acetate can not be attributed to interconversion.

It may be hypothesised from the results of this study, that the effect that lipid has on the digestion of cellulose and probably other insoluble carbohydrates of the grass fibre, is a direct effect on the microbial environment of the rumen liquor, either on specific enzymes of the micro-organisms or directly on the activity and proliferation of the micro-organisms. It is generally agreed that three main types of bacteria in the rumen which degrade cellulose to simple sugars and subsequently to final products of fermentation are the cellulolytic cocci(*Ruminococcus flavifaciens*; *R. albus*), *Bacteroides succinogens*, and *Butyvibrio fibrisolvens*. The fermentation products of the cellulolytic cocci are acetate, lactate, succinate, formate, CO_2, and H_2. *Bacteroides succinogens*

forms succinate and acetate from cellulose, and since succinate is an important fermentation product of this specie, a high proportion of propionic acid is often formed. The third type, Butyvibrio fibrisolvens, forms lactate, formate and acetate but is primarily regarded as one of the chief butyrate producers in the rumen.

The microbial environment effected by lipid probably encourages lipolytic species capable of digesting cellulose, with propionate as the predominant end product. The environment created by the addition of lipid must suppress the activity of micro-organisms found normally in the presence of grass fibre, especially those responsible for producing acetate. The lower pH found in incubations containing lipid may be an essential factor of this environment. The environment also has the consistent effect of depressing the production of NH_3. The results of experiments, in which micro-artificial rumens were used, showed that the peak rate of propionate production, in the presence of lipid, occurred at a time when production of acetate and butyrate was low. This information lends further support to the hypothesis. The effect of the lipid on total VFA is dependent entirely upon the success at engendering this environment; the 1.0g. of added lipid/100ml. rumen liquor, which had the largest depressive effect on total VFA production, may have caused an effect detrimental to other micro-organisms, yet insufficient in quantity to completely procure an environment favourable for the lipolytic-propionate producing micro-organisms. It was suggested by Brooks et. al. (1954), after noticing the reduced cellulose digestion when corn oil was added to dry matter containing 50% cellulose, that the effect of added lipid was for it to coat the fibrous material, thus retarding the bacterial action in cellulose. Whether lipid coats the grass fibre to the extent that a detrimental effect on fermentation occurs, is difficult to

reconcile with the higher gas production and the higher total VFA production that sometimes occurred in the presence of lipid. If an environment favourable for the lipolytic-propionate producing micro-organisms is successfully obtained, the effect of lipid coating fibrous material would cause no detrimental effects on digestion, but rather enhance digestion. If this environment is not achieved, lipid would have the effect of inhibiting fermentation of fibrous material.

SUMMARY

1. When impermeable apparatus was used, the effect of lipid on the total VFA production of rumen liquor incubated with grass fibre was variable. Whether total VFA production was increased or decreased, the changes brought about by lipid were rarely significant.

2. Generally, the levels of lipid added (1.0, 2.0 or 3.0g./100ml. rumen liquor) significantly increased the propionate production and significantly decreased the acetate production of rumen liquor incubated with grass fibre, with increasing levels of lipid accentuating these effects. Butyrate production was increased when lipid was present but required the addition of lipid levels greater than 1.0g. for significant increases to occur. Although lipid levels less than 1.0g. usually increased propionate and butyrate and decreased acetate production, few of these increases or decreases were significant.

3. Results were variable from the experiments in which the micro-artificial rumens were used. However, except for experiment 6a, total VFA production was increased when lipid was added to rumen liquor containing grass fibre.

When lipid was added to the rumen liquor incubations containing grass fibre, the highest rates of propionate production occurred at the same time as the lowest rates of acetate and butyrate production. Conversely, when the rates of acetate and butyrate production were highest, the rates of propionate production were among the lowest that occurred during the incubations.

Problems of detecting the small increments of VFA produced, the frothing of the rumen liquor over into the dialysate, the dialysis of VFA's

from the rumen liquor to the dialysate, and the variation in the activity of the rumen liquor samples made results from the semi-permeable apparatus difficult to determine and compare.

4. Cod liver oil produced more propionate and butyrate and less acetate than when linseed and peanut oils were added to rumen liquor containing grass fibre. When lipids were incubated in rumen liquor alone, the effects upon total VFA, acetate and butyrate were opposite to the effects which occurred when the lipids were added to rumen liquor containing grass fibre.

5. ^{14}C-cellulose was used as a marker to establish the source of VFA's in rumen fermentation of lipid and grass fibre within impermeable apparatus.

Although the incorporation of ^{14}C from ^{14}C-cellulose into total VFA ranged from 38 - 65% in this experiment, the addition of grass fibre and lipid did not affect this incorporation of ^{14}C into total VFA.

The higher level of lipid used (2.0g/100ml. rumen liquor) was found to significantly decrease the production of ^{14}C-acetate and significantly increase the production of ^{14}C-propionate. Lipid had no significant effect on the production of ^{14}C-butyrate.

Since lipid had the above effects, on the individual VFA's formed from ^{14}C-cellulose, it most likely had similar effects on the cellulose and other insoluble carbohydrates of the grass fibre.

In this experiment only 0 - 2% of the ^{14}C from ^{14}C-cellulose was incorporated into CO_2. The addition of grass fibre and lipid did not affect this incorporation of ^{14}C into CO_2.

6. When ^{14}C-cellulose was added to rumen liquor containing grass fibre within the micro-artificial rumens, attempts at determining the relative

production of individual VFA's was unsuccessful because of the difficulty in separating the ^{14}C-VFA's on gas-liquid chromatography columns. This difficulty was probably caused by adsorption of ^{14}C-VFA's on the columns and their subsequent contamination of succeeding VFA samples. An investigation into the problem of adsorption was carried out.

7. When linseed oil was incubated with rumen liquor containing grass fibre, ammonia production was depressed. Often the ammonia production from rumen liquor incubated alone was greater than samples containing grass fibre and lipid.

8. Although the pH of all the rumen liquor fermentations were initially depressed at the commencement of incubation, the pH of rumen liquor was depressed to the greatest extent in the fermentations to which lipid had been added.

9. Approximately 55% of the $(2-^{14}C)$glycerol incubated in rumen liquor was incorporated into VFA's.

Acetate was the major fermentation product when $(2-^{14}C)$glycerol was incubated in rumen liquor and although propionate and butyrate were also produced, together they rarely exceeded one-third of the incorporation of glycerol into acetate.

The addition of grass fibre diversified the production of ^{14}C-VFA's, with increases in propionate and butyrate and decreases in acetate, while the addition of cod liver oil slightly accentuated the effects of grass fibre.

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APPENDICES

APPENDIX 1

(a) Effect of lipid levels and incubation times on the total volatile fatty acid production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	1.09	1.09	0.23	N.S.
Lipid Levels	3	49.70	16.57	3.46	*
Times	3	1895.36	631.79	131.90	**
Lipid Levels x Times	9	46.54	5.17	1.08	N.S.
Residual	15	71.85	4.79		
TOTAL	31	2064.54			

Control v Lipid	1	33.49	33.49	6.99	*
Between Lipid Levels	2	16.21	8.11	1.69	N.S.

D value required = 3.16

(b) Effect of grass fibre levels and incubation times on the total volatile fatty acid production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	175.91	175.91	2.24	N.S.
Fibre Levels	1	5070.86	5070.86	64.51	**
Times	3	864.25	288.08	3.66	N.S.
Fibre Levels x Times	3	204.46	68.15	0.87	N.S.
Residual	7	550.24	78.61		
TOTAL	15	6865.72			

APPENDIX 2

(a) Effect of lipid levels and incubation times on the acetate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	1.12	1.12	0.33	N.S.
Lipid Levels	3	757.70	252.57	74.50	* *
Times	3	328.21	109.40	32.27	* *
Lipid Levels x Times	9	38.62	4.29	1.27	N.S.
Residual	15	50.86	3.39		
TOTAL	31	1176.51			

Control v Lipid	1	556.32	556.32	164.11	* *
Between Lipid Levels	2	201.38	100.69	29.70	* *

D value required = 2.66

(b) Effect of grass fibre levels and incubation times on the acetate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	12.83	12.83	2.63	N.S.
Fibre Levels	1	2053.67	2053.67	420.83	* *
Times	3	198.44	66.15	13.56	* *
Fibre Levels x Times	3	12.77	4.26	0.87	N.S.
Residual	7	34.15	4.88		
TOTAL	15	2311.86			

APPENDIX 3

(a) Effect of lipid levels and incubation times on the propionate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.02	0.02	0.02	N.S.
Lipid Levels	3	498.64	166.21	131.91	* *
Times	3	491.10	163.70	129.92	* *
Lipid Levels x Times	9	44.69	4.97	3.95	* *
Residual	15	18.95	1.26		
TOTAL	31	1053.40			
Control v Lipid	1	232.35	232.35	184.40	* *
Between Lipid Levels	2	266.29	133.15	105.67	* *

D value required = 1.62

(b) Effect of grass fibre levels and incubation times on the propionate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	1.84	1.84	1.44	N.S.
Fibre Levels	1	329.15	329.15	257.15	* *
Times	3	46.34	15.45	12.07	* *
Fibre Levels x Times	3	14.19	4.73	3.70	N.S.
Residual	7	8.95	1.28		
TOTAL	15	400.47			

APPENDIX 4

(a) Effect of lipid levels and incubation times on the butyrate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.02	0.02	0.04	N.S.
Lipid Levels	3	9.25	3.08	5.92	* *
Times	3	11.79	3.93	7.56	* *
Lipid Levels x Times	9	3.39	0.38	0.73	N.S.
Residual	15	7.75	0.52		
TOTAL	31	32.20			

Control v Lipid	1	6.54	6.54	12.58	* *
Between Lipid Levels	2	2.71	1.36	2.62	N.S.

D value required = 1.04

(b) Effect of grass fibre levels and incubation times on the butyrate production (m-mole/l.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.49	0.49	3.27	N.S.
Fibre Levels	1	43.10	43.10	287.33	* *
Times	3	5.07	1.69	11.27	* *
Fibre Levels x Times	3	0.27	0.09	0.60	N.S.
Residual	7	1.06	0.15		
TOTAL	15	49.99			

APPENDIX 5

(a) Effect of lipid levels and incubation times on the ammonia production (mg.NH_3/100ml.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	57.02	57.02	2.04	N.S.
Lipid Levels	3	465.48	155.16	5.54	* *
Times	3	531.83	177.28	6.33	* *
Lipid Levels x Times	9	285.85	31.76	1.13	N.S.
Residual	15	420.08	28.01		
TOTAL	31	1760.26			

Control v Lipid	1	341.75	341.75	12.20	* *
Between Lipid Levels	2	123.73	61.87	2.21	N.S.

D Value required = 7.63

(b) Effect of grass fibre levels and incubation times on the ammonia production (mg.NH_3/100ml.) in Experiment 2.

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	64.47	64.47	2.07	N.S.
Fibre Levels	1	218.35	218.35	7.01	*
Times	3	438.05	146.02	4.69	*
Fibre Levels x Times	3	83.65	27.88	0.90	N.S.
Residual	7	217.90	31.13		
TOTAL	15	1022.42			

APPENDIX 6

Effect of grass fibre and time of incubation on the formation of total VFA, acetate, propionate and butyrate(m-mole/l.) and ammonia(mg.NH_3/100ml.) in Experiment 2.

(a) Total Volatile Fatty Acid.(m-mole/l.) (Mean values)

Fibre Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Fibre Means
0	59.50	66.20	67.75	71.25	66.17
1	90.05	100.15	104.90	109.65	101.19

(b) Acetate Production.(m-mole/l.) (Mean values)

Fibre Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Fibre Means
0	43.30	46.10	48.31	50.72	47.11
1	62.87	69.63	72.02	74.55	69.77

(c) Propionate Production.(m-mole/l.) (Mean values)

Fibre Added(g./100ml.)	TIME OF INCUBATION (hr.) 24	36	48	72	Added Fibre Means
0	11.04	13.49	12.92	13.56	12.75
1	18.36	20.75	23.05	25.19	21.83

(d) Butyrate Production.(m-mole/l.) (Mean values)

Fibre Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Fibre Means
0	5.17	6.61	6.52	6.97	6.32
1	8.83	9.82	9.84	9.91	9.60

(e) Ammonia Production.(mg.NH_3/100ml.) (Mean values)

Fibre Added(g./100ml.)	TIME OF INCUBATION(hr.) 24	36	48	72	Added Fibre Means
0	23.00	26.78	29.03	32.01	27.71
1	26.19	31.62	37.02	41.02	33.96

APPENDIX 7

The effect of linseed oil, cod liver oil and peanut oil on total and individual volatile fatty acid production(m-mole/l.) in Experiment 3.

(i) Total Volatile Fatty Acid Production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.02	0.02	0.086	N.S.
Lipids	3	4.45	1.48	6.34	10%
Residual	3	0.70	0.2333		
TOTAL	7	5.17			

(10%)LSD value required for lipid means = 1.14

(ii) Acetate Production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.04	0.04	0.044	N.S.
Lipids	3	46.47	15.49	17.15	*
Residual	3	2.71	0.9033		
TOTAL	7	49.22			

(5%)LSD value required for lipid means = 3.02

(iii) Propionate Production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.31	0.31	0.66	N.S.
Lipids	3	14.01	4.67	9.94	*
Residual	3	1.42	0.47		
TOTAL	7	15.74			

(5%)LSD value required for lipid means = 2.18

(iv) Butyrate Production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.27	0.27	0.46	N.S.
Lipids	3	9.49	3.16	5.38	10%
Residual	3	1.76	0.587		
TOTAL	7	11.52			

(5%)LSD value required for lipid means = 1.80

APPENDIX 8

The effect of various amounts of cod liver oil on the net production of total and individual volatile fatty acids(m-mole) in Experiment 4.

(a) Net Production of total VFA

(i) Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	28.75	28.75	1.59	N.S.
Lipid Levels	7	111.40	15.91	0.88	N.S.
Residual	7	126.20	18.03		
TOTAL	15	266.35			

(ii) Table of Results

Lipid Added(g./100ml.)	Duplicates		Added Lipid Means
	D_1	D_2	
-	55.10	49.85	52.48
0.2	60.30	57.60	58.95
0.3	52.90	56.70	54.80
0.4	58.20	65.70	61.95
0.5	60.60	50.70	55.65
1.0	57.60	57.10	57.35
1.5	59.50	53.40	56.45
2.0	61.30	53.00	57.15
Duplicate Means	58.19	55.51	$\bar{X}$ = 56.85

S.E. per fermentation bottle = ±4.25 m-mole

(b) Net Production of Acetate

(i) Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	11.96	11.96	1.20	N.S.
Lipid Levels	7	153.51	21.93	2.19	N.S.
Residual	7	70.00	10.00		
TOTAL	15	235.47			

(ii) Table of Results

Lipid Added(g./100ml.)	Duplicates		Added Lipid Means
	D_1	D_2	
-	38.31	34.25	36.28
0.2	39.29	34.95	37.12
0.3	32.30	35.38	33.84
0.4	33.75	40.31	37.03
0.5	36.90	31.21	34.06
1.0	31.20	31.17	31.18
1.5	33.35	28.66	31.00
2.0	30.22	25.56	27.89
Duplicate Means	34.42	32.69	$\bar{X}$ = 33.55

S.E. per fermentation bottle = ±3.16 m-mole

(c) Net Production of Propionate

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	3.28	3.28	1.6	N.S.
Lipid Levels	7	77.30	11.04	5.39	*
Residual	7	14.33	2.05		
TOTAL	15	88.35			

Control v Lipid Levels	1	38.51	38.51	18.79	* *
Between Lipid Levels	6	38.79	6.47	3.16	N.S.

D value required for lipid level means = 5.87

(d) Net Production of Butyrate

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.01	0.01	0.008	N.S.
Lipid Levels	7	40.17	5.74	4.56	*
Residual	7	8.81	1.26		
TOTAL	15	48.99			

Control v Lipid Levels	1	20.57	20.57	16.33	* *
Between Lipid Levels	6	19.60	3.27	2.60	N.S.

D value required for lipid level means = 4.60

APPENDIX 9

The effect of various amounts of cod liver oil on the distribution of ^{14}C (incorporated into VFA from ^{14}C-cellulose) among individual volatile fatty acids (percentages transformed to angles) in Experiment 4.

(a) ^{14}C-acetate production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.03	0.03	0.02	N.S.
Lipid Levels	7	34.29	4.90	3.53	10%
Residual	7	9.74	1.39		
TOTAL	15	44.06			
Control v Lipid	1	15.74	15.74	11.32	*
Between Lipid Levels	6	18.55	3.09	2.22	N.S.

(10%) LSD value for lipid level means = 2.24

(b) ^{14}C-propionate production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.33	0.33	0.52	N.S.
Lipid Levels	7	16.06	2.29	3.58	10%
Residual	7	4.50	0.64		
TOTAL	15	20.89			
Control v Lipid	1	4.66	4.66	7.28	*
Between Lipid Levels	6	11.40	1.90	2.97	10%

(10%) LSD value for lipid level means = 1.52

(c) ^{14}C-butyrate production

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.91	0.91	0.85	N.S.
Lipid Levels	7	13.54	1.93	1.80	N.S.
Residual	7	7.51	1.07		
TOTAL	15	21.96			

APPENDIX 10

The effect of various amounts of cod liver oil on the net production of total and individual volatile fatty acids(m-mole) in Experiment 5.

(a) Net production of total VFA

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	14.85	14.85	1.80	N.S.
Lipid Levels	3	13.12	4.37	0.53	N.S.
Residual	3	24.73	8.24		
TOTAL	7	52.70			

(b) Net production of Acetate

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	0.36	0.36	0.05	N.S.
Lipid Levels	3	32.86	10.95	1.66	N.S.
Residual	3	19.75	6.58		
TOTAL	7	52.97			

(c) Net production of Propionate

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	3.56	3.56	44.50	* *
Lipid Levels	3	14.24	4.75	59.38	* *
Residual	3	0.25	0.08		
TOTAL	7	18.05			

Control v Lipid Levels	1	12.41	12.41	115.13	* *
Between Lipid Levels	2	1.83	0.92	11.50	*

D value required for the lipid level means = 1.37

(d) Net production of Butyrate

Analysis of Variance

Source of Variance	d.f.	S.S.	M.S.	F	Result
Duplicates	1	1.88	1.88	6.71	N.S.
Lipid Levels	3	4.82	1.61	5.75	N.S.
Residual	3	0.85	0.28		
TOTAL	7	7.55			