THE ROLE OF PLANT LIPASES IN THE BOVINE RUMEN

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ABSTRACT

The role of plant lipases in the hydrolysis of dietary lipids in the rumen of pasture-fed ruminants has been investigated by means of *in vitro* and *in vivo* experiments with rumen contents. Lipases present in the leaves of numerous pasture plants remained highly active for at least 5h in the presence of actively metabolising rumen microorganisms, leading to the hydrolysis of triglyceride. Parallel experiments showed that the lipase activity of actively metabolising rumen microorganisms in rumen fluid was very low. A slight increase in lipase activity attributable to microorganisms occurred after about a 4h incubation with autoclaved plant extract at which stage the metabolic activity of the microbial population had passed its peak.

In vivo experiments showed that the lipolytic activity of rumen fluid obtained 0.5h to 5.0h after feeding fresh grass was about twice that of rumen fluid obtained after overnight fasting.

Lipase activity was present in clarified rumen fluid. Paired-feeding experiments with monozygotic twin cows demonstrated that the lipase activity of clarified rumen fluid prepared from the twin 0.5h after feeding fresh pasture was much higher than that of clarified rumen fluid from the 20h-fasted twin.

Further paired-feeding experiments showed that lipase activity was higher in protozoa-free rumen fluid from the pasture-fed than from the hay-fed cow. In both treatments the highest levels of lipolytic activity were observed in the rumen samples removed 0.5h after feeding and there was a steady decline in activity over the sampling periods.

Nevertheless, there was appreciable activity in the rumen samples obtained from the hay-fed animal which is consistent with the presence of lipase activity in the extracts of dried grass.

Multiple forms of plant esterases and phosphatases were present in the soluble fraction of rumen fluid several hours after feeding.

It is concluded that the rapid release of fatty acids which follows the ingestion of pasture is due mainly to the activity of plant lipases and that rumen microorganisms have a subsidiary role in hydrolysing ingested lipid in a pasture-fed ruminant.

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INTRODUCTION

1.1 Lipid metabolism in ruminants and hydrogenation of unsaturated fatty acids in the rumen

The digestive pattern of ruminants differs from that of many other mammals since the food of ruminants is subjected to a microbial fermentation in the rumen before passing into the true stomach. Carbohydrates, proteins, organic acids and many other food constituents are attacked by the microorganisms in the rumen and as a result short-chain fatty acids (VFA's), CO_2, CH_4 and NH_3 are produced as the end products of microbial metabolism. This ruminal fermentation has a considerable effect on the metabolic processes of the animal and the functions of the rumen microorganisms are intimately associated with certain metabolic disorders of the ruminant (Bryant, 1959) such as ketosis and bloat. It is now generally believed that the organisms of functional significance in the rumen are protozoa and bacteria which are capable of growth under the anaerobic conditions prevailing. The rumen provides an ideal anaerobic environment for a large and diverse microbial population at a temperature of 39°-40°C. The pH of the ingesta is slightly acid and the bacteria are adapted to live between pH 5.5 and 7.0 (Hungate, 1966).

It is apparent from earlier studies that the lipids of ruminants differ in several respects from those of non-ruminant herbivorous animals. The occurrence of unusually high proportions of stearic acid, the presence of *trans* acids and isomeric forms of oleic, linoleic and linolenic acid in depot fats and tissue lipids is peculiar to ruminants. Linolenic acid, the predominant fatty acid constituent of pasture

lipids is almost absent in the depot fats of ox and sheep in contrast to non-ruminants which contain a high proportion of linolenic acid in their fats (Shorland, 1952). These differences in the composition of lipids from ruminant and non-ruminant animals may be explained by the fact that dietary lipids undergo extensive changes in the rumen. These changes include the hydrolytic release of esterified fatty acids (section 1.4) (Garton et al., 1958, 1959 and 1961; Wright, 1961) and the hydrogenation of unsaturated fatty acids (Reiser, 1951). Reiser incubated linseed oil with rumen contents and found that the linolenic acid content of the incubation mixture decreased from approximately 30% to 5% and the level of linoleic acid content increased proportionately. This was attributed to the hydrogenation of linolenic acid by rumen bacteria. In subsequent studies, Willey et al. (1952) observed that the depot fat of steers fed cottonseed oil contained more stearic acid and less oleic acid than that from steers which received no oil. The authors suggested that hydrogenation of fatty acids occurred in the rumen, the resulting saturated acids being subsequently absorbed and deposited. Because of the high content of C_{18} unsaturated fatty acids in most common feeds of ruminants, the microbial activity results in the accumulation of free stearic acid as the end product of complete hydrogenation and geometrical and positional isomers of oleic, linoleic and linolenic acids as the end products of incomplete hydrogenation (Shorland et al., 1955 and 1957). A particularly effective hydrogenation in the rumen was observed by Shorland et al. (1955). They found that 50% of pasture linolenic acid was converted into stearic acid. The presence of trans acids in ruminant depot fats (Hartman et al., 1954) was explained on the basis of bacterial action in the rumen. Incubation of linolenic, linoleic and oleic acids under CO_2 with sheep

rumen contents resulted in the production of stearic acid as well as *trans* and positional isomers of unsaturated acids (Shorland *et al.*, 1957).

It has been shown that the development of an active rumen is essential for the process of hydrogenation of unsaturated fatty acids. Depot fats of foetal lambs do not contain such a high proportion of stearic acid as the maternal ewe (Body and Shorland, 1964). The young animals were also found to incorporate dietary unsaturated fatty acids into their depot fats (Sirén, 1962). Ogilvie *et al.* (1961) studied the effect of duodenal administration of linseed oil on the composition of ruminal depot fats and observed that the main fatty constituents of the oil (linoleic and linolenic acids) are capable of being deposited in the depot fats. Normally those fatty acids make up 1 - 2% of the depot fat. Similarly a distinct increase in the proportion of stearic acid was observed in both milk and adipose tissue fat when soybean oil was fed orally to cows, but on intravenous injection of emulsified cottonseed oil, the proportion of poly-unsaturated acids in the milk fat was increased (Tove and Mochrie, 1963).

The low concentration of poly-unsaturated fatty acids in ruminant milk fat and adipose tissue fats is primarily due to biohydrogenation of dietary C_{18} di- and tri- unsaturated fatty acids to more saturated forms by the microorganisms in the rumen. However, there are instances in which it has been shown that the hydrogenating activity in a functional rumen may be inhibited by a high concentration of poly-unsaturated fatty acid. Higher levels of unsaturated fatty acids in milk fat were observed when monozygotic twin milking cows were grazed on young ryegrass (Hawke, 1963) which contained more lipid and more

unsaturated fatty acids than mature ryegrass (McDowall et al., 1961). This increase in unsaturated lipids in the milk fat was related to the higher levels of unsaturated acids in the diet and the consequence of a decrease in the overall hydrogenation in the rumen. On incubation of linoleic acid with rumen contents, the products of hydrogenation by the microorganisms were mainly oleic acid with a small concentration of stearic acid. This was explained on the basis that a high concentration of linoleic acid inhibited the conversion of oleic acid to stearic acid (Moore et al., 1969). Recently it has been shown that lipolysis and hydrogenation in the rumen can be controlled by protecting the lipids from the action of lipases and hydrogenases (Scott et al., 1970). When a formaldehyde treated poly-unsaturated lipid-protein complex was added to the diet of ruminants the proportion of poly-unsaturated acids in the plasma increased from approximately 4% to 25-30% within 24h and led to an increased incorporation of these acids into glycerides of milk and body fats.

Among other aspects of lipid metabolism in the rumen, microbial synthesis of long-chain fatty acids and incorporation of long-chain fatty acids into other lipids have been briefly reviewed below.

Rumen microorganisms are capable of synthesising long-chain fatty acids from short-chain ones. Following 3.5h incubation of ^{14}C-acetate with mixed rumen bacteria, it was observed that ^{14}C was mainly incorporated into monoenoic fatty acids, although a comparatively smaller proportion of radioactivity was detected in dienoic and saturated fatty acids (Viviani and Borgatti, 1967). The authors also demonstrated that rumen protozoa could incorporate ^{14}C-acetate into

long-chain fatty acids in the following order: 18:3, 18:2 and 18:1; the incorporation of ^{14}C-acetate was least into saturated fatty acids.

Long-chain fatty acids released from lipids due to lipolysis are not degraded by rumen microorganisms (Garton et al., 1961). This was supported by Wood et al. (1963) who introduced linoleic acid-1-^{14}C into the rumen of a sheep after the reticulum-omasal orifice had been bound. Of the total dose added, only 0.6 - 1.0% was degraded in the rumen. Furthermore, the authors found very limited evidence for the absorption of long-chain fatty acids from this part of the alimentary tract. But the utilization of long-chain fatty acids by rumen microorganisms for lipid synthesis has been reported recently. About 20% of added ^{14}C-oleic acid was incorporated into phospholipids by a rumen micrococcus (Mills et al., 1970). Following incubation of ^{14}C-linolenic acid with total rumen contents and mixed rumen bacteria, a total of 16-30% of the radioactivity appeared in the sterol ester, triglyceride and polar lipid fractions (Hawke, 1971).

1.2 Relationship between hydrolysis and hydrogenation in the rumen

The two main types of enzymatic reactions which dietary lipid undergoes in the rumen are:

(i) release of the constituent fatty acids (Garton et al., 1958)

(ii) hydrogenation of unsaturated fatty acids (Reiser, 1951)

which are the main fatty acid components of plant lipids (Weenink, 1961). Comparisons of the degree of saturation of the free fatty acids (FFA's) and the esterified lipids of rumen contents suggest that biohydrogenation of the fatty acids does not occur until they are hydrolysed from dietary glycerolipid (Garton et al., 1961; Hawke and Robertson, 1964;

Patton and Kesler, 1967). The requirement for FFA substrates in rumen biohydrogenation was further confirmed by Hawke and Silcock (1969). In more precise studies on the relationship between lipolysis and hydrogenation, a synthetic triglyceride, 2-(1-^{14}C) linolenoyl - 3 - oleoyl - 1 - palmitoylglycerol, was incubated with rumen contents (Hawke and Silcock, 1970). The authors found no detectable hydrogenation products in the triglyceride fraction which remained unhydrolysed and in the partial products of hydrolysis whereas hydrogenation of (1-^{14}C)-linolenic acid occurred in the liberated FFA fraction producing a mixture of ^{14}C- stearic, monoenoic and dienoic acids. From this evidence it was concluded that biohydrogenation of unsaturated fatty acids of dietary lipids in the rumen proceeds only after the fatty acids have been removed from ester combination by lipolysis. Consequently, the extent of hydrogenation of unsaturated fatty acids is dependent on the lipolytic activity of the rumen contents.

1.3 Lipids and related compounds of pasture plants

The food of grazing ruminants is mainly pasture species such as ryegrass and clover and to a lesser extent the leaves of many other plant species. The lipid content of various grass leaves is about 5-9% of the dry matter (Shorland, 1961; Weenink, 1961; Hawke, 1963). Galactolipid in the form of mono- and digalactosyldiglyceride (MGDG and DGDG) accounts for about 60% of the total lipid. The predominant fatty acid component of the galactolipid is linolenic acid (Shorland, 1961; Weenink, 1961; Hawke, 1963). Other lipid constituents of leaf tissue include phospholipids (Weenink, 1964) and sulpholipids (Weenink, 1963) with minor amounts of sterol esters, triglycerides, diglycerides, free sterols, hydrocarbons (Weenink, 1962), wax esters, FFA's, free

fatty alcohols and carotenoids (Body, 1974).

Poly-unsaturated fatty acids, particularly linolenic acid, comprise the major fatty acid content of hay (Ward et al., 1964), artificially dried grass (Garton, 1960) and legume grass silage (Ward and Allen, 1957). All of these feeds had essentially similar fatty acid profiles to those of fresh pasture (Shorland et al., 1955) thus showing that little loss of linolenic acid occurred by antoxidation during the process of drying or ensilaging.

Although the lipid content of leaf tissue is low, the quantity of lipid consumed by adult ruminants is quite significant e.g., a cow eating 9Kg ryegrass on a dry-weight basis will ingest approximately 720g of lipids (Hawke, 1963) and during a period of pregnancy, lactation or stall-feeding it may receive a diet which provides 1Kg of lipids daily (Garton, 1967).

1.4 Investigations on lipolysis in the rumen

That rumen contents can bring about the hydrolysis of triglycerides was first reported by Garton et al. (1958). Following 24h incubation of three naturally occurring triglycerides with sheep rumen contents obtained 3-4h after feeding hay and concentrates, Garton et al. (1961) observed 95, 68 and 40% hydrolysis of the esterified fatty acid residues in linseed oil, olive oil and cocoa butter respectively. Under similar conditions, Allen et al. (1959) have reported between 18-48% hydrolysis of various triglycerides by rumen fluid. Similar variations were found by Wright (1961) who reported 13, 15, 21 and 49% hydrolysis of linseed oil, olive oil, monostearin and tributyrin

respectively. It appears that triglycerides of low molecular weight are more rapidly hydrolysed than those of high molecular weight.

Since no lipase activity was detected in adult sheep saliva or in rumen contents free from microorganisms it was concluded that microorganisms were responsible for lipolysis in the rumen (Garton et al., 1961). Fractionation of rumen contents by differential centrifugation indicated that lipolytic activity was mainly associated with bacteria. The site of lipolysis has been suggested to be within the bacteria or at the point of contact between bacteria and lipid particles (Garton et al., 1961). However, experiments based on fractionating rumen contents demonstrated that lipases were primarily associated with protozoa (Hill et al., 1960). Considerable reduction in lipase activity was observed when penicillin or terramycin were added to rumen fluid, but neomycin and streptomycin had little effect (Wright, 1961). On the basis of the inhibition of lipase activity by antibiotics Wright suggested that bacteria, rather than other microorganisms, were responsible for rumen lipolysis. But reports on the inhibition of lipases in rumen fluid by different antibiotics remains unclear. Hill et al. (1960) found that penicillin, erythromycin and tylosin depressed lipolysis in rumen fluid but Shellenberger (1964) demonstrated that these antibiotics had no effect on the lipolytic activity of rumen fluid. On the other hand, it has been reported that antibiotics also inhibit lipases of other sources apart from their inhibition on lipase producing microorganisms. For example, pancreatic lipase activity is inhibited by aureomycin (Rokos et al., 1958). In the presence of Ca^{++} (Rokos et al., 1958) and Co^{++}, Zn^{++}, Mn^{++}, Ca^{++}, Mg^{++} or Ba^{++} (Rokos et al., 1959) chlorotetracycline inhibited pancreatic lipase

activity and the enzyme inhibition was assumed to be due to the formation of an enzymatically inactive complex of enzyme, metal and antibiotic (Rokos et al., 1959). The activity of phospholipase C is also reported to be strongly inhibited by antibiotics such as bleomycin and polymixin B sulphate (Saito et al., 1972).

Hobson and Mann (1961) reported the isolation of bacteria from the rumen of sheep on diets supplemented with linseed oil, which were capable of hydrolysing linseed oil and tributyrin. These bacteria were anaerobic, Gram-negative, vibrio-shaped organisms. Morphologically they were similar to many types of rumen bacteria, but differed from all known species in their limited fermentation reactions. Subsequent studies have been carried out with a similar strain (Hobson and Summers, 1966). This strain has been reported to produce a lipase mainly secreted into the culture medium and an esterase which is mainly associated with the cells. The organism has been designated Anaerovibrio lipolytica and shown to produce an extracellular lipase during exponential growth in batch culture. No lipase activity was found to be associated with the bacteria or with fragmented bacteria (Henderson, 1971). This lipase was active towards some triglycerides containing short- and long-chain fatty acids but inactive towards galactolipid (Henderson, 1968) and tributyrin (Henderson, 1971) although total rumen contents and extracts of rumen bacterial preparation hydrolyse these substrates (Wright, 1961; Bailey, 1962). Furthermore, bacteria capable of hydrolysing tributyrin have been shown to be abundant in the rumen of pasture-fed cattle (Faruque et al., 1974). Among the three strains of tributyrin-hydrolysing bacteria isolated from the rumen, none hydrolysed triglyceride containing long-chain

fatty acids, but one strain was shown to be capable of deacylating MGDG. These results suggest that the rumen contains strains of bacteria which are relatively specific with respect to the molecular weight or types of lipid which they can hydrolyse.

1.5 Evidence for the involvement of plant lipases in rumen lipolysis

1.5.1 Effect of diet on the lipase activity of rumen contents

Rumen fluid from animals fed on alfalfa pasture showed several times more lipase activity than rumen fluid from animals on dry feeds (Hill et al., 1960). In a similar study it has been shown that the degree of hydrolysis of linseed oil by rumen contents from an animal fed fresh clover was approximately twice that of an animal fed on clover hay (Wright, 1961); but the author could not decide whether this was an animal or dietary difference. It seems possible that there may be a relationship between the lipase activity of rumen contents and the diets of the ruminants.

1.5.2 Effect of homogenisation of rumen contents on the lipase activity

It has been reported that the 500g supernatant of homogenised total rumen contents contained much more lipase activity than that of the unhomogenised rumen contents (Clarke and Hawke, 1970). The authors suggested that lipolytic bacteria were associated with plant particulate material and homogenisation dislodged them i.e. transferred some of the activity into the 500g supernatant. In subsequent studies (Faruque et al., 1974), it was found that homogenisation of rumen fluid obtained from a pasture-fed cow resulted in an overall increase in the lipolytic activity of rumen fluid. These observations indicate a possible role

of plant lipases. Apart from the release of lipolytic bacteria into the 500g supernatant as previously suggested by Clarke and Hawke (1970), alternative possibilities are that homogenisation fragments the digesta so that greater centrifugal forces are required for sedimentation or that plant lipases released from the digesta retain at least part of their activity under rumen conditions.

1.5.3 Lipase activity of cell-free enzyme preparations from rumen contents

Preliminary attempts to obtain a cell-free lipase preparation from rumen microorganisms were not successful (Garton et al., 1961). However, a soluble enzyme preparation from mixed rumen microorganisms which hydrolysed lysolecithin with the production of glycerylphosphoryl-choline was prepared by Dawson (1959). This enzyme preparation showed greater activity at high pH values (7.5-9.5) in sodium-glycine buffer. In subsequent studies, it was shown that cell-free extracts prepared from rumen contents were active in the release of FFA's from tributyrin (Wright, 1961), glyceryltri(oleate-1-^{14}C) (^{14}C-triolein) (Clarke and Hawke, 1970) and triolein and tributyrin (Faruque et al., 1974). The soluble enzyme preparation exhibited pH optima of 8.0-8.5 and 7.0 for the hydrolysis of triolein and tributyrin respectively. At their respective pH optima, hydrolysis of tributyrin was about 30 times faster than triolein (Faruque et al., 1974).

To obtain cell-free extracts, mixed rumen bacteria were prepared from the rumen contents obtained from animals 0.5h (Wright, 1961) and 3h (Clarke and Hawke, 1970; Faruque et al., 1974) after feeding fresh pasture. Under these circumstances, rumen contents contain significant

amounts of particulate plant materials. The larger fragments were removed by low speed centrifugation. Further high speed centrifugation of the supernatant resulted in the sedimentation of bacteria and plant particles of similar density. Sonication, osmotic shock or other standard techniques used for breaking up the cells probably resulted in the release of enzymes both from bacterial cells and from contaminating plant particles. Therefore, cell-free extracts prepared from rumen contents most probably contain enzymes of plant and bacterial origin.

1.6 Plant lipolytic and other hydrolytic enzymes

1.6.1 Lipases

The report of the commission on enzymes of the International Union of Biochemistry (1961) defines lipase (E.C.3.1.1.3) as a "glycerol ester hydrolase" and the use of emulsified substrates is recommended because they are active in heterogeneous systems (Sarda and Desnuelle, 1958) whereas esterases seem to hydrolyse substrates in solution (Aldridge, 1954). This distinction between lipases and esterases does not imply a different catalytic mechanism between the two groups of enzymes (Oosterbaan and Jansz, 1965). The above commission further defines a unit of lipase as being that amount of enzyme which, acting on an ester emulsion under the conditions of the test, liberates 1 micro-equivalent of acid per min. The emulsion should be of such nature that it gives the maximum reaction rate.

Lipases are wide spread in plants, animals and microorganisms (Wills, 1965). Fewer studies have been made on plant lipases than those of the animal origin. Pancreatic lipase has been the most extensively studied and our knowledge of the machanism of lipase action

is almost entirely derived from studies of this enzyme.

Few studies have been made on the distribution of lipases in plant leaf tissues although their presence has been reported in various seeds and fruits e.g. cotton (Olcott and Fontaine, 1941), tung kernels (Johnston and Sell, 1944), millet (Féron and Bouquet, 1948), lucerne, tomato and orange (MacDonnell et al., 1950), coconuts (Sadasivan, 1951), walnuts (Musco and Cruess, 1954), rice bran (Kawai, 1954), lettuce (Rimon, 1957), potato, bean and cabbage (Schwartz et al., 1964), wheat germ, malt, soya beans, castor beans and oat (Oosterbaan and Jansz, 1965).

Of all seed lipases, only castor bean lipase has been investigated in detail with regard to its mechanism of action. The enzyme exhibited an optimum pH 4.3 (Ory et al., 1962) but the optimum pH of the lipases from the same tissue during germination was previously reported to be 6.8 (Yamada, 1957). In this connection, an acid (pH 5.2) and a neutral (pH 7.1) lipase have been found in Douglas Fir seeds and during germination of the seeds, the activities of both types of lipase increased sevenfold and fourfold respectively (Ching, 1968). The germinating cotton seed lipase showed a pH optimum 7 to 8 (Olcott and Fontaine, 1941). Lipases from many other seeds (e.g., wheat, buckwheat, oats and corn) were most active between pH 7.0 and 8.0 (Wills, 1965).

Hydrolysis of coconut oil was more rapid than animal fats by castor bean lipase (Ahmad and Sareen, 1946). The slow hydrolysis of animal fats could be attributed to the inaccessibility of the substrate

for the enzyme. Recently it has been shown that triglycerides containing saturated fatty acids with chain length between C_4 and C_8 are hydrolysed most rapidly (Ory et al., 1962) although castor oil, its natural endogenous substrate is also hydrolysed rapidly (Ory et al., 1960). Like pancreatic lipase (Savary and Desnuelle, 1956), castor bean lipases hydrolyse primarily the ester bonds in 1- and 3- positions of synthetic triglycerides and the fatty acids from the 2- positions appear in the reaction products only at a much later stage (Ory et al., 1969). These findings on the positional specificity of castor bean lipases contradict the earlier observations of Savary et al.,(1958) who showed that castor bean lipases were nonspecific for positions in the triglyceride.

1.6.2 Galactolipases

A number of highly active lipolytic enzymes are released from leaf tissues on masceration and homogenisation which rapidly degrade the normal cellular lipids into their constituent parts. These include galactolipases, phospholipases and sulpholipases. These lipolytic enzymes are so active that the principal leaf lipids cannot be detected in water homogenates and chloroplast preparations from leaves although a high concentration of lipids is present in intact leaves (Sastry and Kates, 1963, 1964a, b). The released enzymes from the disrupted cells begin to act on the appropriate substrates as soon as the cells are broken.

Galactolipid-hydrolysing enzymes have been found to be present in a wide range of Phaseolus species (Sastry and Kates, 1964b; McCarty and Jagendorf, 1965; Helmsing, 1969), spinach leaves (Sastry and Kates,

1964b; McCarty and Jagendorf, 1965; Helmsing, 1967; Wintermans et al., 1969) and potato tubers (Galliard, 1970, 1971a, b).

Attempts have been made by investigators to purify galactolipases from runner-bean leaves (Sastry and Kates, 1964b). In an attempt to investigate the distribution of enzymes, various leaf cell fractions were incubated with MGDG and DGDG at pH 7.0 and it was observed that most of the activity was associated with the broken-chloroplast fraction and to a lesser extent with the cell-sap cytoplasm, relatively little activity was associated with the microsome pellet. The $(NH_4)_2SO_4$ precipitate from the soluble fraction of leaf extract exhibited pH optima of 7.0 and 5.6 for the hydrolysis of MGDG and DGDG respectively. At these pH optima, the enzyme activity was greater toward DGDG than toward MGDG. The enzyme activity toward DGDG decreased markedly on storage at $4^{\circ}C$ for several days, but the activity decreased only slightly toward MGDG. The enzyme preparation was active only towards natural unsaturated galactolipids since no significant hydrolysis of saturated galactolipids occurred. This was attributed to the inaccessibility of the substrate for the enzyme.

During hydrolysis of galactolipids by runner-bean galactolipases, mono- and digalactosyl glycerols were accumulated but no evidence for the formation of lysogalactolipids was obtained (Sastry and Kates, 1964b; Helmsing, 1969). This indicates that both fatty acid residues are released from the galactolipid with equal rapidity.

Strong reductants such as sodium dithionite and sodium metabisulphite stimulated galactolipase activity, possibly by reducing

endogenous quinones which would normally inhibit the enzyme (Helmsing, 1969).

1.6.3 Phospholipases

Direct evidence for the presence of phospholipase A in plant tissues is sparse. However, extracts from the green alga Scenedesmus obliquus hydrolysed plant phospholipids with the production of corresponding lysophosphatides (Yagi and Benson, 1962), and phospholipase B activity has been reported in rice grains (Contardi and Ercoli, 1933), barley (Acker and Bücking, 1957) and at the cell surface of Chlorella ellipsoidea (Miyachi et al., 1965). Since no lysophospholipids were accumulated during hydrolysis of phospholipid by potato tuber phospholipase, it has been suggested that the enzyme involved was phospholipase B type or a combined activity of phospholipases A and B (Galliard, 1970).

Phospholipase D catalyses the release of choline, ethanolamine, serine and glycerol from their respective phosphoglycerides with the formation of phosphatidic acid. This enzyme has not been detected in animal or bacterial material so far, but has been found only in plant tissues and in all the leaf tissues examined (Kates, 1970). During isolation of chloroplasts from leaf tissues, the enzyme activity is very high. Within a very short period it causes a substantial degradation of endogenous lecithin and phosphatidylethanolamine with the formation of phosphatidic acid (Sastry and Kates, 1964b). The enzyme has been found to be associated with plastid preparations from plant tissues such as spinach, sugar beet, cabbage leaves and carrot root (Kates, 1954). In addition, it was isolated in soluble form from the leaves of cabbage, carrot, cauliflower, celery and lettuce (Davidson and Long, 1958;

Quarles and Dawson, 1969). The plastid-associated enzyme was relatively stable between 25 and 40°C but completely inactivated after 10min at 70°C (Kates, 1954). The soluble enzyme was found to be more stable in the crude state (Davidson and Long, 1958). Like galactolipases, phospholipase D hydrolyses highly unsaturated phospholipids at a much greater rate than phospholipids containing saturated fatty acids (Kates, 1956).

Diethyl ether was found to be a potent stimulator of the enzyme; the activity was enhanced by other solvents such as n-propyl ketone and butyl acetate as well (Kates, 1970). Presence of these solvents possibly gives a better dispersion of the substrate for the enzyme action but at the same time do not denature the enzyme.

1.6.4 Sulpholipases

Sulpholipids are degraded to lysosulpholipid and then completely deacylated to sulphoquinovosylglycerol by sulpholipases. These enzymes are widespread in plants and are released on disruption of leaf tissues. Deacylation of sulpholipid was catalysed by Scenedesmus extracts, Chlorella extract, alfalfa leaf and root extracts, corn-root extract and by emulsin preparations from almonds (Yagi and Benson, 1962). Absence of sulpholipid in many plant lipid preparation (e.g. pelleted alfalfa) is attributed to the enzymatic degradation of the compound during isolation procedures (Benson, 1963).

1.6.5 Galactosidases

Both α-and β- galactosidases are widely distributed in higher plants (Wallenfels and Malhotra, 1961). During the hydrolysis of

mono- and digalactolipids by galactolipases, mono- and digalactosylglycerols accumulate. α- and β- galactosidases which have been isolated from runner-bean leaves further degrade these galactosylglycerols to free galactose and glycerol (Sastry and Kates, 1964b). α-galactosidases have been purified from germinating seeds (Dey and Pridham, 1969) and from Pisum sativum (Barham et al., 1971). Both α- and β-galactosidases have been purified from germinating seeds (Agrawal and Bahl, 1968) and from spinach leaves (Gatt and Baker, 1970). The purified β-galactosidase hydrolysed galactose from MGDG at a slower rate than the p-nitrophenyl substrates (Gatt and Baker, 1970).

1.6.6 Esterases

Occurrence of esterases in higher plants has been reported by many investigators. It was shown that esterases isolated from alfalfa, tomato and orange catalysed the hydrolysis of a large number of simple esters. The extent of hydrolysis was greater at pH 6.8 than at 4.5 (MacDonnell et al., 1950). Complex systems of ester hydrolases were found to be present in wheat seeds, cucumber, soybean and corn seedings (Jooste and Moreland, 1963); these enzymes were partially and tentatively identified by applying criteria established for animal serum enzymes. Purified galactosidase preparations have been shown to possess esterase activity e.g., α-galactosidase preparation from spinach leaves variably hydrolysed p-nitrophenyl esters of butyrate, caprylate, caproate, laurate and palmitate. In addition, the enzyme preparation showed phosphatase and sulphatase activities by hydrolysing p-nitrophenyl phosphate and p-nitrophenyl sulphate respectively. 1000-fold purified β- galactosidase preparation hydrolysed p-nitrophenyl butyrate and p-nitrophenyl sulphate only (Gatt and Baker, 1970).

Electrophoretic techniques have been applied to the study of esterases in higher plants. Enzyme extracts prepared from various plants showed a multiplicity of esterases active against 1-naphthyl acetate and the enzyme pattern differed in different plant species, in different strains of the same species and even in different parts of the same plant (Schwartz et al., 1964). These authors made a survey of the esterase activity of a number of plants belonging to the Cucurbitaceae using indophenylacetate as the substrate. Multiple forms of esterases were demonstrated by electrophoresis in polyacrylamide gel. Bean leaves contained 9 and 7 esterase bands active towards 1-naphthylacetate and 1-naphthylbutyrate respectively (Rudolph and Stahmann, 1966).

In view of these numerous reports of high lipase activity in disrupted leaves, the existence of a wide variety of lipases in plants and the rapid release of FFA's in the rumen following ingestion of herbage containing lipids (Hawke and Robertson, 1964) it is surprising that attempts have not been made to establish whether or not plant lipases play a role in the rumen metabolism of lipids.

1.7 Release of plant protein in the rumen following mastication and during rumination

A large proportion of the plant cells in green feed are effected by the grinding and crushing action of the ruminants' jaws; in some cases, complete rupture of the cell wall takes place and as a result the entire cell constituents are released. The rate of release of the cell constituents depends on the condition of the leaf tissue and the duration and vigour of chewing by the animal. Cells from fresh grass are ruptured more readily than those of flaccid leaves. The proportion

of soluble leaf protein and chloroplastic material released by ruminants eating red clover has been estimated to be 55-65% and 26-28% respectively (Reid et al., 1962). One of the most distinctive features of ruminants is that they rechew their food or chew the cud. A series of acts such as regurgitation, remastication, reinsalivation and deglutition occur during this process (Bell, 1961). Initial mastication of plant material during feeding is not thorough, but rechewing during rumination, when the jaws of the animal operate with a lateral grinding motion, masticates food particles more thoroughly (Hungate, 1966). Downie (1954) and Gordon (1958) observed 56.4 and 78 chews per bolus respectively. The actual amount of chewing depends on the individual animal and the nature of the feed. Digesta containing coarse food material is rechewed more than that from less resistant food (Downie, 1954; Gordon, 1958). Almost immediately after swallowing the rechewed bolus, another bolus is regurgitated (Hungate, 1966). Rumination normally continues for a period of about 8h per day (Hughes and Reid, 1951).

The process of eating and rumination provide an efficient natural mechanism for breaking down plant cells and releasing their enzymes and substrates. It is probable that the lipolytic enzymes released actively hydrolyse their respective substrates in the same way as they would do in leaf extracts prepared in the laboratory.

1.8 Proteolytic activity of rumen microorganisms and factors which affect the resistance of dietary proteins to fermentative digestion in the rumen

The presence of active proteases in rumen contents, considered to be of microbial origin, was first demonstrated by Sym (1938). The author followed the disappearance of casein (0.5% $^{W}/_{V}$) during *in vitro*

incubation with rumen contents and found that within 3h, 44% of the casein was no longer acid precipitable. In subsequent studies other workers (Pearson and Smith, 1943; McDonald, 1952; Annison, 1956; McDonald and Hall, 1957; Blackburn and Hobson, 1960a) confirmed and extended Sym's findings. Blackburn and Hobson (1960a) showed that proteolytic activity was associated with a wide range of rumen micro-organisms including protozoa and small and large bacteria. Little extracellular proteolytic enzyme was found in the rumen.

The solubility of proteins under the rumen conditions has been considered to be the main factor which influences the rate of digestion of protein within the rumen. Most of the evidence for this was obtained by relating high concentrations of NH_3 in the rumen of animals fed diets containing soluble protein (Chalmers et al., 1954; Chalmers and Synge, 1954; el-Shazly, 1958). However, the disappearance of nitrogen in the form of soluble protein and the appearance of peptide and α-amino nitrogen in the rumen were also measured (Annison, 1956; Blackburn and Hobson, 1960b, c). In vitro studies of Annison (1956) have demonstrated that casein, arachin and soya bean protein are extensively degraded by rumen microorganisms whereas bovine albumin, wheat glutin and zein are only slightly attacked. Subsequent in vitro studies have shown that the rate of digestion of proteins by rumen bacteria varies widely and is correlated with the solubility of proteins in salt solutions (Henderickx and Martin, 1963).

Many proteins which are not included in the natural diets of the ruminants have been extensively used in vitro or in feeding experiments to assess the proteolytic activity of rumen microorganisms. Rather

less work has been carried out on the extent of degradation of the proteins of plant origin in the rumen. However, recent work has shown that a good portion of the ruminants natural dietary protein is resistant to fermentative digestion in the rumen. Hogan and Weston (1970) and Coelho da Silva et al. (1972) have demonstrated that considerable amounts of dietary protein escape microbial fermentation in the rumen when sheep are fed on dried herbage of high nitrogen content. More recently, Ulyatt et al. (1974) studied the extent of microbial digestion of protein from fresh pastures in the rumen of sheep. These authors found that 46.5, 33.4 and 37.4% of protein entering the duodenum of sheep fed on perennial ryegrass, short-rotation ryegrass and white clover were dietary protein which had escaped degradation in the rumen. It took about 3-4h for the movement of the digesta from the rumen into the duodenum (Ulyatt, personal communication). In contrast to the extent of protein degradation in the rumen, Ulyatt and MacRae (1974) found that approximately 93% of water-soluble sugars, 85% digestible hemicellulose and 90% of digestible cellulose were digested in the rumen. Further evidence regarding the extent of ruminal digestion of protein and carbohydrate comes from Hogan and Weston (1969) who observed that the protein component of the diet was less digestible in the rumen than the carbohydrate component.

1.9 The aim of the present study

The modifications of dietary lipids in the rumen are of interest in so far as these influence the composition of depot and milk fat of ruminants. These modifications include hydrolytic release of esterified fatty acids from the dietary lipids and hydrogenation of unsaturated fatty acids. It has been firmly established that biohydro-

genation in the rumen takes place only after the constituent fatty acids of dietary lipids are released by lipolysis.

Although lipolytic activity in the rumen has been known for many years, a thorough investigation of the sources of these lipolytic enzymes has not been carried out. Despite the numerous reports of high lipase activity in disrupted leaves, the existence of a wide variety of lipases in plants and the rapid release of FFA's in the rumen following ingestion of herbage containing lipids, attempts have not been made to establish whether or not plant lipases play a role in rumen lipolysis. The work reported in the present study was undertaken in an attempt to measure the stability and activity of lipases of pasture species under the conditions which are encountered in the rumen and to assess the relative contribution which plant and microbial lipases make to lipolysis in the rumen.

CHAPTER 2

MATERIALS, ANALYTICAL TECHNIQUES AND EXPERIMENTAL PROCEDURES

2.1 Solvents and reagents

All solvents used were purified by distillation and reagents were of Analar or Reagent grade unless otherwise mentioned. Glyceryl tri (oleate - 1 - ^{14}C) (34.1 mc/mmol) and $^{14}CH_3COONa$ (56 mc/mmol) were obtained from the Radiochemical Centre, Amersham, England. Glyceryl tri (oleate - 1 - ^{14}C) was purified by preparative thin-layer chromatography. ^{14}C-labelled lipids which were extracted from spinach leaves after the plant had been grown in $^{14}CO_2$ for 24h, was kindly supplied by Dr. B.A. Tapper of the D.S.I.R., Palmerston North. ^{14}C - MGDG was purified from this crude extract by preparative thin-layer chromatography.

2.2 Thin-layer chromatography

2.2.1 Preparation of chromatographic plates

Silica gel G (E. Merck AG, Darmstadt, Germany) was mixed with water (1:2, $\frac{W}{V}$) to give a slurry which was spread at a thickness of 0.25 - 0.5 mm on glass plates (5 x 20 cm and 10 x 20 cm) using a commercial spreader. After setting at room temperature, the layers were activated in an oven at 110°C for about 1h. No special conditions were used for the storage of the prepared plates.

2.2.2 Development of chromatograms; identification and extraction of lipids

The samples dissolved in hexane or $CHCl_3$ were applied to the chromatographic layer about 1.5 cm from the edge of the plate. The chromatograms were developed in tanks lined with filter paper to assist

equilibriation of solvents, air-dried, sprayed with 0.1% ($\frac{W}{V}$) solution of 2',7'-dichlorofluorescein in MeOH and then viewed under UV-light. Lipid components were identified by comparison with known compounds chromatographed simultaneously. In preparative procedures, the required lipid components were obtained by scraping the appropriate zones into centrifuge tubes and extracting with diethyl ether or $CHCl_3$-MeOH (2:1 and 1:2, $\frac{V}{V}$) by shaking on a vortex mixer followed by centrifugation and decantation. The solvents were evaporated to dryness in a stream of N_2.

2.2.3 Elution solvents

Of the solvents used to develop the chromatograms, hexane (B.P. 66-70°C) and diethyl ether were dried over anhydrous sodium sulphate before use.

Compounds separated	Solvent mixture
Long-chain fatty acids, mono-, di- and triglycerides; long-chain fatty acids from ^{14}C-MGDG	Hexane-diethyl ether - glacial acetic acid (70:30:1, $\frac{V}{V}$)
Purification of ^{14}C-triolein	Hexane-diethyl ether (70:30, $\frac{V}{V}$)
Isolation of ^{14}C-MGDG from ^{14}C-lipid extract	Toluene-ethyl acetate- 95% ethanol (2:1:1, $\frac{V}{V}$)

2.3 Radioisotope counting

Aliquots of radioactive samples in hexane, diethyl ether or $CHCl_3$-MeOH were evaporated to dryness under N_2 in counting vials or radioactive components from thin-layer plates were scraped into counting

vials (sub-section 2.12.4), to which 10ml scintillation fluid (toluene containing 2,5 diphenyloxazole (0.6%) and 1,4-bis-2 (5-phenyloxazolyl) - benzene (0.05%)) was added. The radioactivity was measured in a tri-carb liquid scintillation spectrometer (Packard, Model 3375). All lipid samples counted fell in the Automatic External Standardisation (AES) range of 0.28-0.9. A curve of AES against counting efficiency, as constructed by counting a measured weight of ^{14}C-hexadecane in the presence of increasing amounts of quenching agent, has shown that AES and efficiency are equivalent in the AES range of 0.2-0.65 (Bycroft, 1972). Radioactivity measurements of all lipid samples which fell in this AES range were corrected for quenching by dividing the counts per minute by the AES value. Lipid samples which fell above this AES range were also corrected for quenching as above since at this stage the difference between the AES values obtained for different lipid components was small.

2.4 Gas-liquid Chromatography

Individual VFA's were estimated on a Packard gas chromatograph. Aqueous solutions (usually 5 - 20 µl) of an incubation mixture or rumen fluid containing VFA's were injected directly into a coiled glass column (200cm x 0.4cm) packed with 20% ($\frac{W}{W}$) FFAP (carbowax 20M - 2 nitroterephthalic acid complex, Varian Aerograph) on chromosorb W (60 - 70 mesh). The column was operated at 136°C. N_2 (40 ml/min) was bubbled through HCOOH before passing to the column (Ackman and Burgher, 1963). Typical gas-liquid chromatographic charts demonstrating the separation of VFA's following the above mentioned conditions are shown in Fig. 1. The proportions of each VFA by weight was obtained from planimetric measurements of peak areas and comparing these with those obtained after chromatographing standardised (by weight) VFA mixtures.

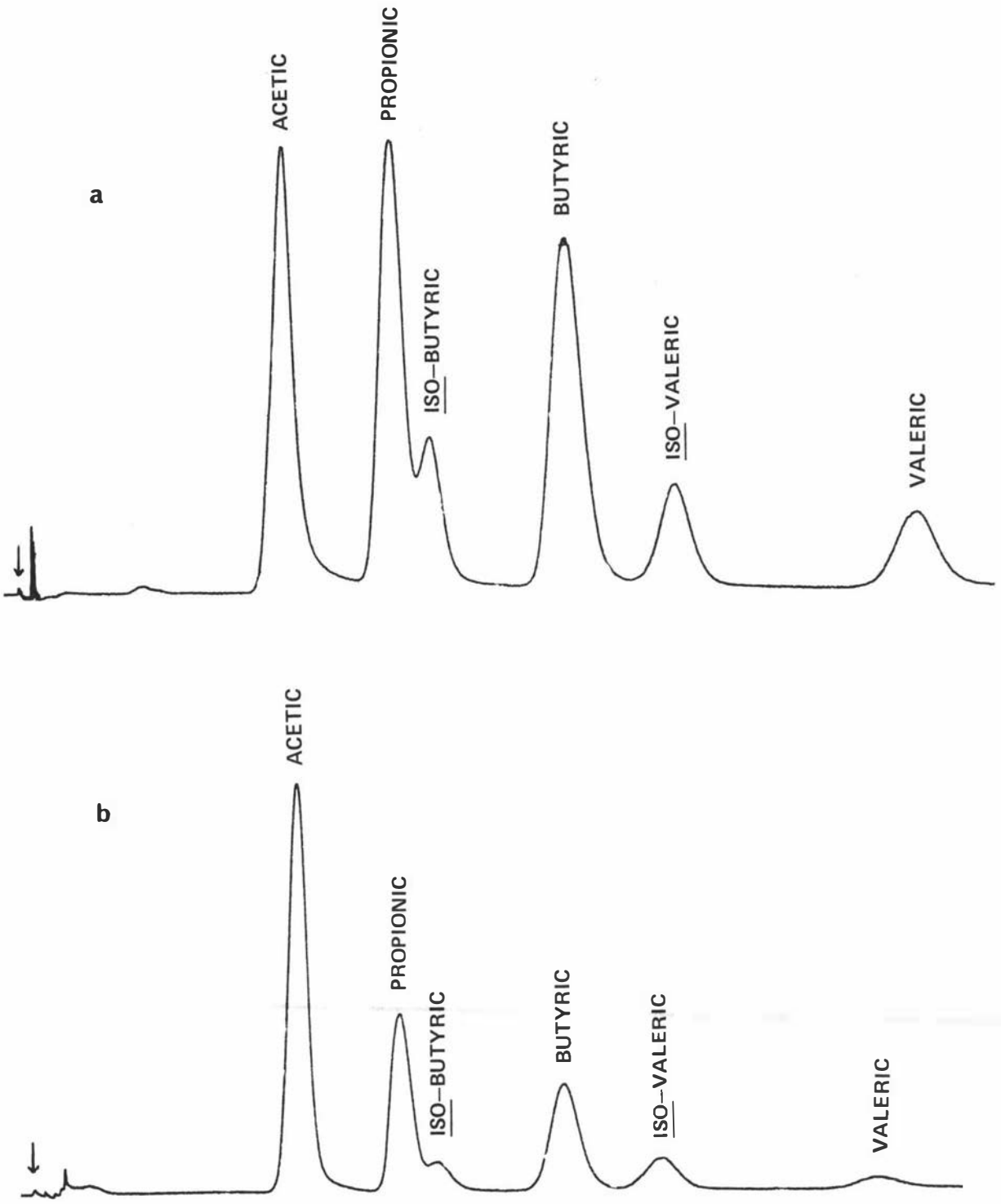


Figure 1. Separation of (a) a mixture of volatile fatty acids of known composition and (b) volatile fatty acids in rumen fluid by gas-liquid chromatography.

See section 2.4 for conditions of analysis.

Key: Arrow indicates the point of injection of sample into the chromatogram.

2.5 Polyacrylamide gel electrophoresis

The apparatus used for the vertical flat-sheet polyacrylamide gel electrophoresis and the gel slab assembly were basically the same as those used by Reid and Bieleski (1968).

2.5.1 Preparation of gel slab

The two glass plates of the gel slab assembly were cleaned thoroughly and rinsed in dilute aqueous detergent (0.01% ($\frac{V}{V}$) Teepol, Shell Co. Ltd., N.Z.). During the preparation of the gel slab, the plates were arranged according to the procedure described by Reid and Bieleski (1968). A 15ml aliquot of a solution containing 15% ($\frac{W}{V}$) acrylamide and 0.3% ($\frac{W}{V}$) bisacrylamide in distilled water was mixed with 15ml 0.1M-Tris/0.1M-glycine buffer (pH 8.5) in a flask and closed with a ground glass stopper. The mixture was deaerated under vacuum for about 1min. Then 0.3ml each of 10% ($\frac{W}{V}$) ammonium persulphate and 10% ($\frac{V}{V}$) N,N,N′, N′-tetramethylethylenediamine (Koch-Light Lab. Ltd., England) solution was added. The resultant mixture was immediately poured into the gel slab assembly through the slot in the glass plate. The assembly was tilted slightly during filling so that no air bubbles were entrapped on the well-forming mould. Usually it took 15 - 20 min for the gel to set at room temperature.

2.5.2 Electrophoresis of Samples

The protein samples (15-50µl) in 8% sucrose were layered by means of a microsyringe fitted with narrow polyvinylchloride tubing directly into the bottom of each well which had been previously filled with the electrode buffer (0.1M-Tris/0.1M-glycine, pH 8.5). Electrophoresis was carried out at 12 mA for the 15 min to concentrate the proteins and

then at 25 mA for 3h 15 min. Bromophenol blue was used as marker compound. All operations were carried out at 3°C in a cold room.

2.5.3 Localization of protein bands

To reveal protein bands, the gel was stained in 0.025% ($^{W}/V$) solution of Coomassie Brilliant Blue (Sigma Chemical Co. Ltd., U.S.A.) in MeOH : HOAC : H_2O (25:7:68, $^{V}/V$) for 30 min with occasional gentle agitation. This solvent mixture without Coomassie Blue was used for destaining the gel until the background was colourless and for storing until photographed.

2.5.4 Localization of enzyme bands

After electrophoresis, the gel sheet was immersed in 0.05 M-Tris/HCl buffer (pH 6.5) for 15 min at 3°C to remove soluble gel constituents such as buffer salts and NH_4^+ (Reid and Bieleski, 1968) which may interfere with the specific enzyme tests. The following methods were used for the detection of specific enzyme bands in the gels.

(a) Esterases

Esterases were located (Burstone, 1962) by incubating the gel for 1h at 3°C in a reaction mixture containing 100mg Fast Garnet GBC Salt (Sigma Chemical Co. Ltd., U.S.A.) in 100ml 0.05M-Tris/HCl (pH 6.5) and 20mg 1- or 2- naphthylacetate in 2ml acetone. The gel was rinsed twice and stored in distilled water until photographed.

(b) Phosphatases

Phosphatases were detected (Burstone, 1962) in gels by the same diazonium dye method used for esterases except that 1- or 2- naphthylacetate in 2ml acetone was replaced by 10mg 1-naphthylphosphate (Na-salt) (Sigma Chemical Co. Ltd., U.S.A.) and the reaction was carried

out at 30°C for 1.5h.

(c) Lipases

(I) Diazonium dye method as described for esterases was used except that 1- or 2- naphthylacetate was replaced by 1-naphthyl-oleate (a gift from Mr. R. Norris), 2-naphthyloleate or 1-naphthyl-stearate (Sigma Chemical Co. Ltd., U.S.A.) and the incubation of the gel in the reaction mixture at 3°C or 39°C was extended up to 14h.

(II) Gomori's Tween procedure (Burstone, 1962) was used with a modified dye technique (Takeuchi and Furuta, 1956).

2.6 Purification of insoluble polyvinylpyrrolidone (Polyclar AT) used to inhibit polyphenol oxidases

Commercially available Polyclar AT (Mauri Bros. and Thomson Ltd., N.Z.) was purified before use according to the procedure described by Loomis and Battaile (1966). Polyclar AT was suspended in a 10% ($^{V}/V$) solution of HCl and boiled for 10 min. Washing with distilled water was continued until free of Cl^{-}. Fines were discarded during the washing procedure. Finally, Polyclar AT was washed with acetone and dried first at room temperature and then under vacuum.

2.7 Determination of protein

The protein content of soluble enzyme preparations from plant and rumen fluid sources was estimated by the Biuret method using bovine serum albumin (Sigma Chemical Co. Ltd., U.S.A.) as a standard protein.

2.8 Plant tissues

Tall fescue (*Festuca arundinacea mediterranean*), short-rotation ryegrass (*Lolium (perenne xmultiflorum)* cv. Manawa), lucerne (*Medicago*

sativa L.), Yorkshire fog (Holcus lanatus L.), phalaris (Phalaris arundinacea x tuberosa), timothy ((Phleum pratense L.) cv. Kahu), browntop (Agrostis tenuis) and paspalum (Paspalum dilatatum) leaf tissue was collected from clones grown on plots managed by the Agronomy Department, Massey University. Ariki ryegrass (Lolium perenne x (L. perenne x L. multiflorum)) and perennial ryegrass (Lolium perenne) leaf tissues were obtained from fields managed by the Dairy Husbandry Department, Massey University. Leaves of Cocksfoot (Dactylis glomerata L.) were obtained from Massey University grounds. A crude protein extract from white clover (Trifolium repens L.) used as source of lipase was kindly supplied by Dr. W.T. Jones of the D.S.I.R., Palmerston North.

2.9 Preparation of dried ryegrass

400g Ariki ryegrass was sun-dried (28-30°C) in 3 stages for a total of 24h. The dried product, which was brittle and retained much of its green colour, was stored in a plastic bag at room temperature. The moisture content of the dried grass determined by drying in an oven at 110°C for 18h was found to be 9.1% as compared with fresh Ariki ryegrass which contained 80.5% moisture.

2.10 Treatment of animals, sampling from the rumen and fractionation of rumen fluid

Rumen contents were obtained via rumen fistula from Jersey Cows which had been grazing on fresh pasture consisting mainly of ryegrass with some clover before being fasted approximately 18h (except where otherwise mentioned). The contents in the rumen were well mixed by hand prior to sampling. The samples were taken from below the fistula

in the centre of the rumen and strained through two layers of cheese-cloth immediately after sampling. The strained fluid was placed directly into a previously warmed thermos flask. This fluid was used for incubation with plant extracts as prepared in sub-section 2.11.1.

For investigation of the lipase activity of protozoa-free rumen fluid, protozoa and plant debris were removed from cheese-cloth strained rumen fluid by centrifugation at 500g for 10 min (Wright, 1961). When necessary this supernatant suspension was autoclaved for 15 min at 15 psi. Clarified rumen fluid was prepared by centrifuging strained rumen fluid at 35,000g for 30 min at 0^{o}C.

2.11 Enzyme preparations

2.11.1 Crude enzyme extracts from leaf tissue

Surface moisture on fresh leaves was removed by drying with absorbent tissue paper and the leaves were chilled in a cold room at 3^{o}C for about 30 min. A known weight of leaves was cut cross-sectionally into small pieces (0.3 - 0.5 cm) and ground in a mortar with an equal weight of acid-washed sand (May and Baker Ltd., England) in 0.06M-phosphate buffer (pH 7.0) to give a fine slurry. The homogenate was strained through one layer of Miracloth (supplied by Calbiochem, California, U.S.A.). Routinely, this strained extract with or without autoclaving at 15 psi for 15 min was used for incubation with rumen fluid to follow lipolysis and fermentation. In order to investigate the distribution of lipases between the chloroplastic fraction and the cytoplasm, the strained fluid was centrifuged at 16,000g for 20 min in a Sorvall model RC2-B using a SS-34 rotor at 0^{o}C to precipitate the whole and broken chloroplasts (Sastry and Kates, 1964b).

2.11.2 Soluble proteins from leaf tissue and rumen fluid for electrophoretic separation on polyacrylamide gel

Soluble proteins were isolated from leaf tissue according to the following procedure. Leaf tissue with equal weights of Polyclar AT and sand were ground in 0.06M-phosphate buffer (pH 7.0) containing 0.01M-sodium diethyldithiocarbamate in order to absorb oxidation products of endogenous phenolic compounds (Loomis and Battaile, 1966). The homogenate was centrifuged at 35,000g for 30 min at 0°C; powdered $(NH_4)_2SO_4$ was added to the supernatant and the precipitate formed at 0 - 0.85 saturation was collected by further centrifuging the mixture at 20,000g for 20 min at 0°C. The precipitate was redissolved in a suitable volume of phosphate buffer (I.S. 0.2; pH 7.5) and dialyzed against 15 litre of the same buffer for 40h. Dialysis was carried out in cellulose tubing (Visking Co., U.S.A.) with continuous stirring of the buffer in a cold room (3°C). The dialyzed material was stored at -20°C until required for analysis.

Soluble enzymes were isolated from rumen fluid obtained from a cow at different times after feeding ryegrass. Each rumen sample (50ml) was strained through cheese-cloth into a stoppered container (previously flushed with 10% CO_2 in N_2) containing 5g Polyclar AT and 0.01M-sodium diethyldithiocarbamate. The contents were mixed by inversion and centrifuged immediately at 35,000g for 30 min at 0°C. Proteins from the supernatant were isolated by $(NH_4)_2SO_4$ precipitation according to the procedure described above and stored at -20°C until required for analysis.

2.12 Procedures for following lipolysis

2.12.1 Preparation of lipid substrates for incubation

Oil/water emulsions used as substrates in the assay of lipases were prepared as follows: 0.5g gum acacia (British Drug House Ltd., England) was thoroughly mixed with 4ml distilled water in a glass tube. Safflower oil (54mg), tributyrin (0.66g) or triacetin (0.65g) was added and the resultant mixture was subjected to sonication in a 100 watt ultrasonic disintegrater (Measuring and Scientific Equipment Ltd., England) for 30 sec at a power output of 8 microns peak-to-peak. The process was repeated twice after cooling in ice to give a fine dispersion of the oil. This intermittent procedure of sonication with cooling was adopted in order to avoid nonenzymatic hydrolysis of the oil by the heat produced during sonication. Water was added to give a final volume of 5ml. The emulsified triglycerides were used on the day they were prepared. Autoclaved ryegrass extracts were used as the source of total endogenous plant lipids.

MGDG have a very high melting point and cannot be prepared as a stable emulsion for a sufficient length of time to allow a reproducible rate of lipolysis. The less satisfactory alternative method of adding the substrate as a hexane solution to the incubation mixture was adopted after preliminary incubations of plant extracts with various solvents containing ^{14}C-MGDG had shown that the release of ^{14}C-fatty acids occurs in the order: hexane > diethyl ether + MeOH > MeOH. Glyceryl tri (oleate - 1 - ^{14}C) was added to the incubation mixture (Clarke and Hawke, 1970) as a solution in hexane.

2.12.2 Methods of incubation

All incubations with or without added substrates were carried out in 50ml Erlenmeyer flasks in a water bath at 39°C. To maintain anaerobiosis, the flasks were flushed with a mixture of 10% CO_2 in N_2 before and after addition of the incubation mixture. To follow lipolysis, substrates were added to the incubation mixture either in emulsified form (safflower oil, tributyrin and triacetin) or as a hexane solution (^{14}C-triolein and ^{14}C-MGDG). Mild shaking of the reaction mixture was continued throughout the incubation period by means of a shaking system attached to the water bath.

2.12.3 Termination of lipolysis and extraction of lipids for analysis

When ^{14}C-triolein or ^{14}C-MGDG was used as a substrate for the assay of lipase activity, the reaction was stopped by adding an equal volume of ethanol to the total incubation mixture and heating for 5 min in a water bath at 70 - 80°C (Clarke and Hawke, 1970). When cooled, the pH of the incubation mixture was lowered to 4.0 by addition of 0.1M-HCl. The resultant mixture was transferred to a separating funnel and lipids were extracted with $CHCl_3$ (3 separate extractions). The combined extracts were washed with distilled water until the washes were neutral and evaporated to dryness *in vacuo* on a rotary evaporator. The dried lipids were dissolved in a known volume of $CHCl_3$ and stored in a refrigerator at 4°C until required for analysis.

When unlabelled substrates such as safflower oil and total plant lipids were used to follow lipolysis, termination of reaction and extraction of lipids from the incubation mixture were carried out

according to the procedure described by Dole and Meinertz (1960) 1ml aliquots from the incubation mixture were transferred to 5ml of an extraction mixture consisting of heptane - iso-propanol - 0.5M-H_2SO_4 (1:4:0.1, $^{V}/_{V}$) in ground glass tubes and stoppered. The mixture was shaken vigorously for 2 min and allowed to stand at room temperature for at least 5 min before adding 3ml heptane; finally 2ml 0.01 to 0.02M-H_2SO_4 was added to bring pH to between 2.0 and 2.2. The mixture was shaken for 3 min. After a distinct separation between the two phases, VFA's were removed from aliquots of the upper phase according to the procedure described below:

The VFA's in rumen contents interfere with the colorimetric estimation of long-chain fatty acids. It was found that VFA's were partly extracted into the heptane layer during the normal Dole and Meinertz's (1960) extraction procedure and gave a positive reaction with the colorimetric reagents used for the analysis of long-chain fatty acids. The most convenient method of overcoming this interference was to remove VFA's from the heptane solution of the FFA's by evaporating the extract to dryness under N_2 at room temperature. VFA's added to plant extracts and subjected to the above procedure could be completely removed. Consequently, to remove VFA's from the extracted lipids of the reaction mixture, a suitable aliquot from the heptane layer was transferred to another tube and evaporated to dryness under N_2. The residual lipids were redissolved in a further aliquot of heptane and again evaporated to dryness. The dried lipids were dissolved in an appropriate volume of heptane for colorimetry.

2.12.4 Radiochemical analysis of hydrolysis products

In order to determine the distribution of radioactivity between MGDG, FFA's, mono-, di- and triglycerides, the lipids of the reaction mixture were separated and identified by thin-layer chromatography as described under section 2.2.

(a) Removal of zones

When ^{14}C-triolein was used as the substrate for lipolytic enzymes, the radioactivity of the hydrolysis products was determined by removing appropriate zones from chromatograms into counting vials and measuring the radioactivity of the silicic acid suspension (Clarke and Hawke, 1970) in toluene scintillation fluid as described in section 2.3. However, MGDG becomes bound to silicic acid in the toluene scintillation fluid and quantitative measurements of radioactivity under these conditions were not obtained. Consequently, when ^{14}C-MGDG was the substrate for lipases, radioactive lipids were extracted from the appropriate zones of the chromatogram by $CHCl_3$-MeOH (2:1, $^{V}/_{V}$) (twice) and $CHCl_3$-MeOH (1:2, $^{V}/_{V}$) (twice). The combined extracts were evaporated to dryness under N_2 in counting vials and counted as described in section 2.3. Unless otherwise mentioned, measurement of radioactivity of all lipid samples was carried out by the procedure described above.

(b) Scanning

Alternatively, the distribution of radioactivity between lipid components on thin-layer chromatographic plates was determined with a Radiochromatogram Scanner (Packard Model 7200) modified to take thin-layer plates (5cm x 20cm) using the following settings: Voltage, 300; time constant, 30 sec; chart speed, 6cm/h; gas (98.7% helium, 1.3% iso-butane) flow, 110ml/min (Clarke and Hawke, 1970). Relative estimates of the proportion of the radioactivity present in each lipid

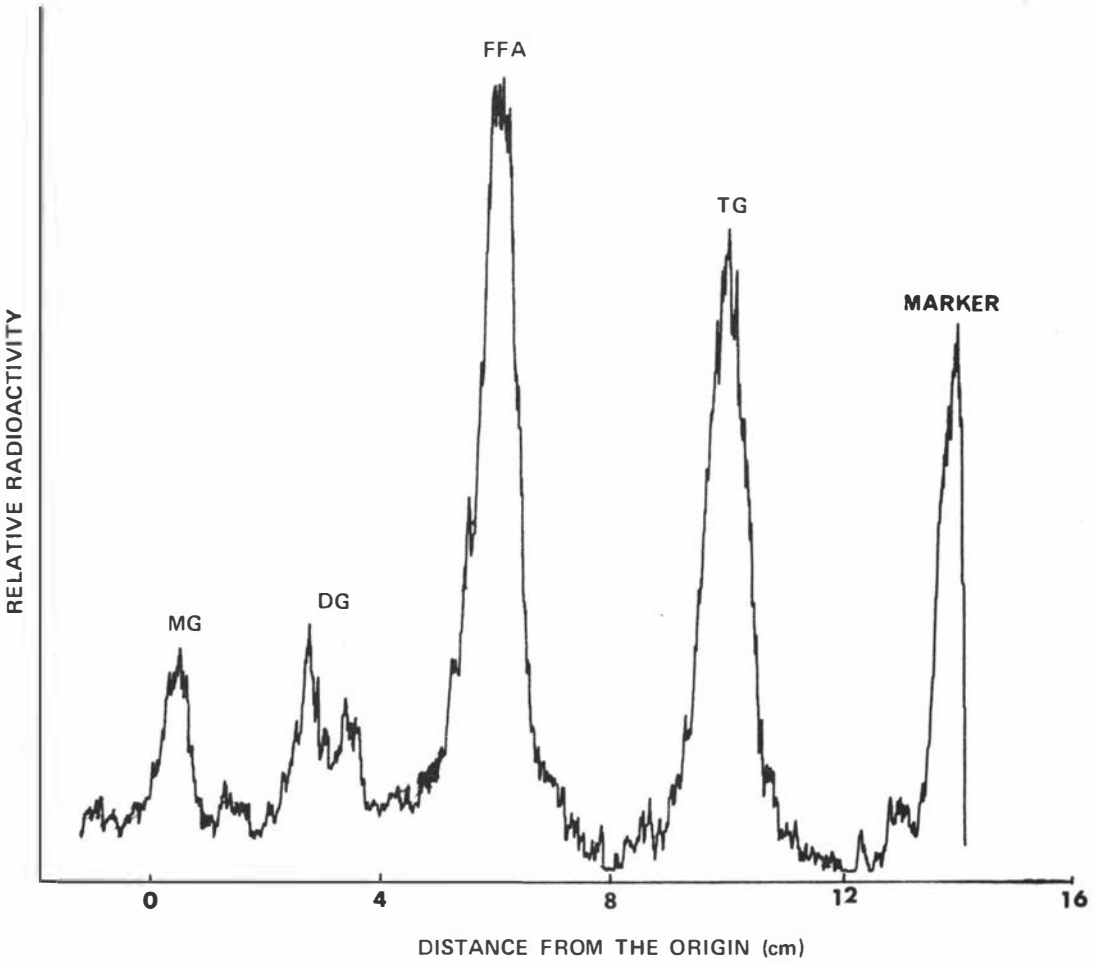


Figure 2. Radioscan of a thin-layer chromatogram of the lipid products following incubation of ^{14}C-triolein with a perennial ryegrass extract.

The chromatogram was run on silica gel G in hexane-diethyl ether-acetic acid (70:30:1, by vol.).

See sub-section 2.12.4. and Table 1 for scanning and incubation conditions respectively.

Key: MG = monoglyceride, DG = diglyceride, TG = triglyceride, FFA = free fatty acid.

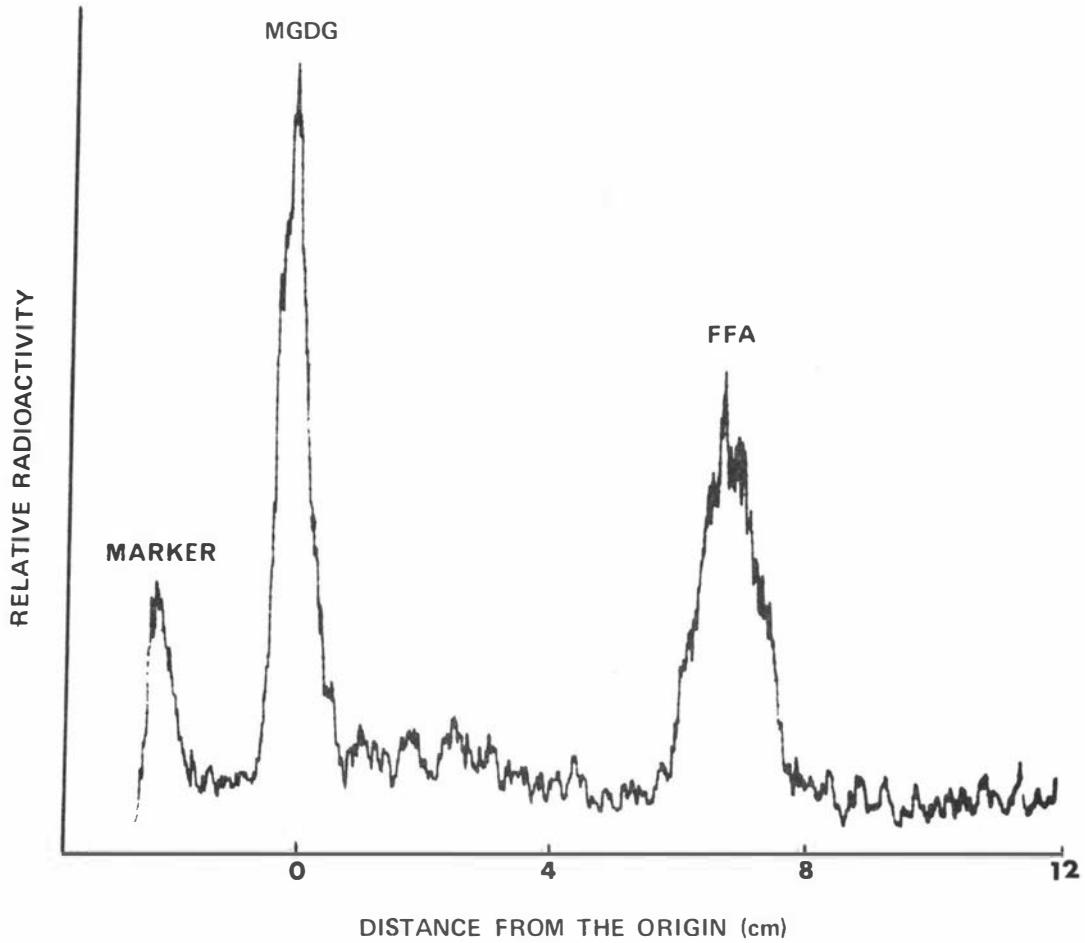


Figure 3 Radioscan of a thin-layer chromatogram of the lipid products following incubation of ^{14}C-monogalactosyldiglyceride with a perennial ryegrass extract.

The chromatogram was run on silica gel G in hexane-diethyl ether-acetic acid (70:30:1, by vol.).

See sub-section 2.12.4 for scanning conditions.

Reaction mixture: 5ml* of 16,000g supernatant of the total ryegrass extract; 2.5ml autocalved rumen fluid; ^{14}C-monogalactosyldiglyceride (1.9×10^4 dpm) in 25 µl hexane; the incubation was carried out at 39°C for 15min under 10% CO_2 in N_2 with constant shaking.

Key: FFA = free fatty acid; MGDG = monogalactosyldiglyceride

* equivalent to 1.63g fresh leaf tissue.

component were obtained by planimetry. The distribution of radioactivity between the products of lipolysis and unhydrolysed lipids after incubation of ^{14}C-triolein and ^{14}C-MGDG with perennial ryegrass extracts was shown by the radioscan of thin-layer chromatograms, as illustrated in Fig. 2 and Fig. 3 respectively.

2.12.5 Colorimetric analysis of long-chain fatty acids

(a) Removal of long-chain fatty acids from plant extracts

Extracts prepared from leaf tissue by grinding in buffer (subsection 2.11.1) contained long-chain fatty acids released by the action of lipolytic enzymes on endogenous lipids. When long-chain fatty acid-free plant pigments were required for the preparation of a standard curve of palmitic acid colorimetrically, the long-chain fatty acids were removed by washing a leaf tissue extract with 1M-NaOH (5 times) and then with water until the washes were neutral. Plant pigments were extracted in heptane according to the procedures described in subsection 2.12.3.

(b) Preparation of standard curves with palmitic acid

The method described by Mosinger (1965) was used to prepare standard curves of palmitic acid (British Drug Houses Ltd., England, specially pure grade) in the range of 0 - 328 nmol in heptane (Fig. 4). To 1ml solution of fatty acid in heptane or heptane alone for the blank, Mosinger's 1.5ml barbital-phenol red buffer was added. The optical density values were read at 560 nm against heptane-ethanol (Fig. 4, ■ —■). In order to correct for the endogenous plant pigments extracted in heptane from the incubation mixture, a second standard curve was prepared by mixing 0.2ml plant pigments solution in heptane with 0.8ml fatty acid solution (Fig. 4, ● —●). 0.2ml plant pigment gave an

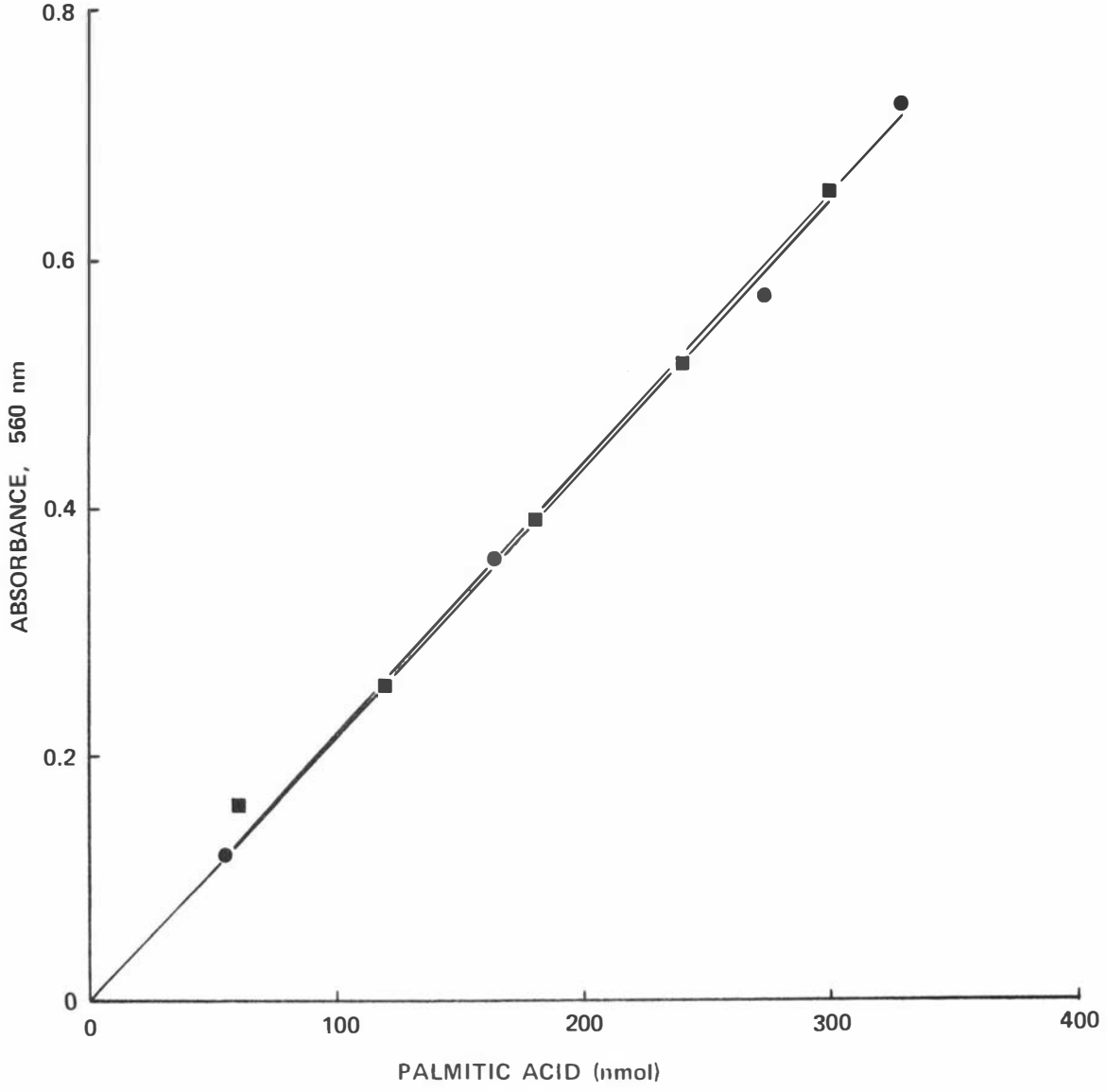


Figure 4. Relationship between absorbance and the concentration of palmitic acid.

To 1 ml heptane containing 0-300 nmol palmitic acid and to 0.8 ml heptane containing 0-328 nmol palmitic acid and 0.2 ml plant pigment solution in heptane, 1.5 ml barbital-phenol red buffer (Mosinger, 1965) was added. The optical density values were read at 560 nm against heptane-ethanol. Differences between sample and blank optical density values were plotted to obtain standard curves. See text for other conditions.

Key: With plant pigments (● —●); without plant pigments (■ — ■).

absorbance of 0.025 at 560 nm against heptane-ethanol. No significant difference between the two standard curves was obtained and both gave a linear relationship between concentration and absorbance. Consequently, 0.1 - 0.25ml aliquots of lipid extracts from the incubation mixtures were used routinely for FFA analysis and the fatty acid contents were estimated from the curve presented in Fig. 4, ●—● .

2.13 Procedures for following the incorporation of acetate by rumen microorganisms into long-chain fatty acids

To investigate rumen microbial synthesis of long-chain fatty acids from acetate, an aqueous solution of ^{14}C-acetate was mixed with rumen fluid containing autoclaved plant extract. General conditions of incubation were the same as those described in sub-section 2.12.2. The amount of acetate formed in the reaction mixture after different times of incubation was quantitatively estimated by gas-liquid chromatography (section 2.4). 1ml aliquots from the incubation mixture were withdrawn at different time intervals. Termination of the reaction and extraction of lipid were carried out according to the procedures described in sub-section 2.12.3. Long-chain fatty acids were separated by thin-layer chromatography (section 2.2) and radioactivity was measured as in section 2.3.

2.14 Procedure for following the fermentation-rate of rumen microorganisms

The rate at which plant extract or autoclaved plant extract was fermented by rumen contents was followed by measuring the rate of gas production. The procedure described by el-Shazly and Hungate (1965) was adopted. Incubations were carried out at 39^{o}C under a mixture of

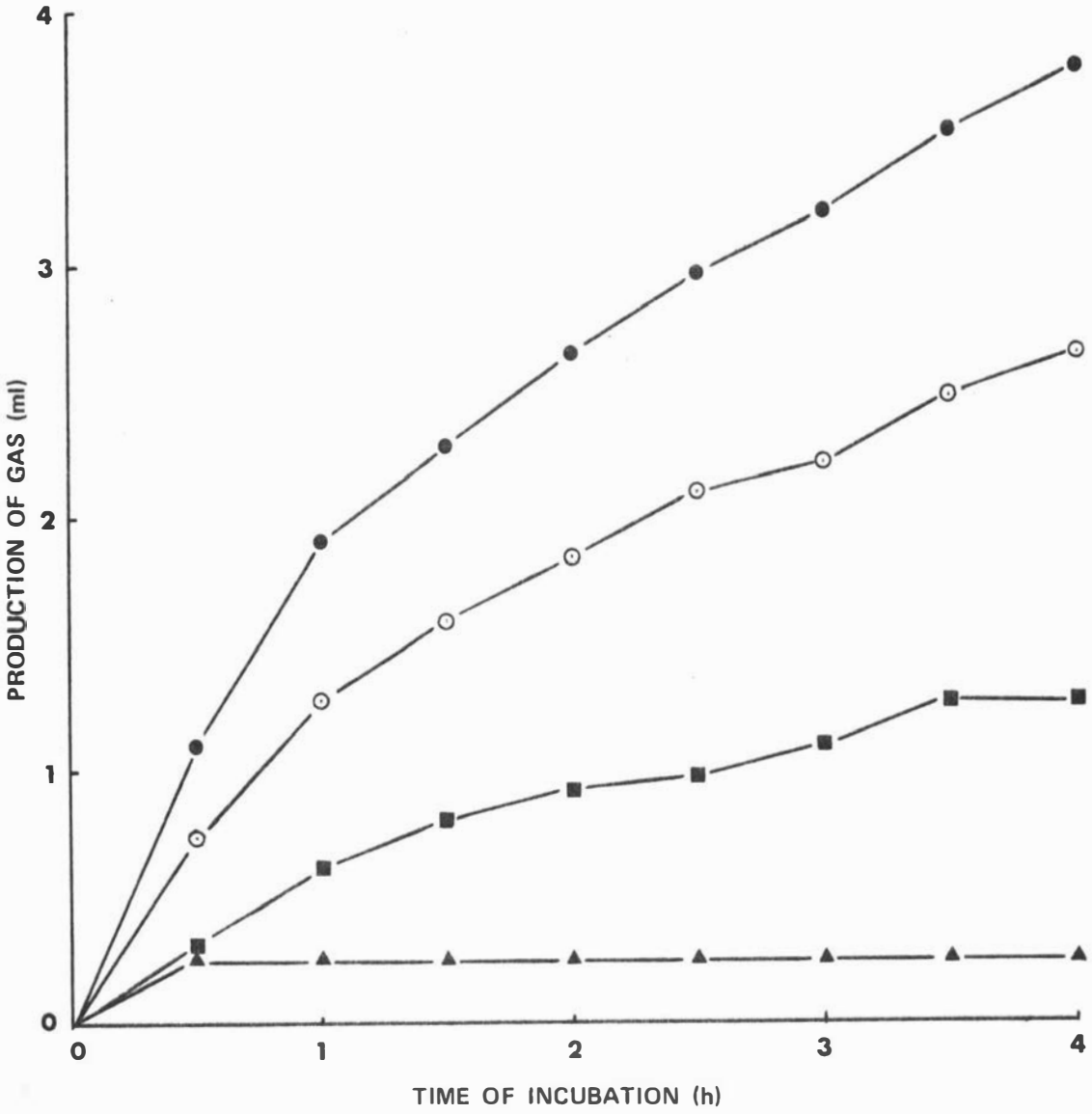


Figure 5. Effect of increasing the concentration of autoclaved fescue extract on the rate of gas production by rumen microorgamisms.

Conditions of incubation: Each incubation contained 0, 1*, 2 and 3 ml autoclaved fescue extract and 5, 4, 3 and 2 ml 0.06 M-phosphate buffer (pH 7.0) respectively and 2 ml rumen fluid. Incubations were carried out at 39°C under 10% CO_2 in N_2 with occasional gentle agitation of the incubation mixture.

Key: Gas produced by rumen fluid in the presence of 0.0ml (▲—▲), 1.0 ml (■—■), 2.0 ml (○—○) and 3.0 ml (●—●) autoclaved plant extract.

* equivalent to 0.25g fresh leaf tissue.

10% CO_2 in N_2 in 25ml conical flasks closed with butyl rubber stoppers. The flasks were agitated gently during the incubation. The rate of gas production was measured by inserting the needle of a 2ml water-lubricated syringe through the stopper and reading the volume of the gas forced into the syringe at different time intervals. Fig. 5 shows the rate of gas production by rumen microorganisms in the presence of varying amounts of autoclaved fescue extract.. The production of gas increased with the increase in the concentration of autoclaved plant extract.

2.15 Discussion of the procedures used to measure lipase activity

The radiochemical techniques recently developed for determining the lipolytic activity involve separation of the products of lipolysis of radioactive triglyceride and unreacted triglyceride followed by the measurement of radioactivity of individual lipid components. ^{14}C-triolein has been used as test substrate since it is readily emulsified and is available commercially. Chino and Gilbert (1965) used a Florisil column to separate FFA's, mono-, di- and triglyceride. Clarke and Hawke (1970) separated the products by thin-layer chromatography on silica gel. Well-separated radioactive lipids from the reaction mixture on a thin-layer chromatographic plate could be scanned directly, or alternately appropriate areas from the chromatogram could be transferred into counting vials and the radioactivity measured in a scintillation spectrometer. In the present study, the latter technique was preferred since it was found to be more sensitive than the former. This method was satisfactorily used to study the lipase activity of various pasture plants under conditions similar to those found in the rumen. In vitro investigations of the stability of plant lipases in

the rumen were also performed with this technique.

The majority of methods described for measuring lipase activity are based on the titration of liberated fatty acids with alcoholic NaOH solution (Dole, 1956). This method was used to investigate the activity of microbial lipases (Alford and Pierce, 1963; Vadehra and Harmon, 1965). One disadvantage with titrimetric methods of FFA estimation is the difficulty in determining the end-point of the titration in coloured samples. However, the continuous automatic titration of the fatty acid produced by the action of lipase on an appropriate substrate in a pH - stat has been used by many investigators (Tirunarayanan and Lundbeck, 1968; Downey and Andrews, 1969). The major advantage of this procedure is that the initial velocity of lipolysis can be measured within a short period of incubation and no extraction of FFA's is involved. However, the method has certain drawbacks e.g., it is difficult to measure lipase activity at pH < 7.0 which could be due to incomplete titration of long-chain fatty acids and the method cannot be used to follow lipolysis in a culture medium (Lawrence, 1967). The formation of acidic end products of microbial metabolism also limits the application of such methods in microbial systems.

The methods described for the colorimetric micro-determination of fatty acids depend on the formation of a copper soap of the fatty acid and its extraction into an organic solvent followed by the estimation of copper (Iwayama, 1959; Baker, 1964; Duncombe, 1963). These methods cannot be used in some circumstances. For instance, there would be appreciable interference of phospholipids and the presence of relatively high concentration of triglyceride, cholesterol

and cholesterol esters could interfere with the FFA estimation (Duncombe, 1963). In the present study, fatty acids from the incubation mixture were extracted into heptane according to the procedure described by Dole and Meinertz (1960), short-chain fatty acids were removed from the total extracts by evaporating to dryness under N_2 (sub-section 2.12.3) and FFA's were determined colorimetrically (sub-section 2.12.5) according to the procedure described by Mosinger (1965). The method is based on the measurement of colour changes due to varying concentrations of FFA's in phenol red-barbital buffer in heptane-ethanol. This technique of FFA estimation was satisfactorily used for the determination of lipase activity of ryegrass extracts and for the *in vivo* investigations of the contribution of plant lipolytic enzymes towards lipid hydrolysis in the rumen.

CHAPTER 3

EXPERIMENTAL AND RESULTS

3.1 Lipolytic activity of leaf extracts of pasture plants

The ability of extracts of a number of pasture species to remove fatty acids from ester combination was examined under conditions similar to those found in the rumen (Table 1). Enzyme extracts prepared from all pasture species examined released ^{14}C-labelled hydrolysis products from ^{14}C-triolein when incubations were carried out at neutral pH in the presence of autoclaved rumen fluid. Total extracts prepared from Ariki ryegrass removed 89.1% of the total ^{14}C-label from the ^{14}C-triglyceride into the FFA fraction; on fractionation of the total extracts by centrifugation, it was found that lipase activity was associated with both chloroplastic and cytoplasmic fractions. Moreover, substantial lipase activity was obtained in extracts prepared from dried Ariki ryegrass indicating that lipases were stable under conditions experienced during drying (Table 1). A 30 min incubation of ^{14}C-triolein with 30mg white clover protein in 0.06M-phosphate buffer (pH 7.0) resulted in the release of 20.4% ^{14}C-label into FFA (radioactive products were measured from a radiochromatogram scan). A control incubation of ^{14}C-triolein with autoclaved plant extract for 2h gave a negligible release of radioactivity into FFA (1.5%).

The extent of release of radioactivity from the added substrate by different leaf extracts was variable (Table 1) but no special relevance should be given to these variations because a variable amount of endogenous lipid present in different plant extracts was undergoing simultaneous lipolysis. However, appreciable lipase activity in all

TABLE 1

Formation of ^{14}C-labelled hydrolysis products from ^{14}C-triolein by leaf extracts of various pasture plants.

Conditions of lipase assay except otherwise mentioned: Each incubation mixture contained 7.5ml total leaf extract or corresponding centrifugate or supernatant (equivalent to 2.0g fresh leaf tissue), 3.7ml autoclaved rumen fluid and ^{14}C-triolein (9.0×10^4 - 1.2×10^5dpm; 1.2 - 1.63 nmol) in 25 µl hexane. Incubations were performed at 39°C for 30min under 10% CO_2 in N_2 with continuous shaking.

Plant tissue	Enzyme source	Distribution of radioactivity (% of total)			
		Triglyceride	Diglyceride	Monoglyceride	Free fatty acid
Ariki ryegrass	Total leaf extract	8.3	1.8	0.8	89.1
Ariki ryegrass	16000g centrifugate + autoclaved 16000g supernatant	52.7	8.8	0.4	38.1
Ariki ryegrass	16000g supernatant	20.3	6.0	1.9	71.8
Dried Ariki ryegrass	Total leaf extract	48.6	12.2	1.3	37.9
Lucerne	Total leaf extract	91.1	2.4	0.4	6.1
Yorkshire fog	Total leaf extract	21.3	6.7	1.3	70.7
Phalaris	Total leaf extract	7.3	8.4	0.6	83.7
Timothy	Total leaf extract	49.3	6.8	1.1	42.8
Browntop	Total leaf extract	41.7	7.6	0.8	49.9
Paspalum	Total leaf extract	54.3	14.5	1.4	29.8
Perennial ryegrass*	5ml total leaf extract + 2.5ml autoclaved rumen fluid	31.4	11.2	2.3	55.1
Fescue†	5ml total leaf extract + 2.5ml autoclaved rumen fluid	20.4	6.5	1.9	71.2
Short-rotation ryegrass*	5ml 16000g supernatant	46.9	8.9	3.8	40.4
Cocksfoot ‡	7ml 16000g supernatant	64.7	7.3	0.4	27.6

* equivalent to 1.66g fresh leaf tissue; 10min incubation.
† equivalent to 1.61g fresh leaf tissue; 1h incubation.
‡ equivalent to 2.2g fresh leaf tissue; 20min incubation.

extracts has been established which indicates that pasture species may be potential sources of lipase activity in the rumen.

3.2 Lipolysis of natural and synthetic lipids by leaf extracts from fescue and ryegrass

(a) ^{14}C-triolein

The lipolytic activity of fescue extracts in the presence of autoclaved rumen fluid was examined more fully by following the release of FFA's, mono- and diglycerides from ^{14}C-triolein after different times of incubation. The incubation of 5ml plant extracts with the ^{14}C-substrate was carried out for 0-45min (Fig. 6) and 0-12min (Fig. 7) in the presence of 2.5ml inactivated rumen fluid. An abrupt rise in the distribution of the ^{14}C-label in the FFA fraction (58%) was observed after 15min incubation (Fig. 6) and reached a maximum of 76.5% after 45min. Mono- and diglyceride did not accumulate to any appreciable extent during incubation.

Because such a high proportion of the ^{14}C-label was released as FFA's in 15min (Fig. 6), the release of FFA from ester combination was examined over a shorter time of incubation (Fig. 7). About 49% of the radioactivity appeared as FFA's after 3min incubation and thereafter there was an almost linear increase in the formation of FFA's up to 12min. Again, the formation of mono- and diglyceride at different incubation times remained approximately the same. Therefore, the decrease in the residual triglyceride over the same time interval was also approximately linear (Fig. 7).

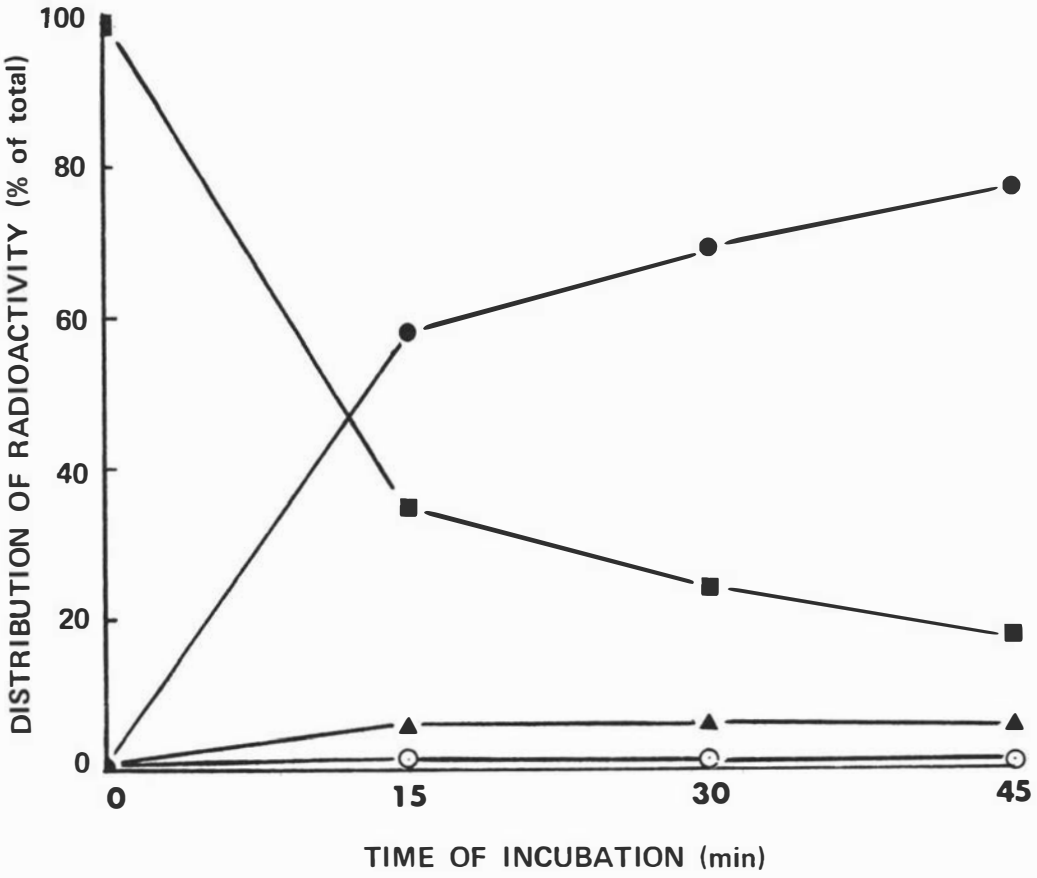


Figure 6. Effect of time on the enzymic hydrolysis of ^{14}C-triolein by extracts of fescue leaves in the presence of autoclaved rumen fluid.

Conditions of lipase assay: Each incubation contained 5 ml* fescue extract, 2.5 ml autoclaved rumen fluid and ^{14}C-triolein (1.25×10^5 dpm; 1.67 nmol) in 25 µl hexane; incubations were carried out at 39°C under 10% CO_2 in N_2 with constant shaking.

Key: Triglyceride (■ — ■); diglyceride (▲ — ▲); monoglyceride (o — o); free fatty acid (● — ●).

* equivalent to 1.63g fresh leaf tissue.

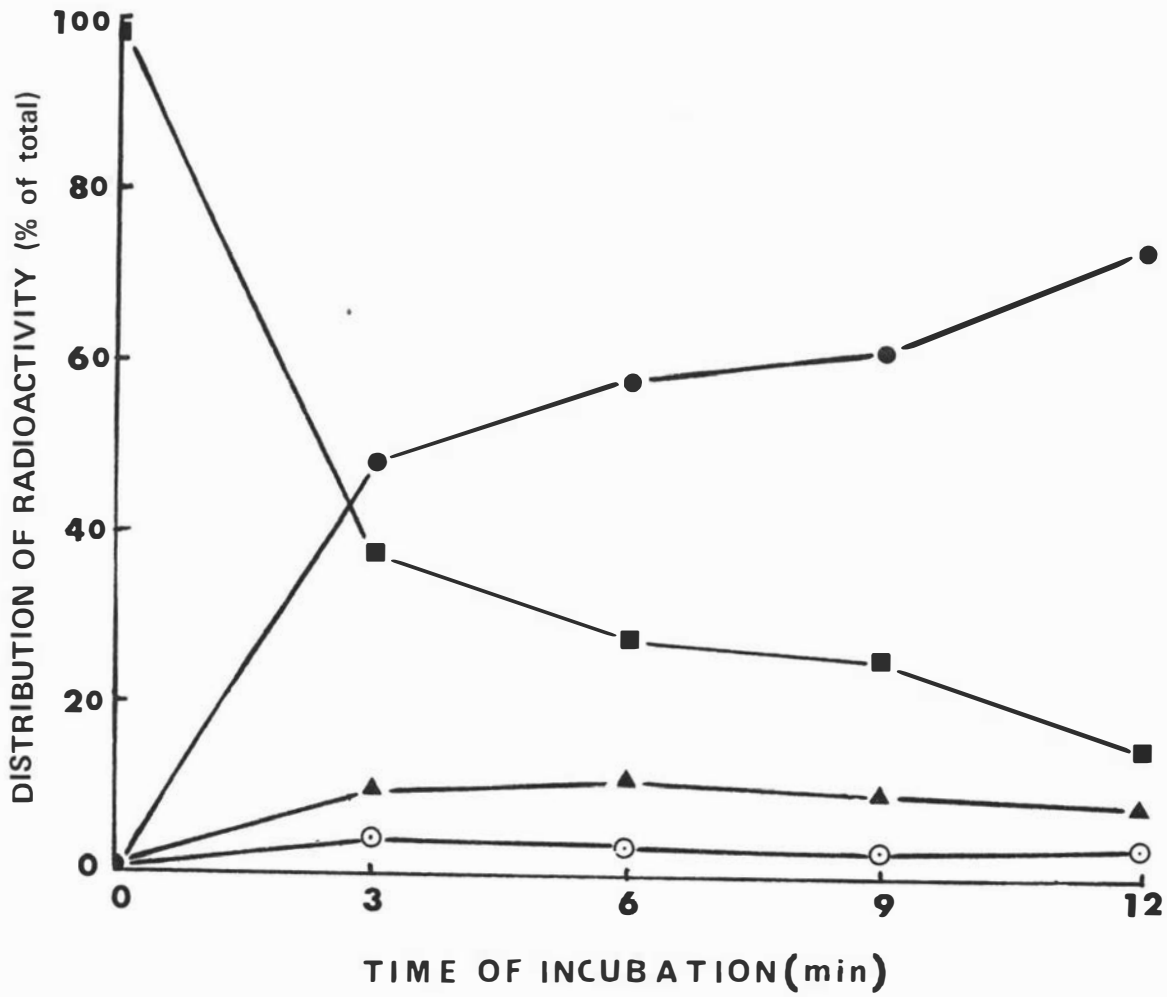


Figure 7. Effect of time on the enzymic hydrolysis of ^{14}C-triolein by extracts of fescue leaves in the presence of autoclaved rumen fluid.

Conditions of lipase assay: For each incubation 25 µl hexane containing ^{14}C-triolein (1.22×10^5 dpm; 1.63 nmol) was added to 5 ml* fescue extract and 2.5 ml autoclaved rumen fluid; incubations were performed in the presence of a mixture of 10% CO_2 in N_2 with constant shaking.

Key: Triglyceride (■ — ■); diglyceride (▲ — ▲); monoglyceride (○ — ○); free fatty acid (● — ●).

* equivalent to 1.7g fresh leaf tissue.

(b) ^{14}C-monogalactosyldiglyceride

When similar experiments were carried out using ^{14}C-MGDG in place of ^{14}C-triolein, 49.2% of the radioactivity was present in the FFA fraction after a 15min incubation but further increase in the incubation time up to 45min resulted only in a very slow increase in the formation of FFA's (Fig. 8). However, when the length of incubation time was shortened (Fig. 9), 27.2% of the total radioactivity was recovered in the FFA fraction after 3min incubation and reached a maximum of about 35% after 12min.

Furthermore, the effect of varying quantities of enzymatically active leaf extracts on the rate of FFA production from ^{14}C-MGDG was studied under the conditions described in Fig. 10. Autoclaved plant extract was used to maintain a constant amount of total plant extract in the reaction mixtures. There was a linear relationship between the amount of enzyme extracts and the release of FFA from a constant amount of ^{14}C-MGDG (Fig. 10).

(c) Safflower oil and endogenous plant lipids

Lipolysis of safflower oil and the total endogenous plant lipids by ryegrass extracts was investigated by following the release of FFA's colorimetrically (sub-section 2.12.5). 5ml plant extract was incubated with 0.5ml safflower oil emulsion in the presence of 5ml inactivated rumen fluid under the conditions described in Fig. 11. Because of the presence of endogenous FFA's both in rumen fluid and in plant extracts, considerable amounts (31.61 μmol) of FFA's was found at zero time. However, the formation of FFA increased from 31.61 μmol to 48.57 μmol during the 2h incubation. When the FFA values formed at different

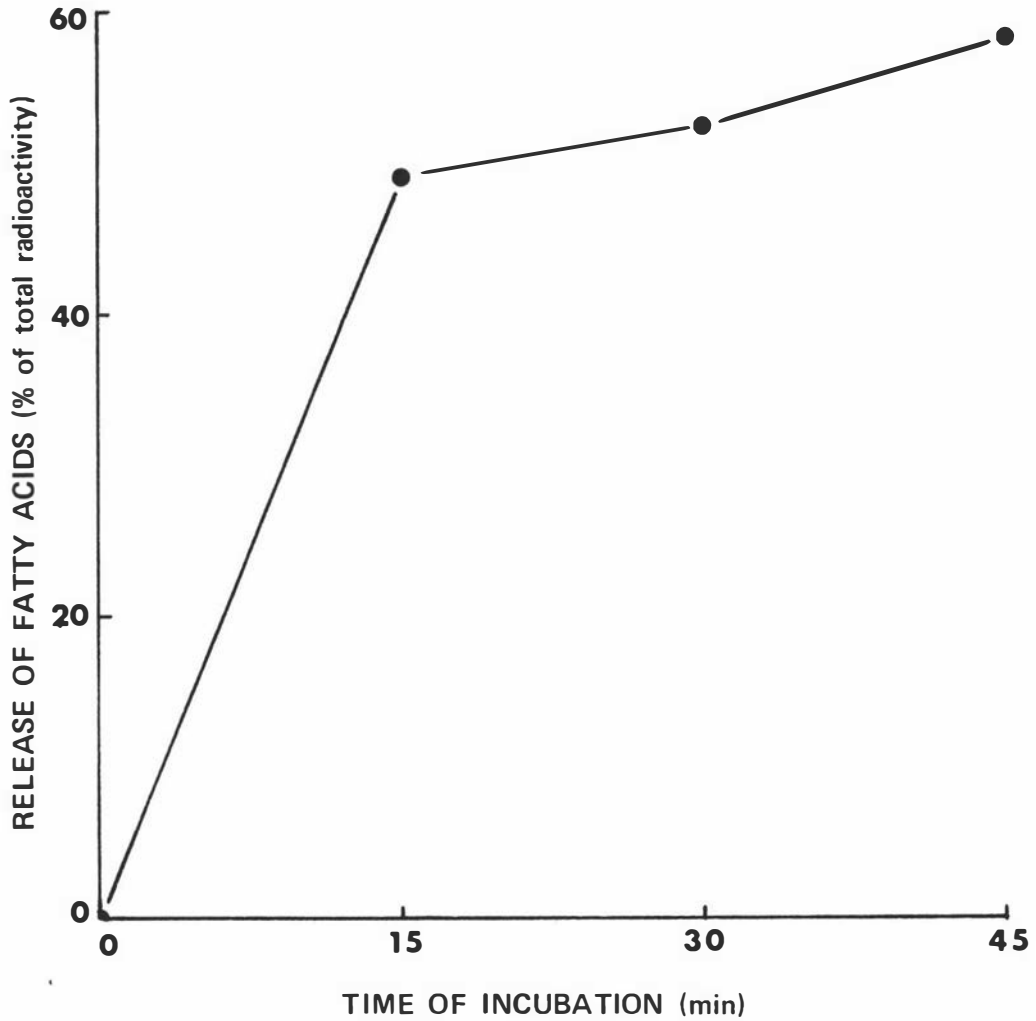


Figure 8. Effect of time on the release of ^{14}C-labelled fatty acids during incubation of ^{14}C-monogalactosyldiglyceride with fescue extract in the presence of autoclaved rumen fluid.

Conditions of incubation: As described in Fig. 6 except that ^{14}C-triolein was replaced by ^{14}C-monogalactosyldiglyceride (1.04×10^5 dpm) in 25 µl hexane.

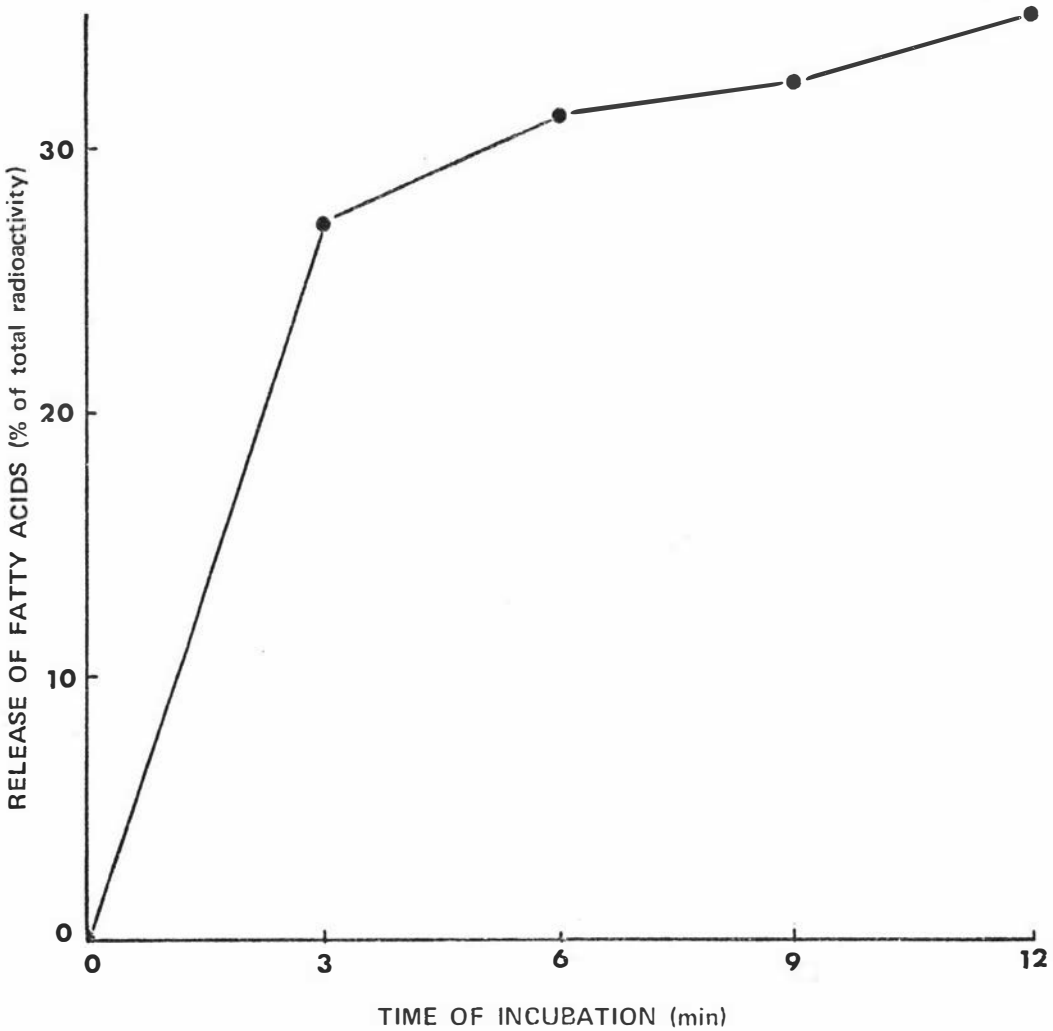


Figure 9. Effect of time on the release of ^{14}C-labelled fatty acids during incubation of ^{14}C-monogalactosyldiglyceride with fescue extract in the presence of autoclaved rumen fluid.

Condition of incubation: As described in Fig. 7 except that ^{14}C-triolein was replaced by ^{14}C-monogalactosyldiglyceride (7.5×10^4 dpm) in 25 µl hexane.

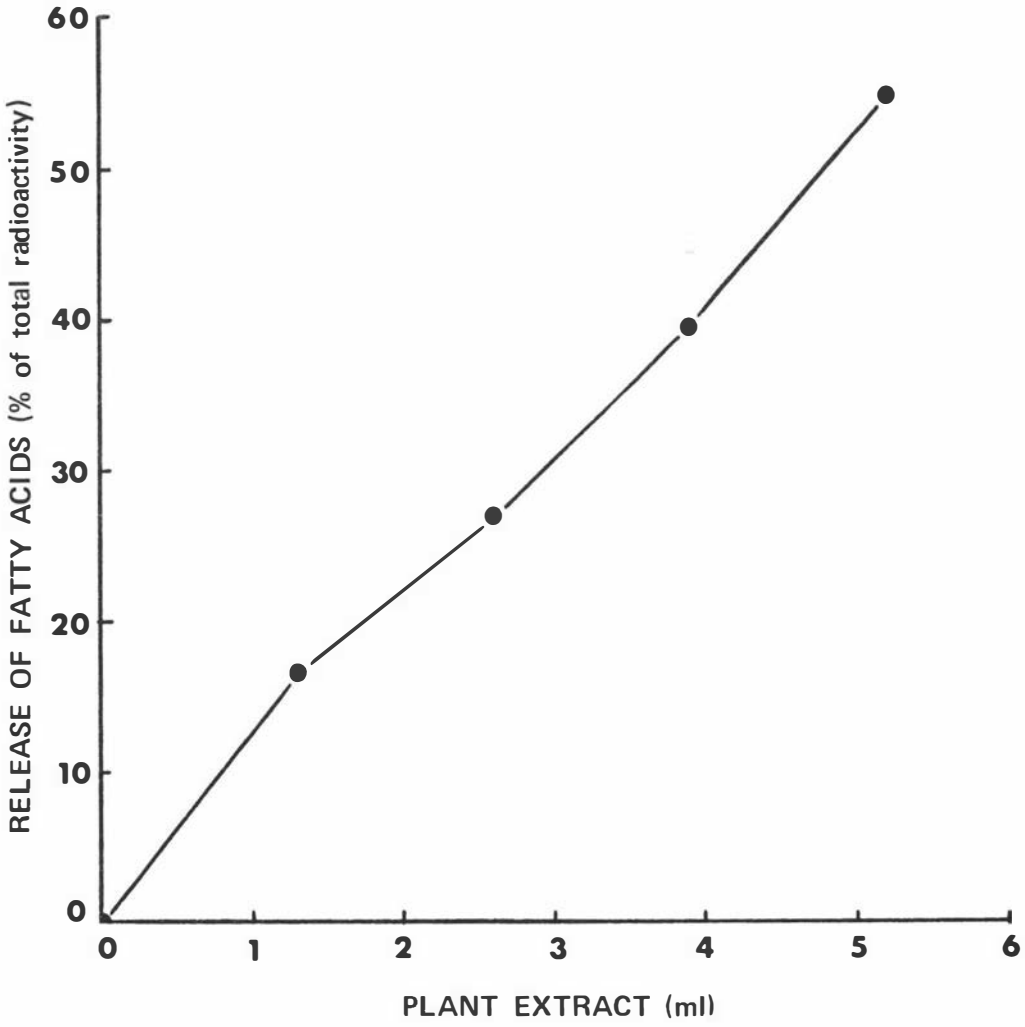


Figure 10. Effect of increasing the concentration of fescue extract on the release of ^{14}C-labelled fatty acids from ^{14}C-monogalactosyldiglyceride.

Conditions of incubation: For each incubation 0, 1.3*, 2.6, 3.9 and 5.2ml fescue extract was mixed with 5.2, 3.9, 2.6, 1.3 and 0ml autoclaved fescue extract respectively; 2.8ml autoclaved rumen fluid and 25 µl hexane containing ^{14}C-monogalactosyldiglyceride (6.6×10^4 dpm) were added. Incubations were carried out for 30min at 39°C under 10% CO_2 in N_2 with constant shaking.

* equivalent to 0.39g fresh leaf tissue.

times of incubation was corrected for the zero time values, a net formation of 6.66 µmol FFA's were observed after 15min incubation, 10.42 µmol after 30min and reached a maximum of 16.96 µmol after 120min (Fig. 11). This net increase of 16.96 µmol FFA represented about 27% hydrolysis of endogenous and added substrate.

(d) Low molecular weight triglycerides

Lipolytic activity of ryegrass extracts towards low molecular weight triglycerides was investigated by following the release of VFA's from emulsified tributyrin and triacetin under the conditions described in Fig. 12. It was not possible to perform the incubation in the presence of rumen fluid because it would have been impossible to distinguish between VFA released from the substrates and endogenous VFA's present in the rumen fluid. Consequently, incubation of only 2.5ml plant extract with 0.2ml triacetin or tributyrin emulsion was carried out for 0-60min.

About 8 µmol acetic acid was present in the incubation mixture containing emulsified triacetin at zero time, indicating that there was some unesterified acetic acid in the triacetin emulsion as contamination, since plant extracts did not contain any endogenous acetate. However, an almost linear increase in the formation of acetic acid with respect to the time of incubation was observed. When acetic acid values formed after different times of incubation were corrected for zero time value, a net formation of 32.54 µmol acetic acid was observed after 45min incubation (Fig.12); this represented 9.5% hydrolysis of the substrate. A linear increase in the formation of butyric acid with the time of incubation was also observed when tributyrin was used as substrate (Fig. 12).

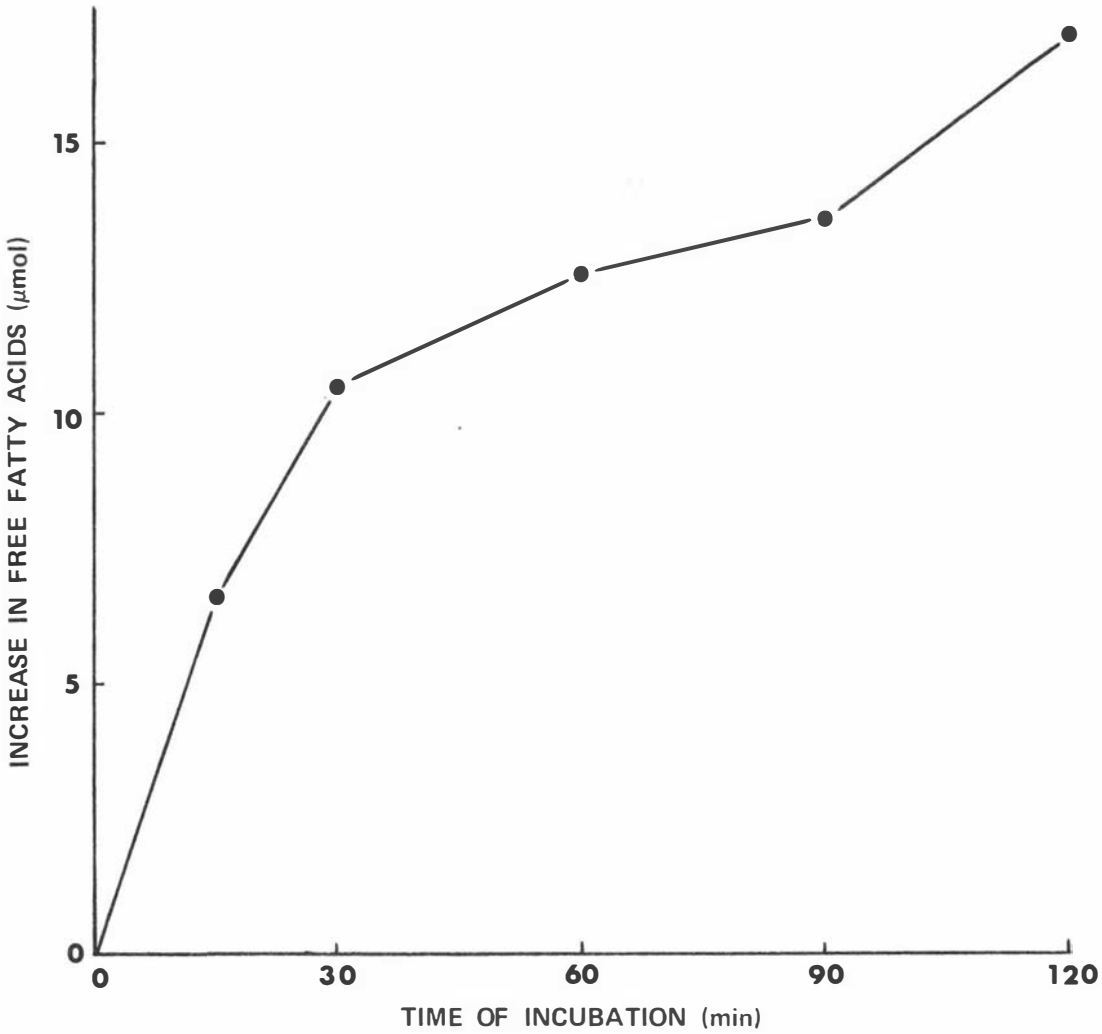


Figure 11. Release of fatty acids from emulisified safflower oil and endogenous lipids by a leaf extract of ryegrass in the presence of autoclaved rumen fluid.

Conditions of lipase assay: The assay was performed by mixing 5 ml* ryegrass extract with 5 ml autoclaved rumen fluid and 0.5 ml safflower oil emulsion (equivalent to 18.43 μmol esterified fatty acids) and incubating at 39°C in the presence of 10% CO_2 in N_2 with continuous shaking.

* equivalent to 2.1 g fresh leaf tissue which contained 45.31 μmol esterified fatty acids.

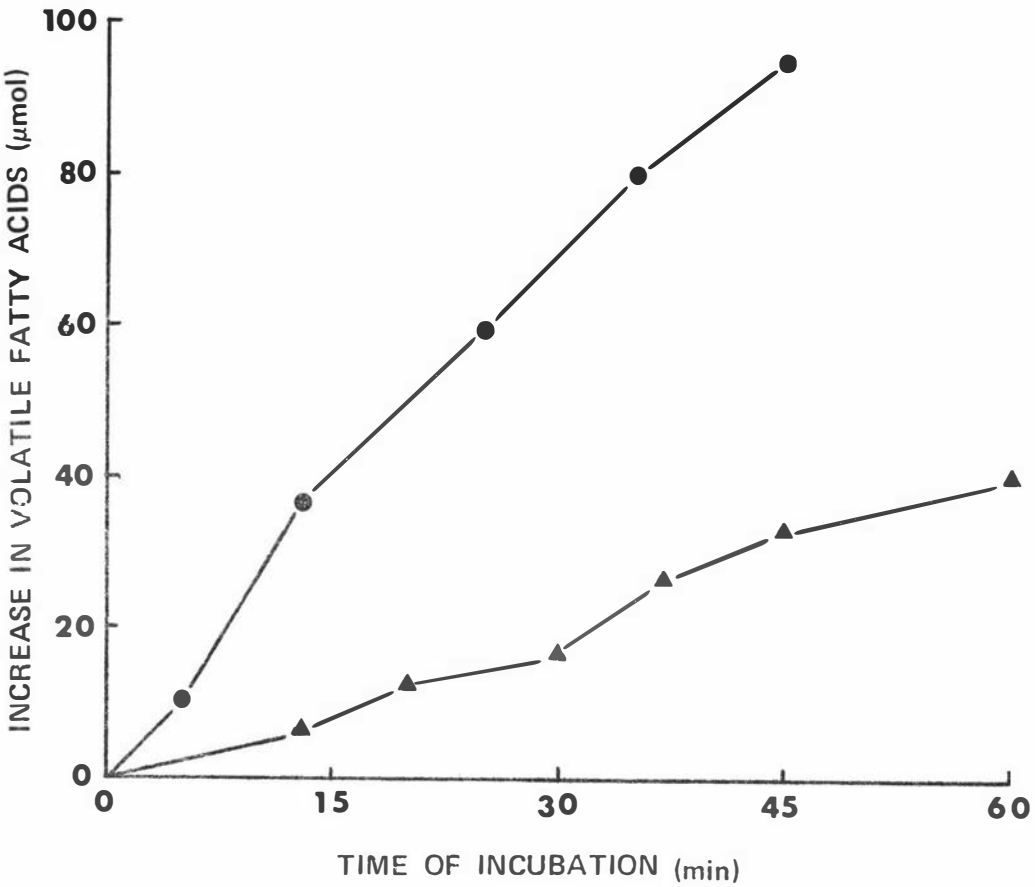


Figure 12. The enzymic hydrolysis of emulsified tributyrin and triacetin by leaf extracts of ryegrass.

Conditions of incubation: 2.5 ml* aliquots of ryegrass extract were mixed with 0.2 ml tributyrin (equivalent to 262 µmol esterified butyric acid) or 0.2 ml triacetin (equivalent to 344.2 µmol esterified acetic acid) emulsion. Incubations were performed at 39°C under 10% CO_2 in N_2 with constant shaking.

Production of VFA's from the appropriate substrates after different times of incubation was determined by analysing 5-20 µl aliquots of the incubation mixture by gas-liquid chromatography (section 2.4).

Key: Butyric acid (● — ●); acetic acid (▲ — ▲).

* equivalent to 0.66g fresh leaf tissue.

No butyric acid was present in the reaction mixture at zero time incubation. Formation of 94.45 μmol butyric acid after 45min incubation represented 36% hydrolysis of tributyrin.

3.3 Lipolytic activities of ryegrass extracts in the presence of rumen microorganisms

Having established that leaf extracts from a wide variety of pasture species possessed lipolytic activity under conditions similar to those found in the rumen, varying quantities of ryegrass extracts were incubated with ^{14}C-triolein in the presence of rumen microorganisms and the formation of ^{14}C-labelled hydrolysis products from ^{14}C-triglyceride was followed (Fig. 13).

When rumen fluid was incubated with ^{14}C-triglyceride in the absence of active plant enzymes, only 2.0% of the total ^{14}C-label was recovered in the FFA fraction during a 20min incubation (Fig. 13). In contrast, the addition of only 2ml plant extract to rumen fluid resulted in the release of 55.6% of the total radioactivity in the FFA fraction, thereby indicating that lipase activity of rumen fluid in the absence of active plant enzymes was very low and this increase in ^{14}C-label in the fatty acid fraction was due to the enzymes of plant origin. The plant source of the lipases was further confirmed by varying the amount of plant extract. The formation of FFA increased with increasing enzyme concentration. FFA's were the major radioactive product of hydrolysed ^{14}C-triglyceride, although low ^{14}C-label was detected in mono- and diglyceride components. The corresponding decrease in the ^{14}C-label in the unhydrolysed triglyceride is also shown in Fig. 13.

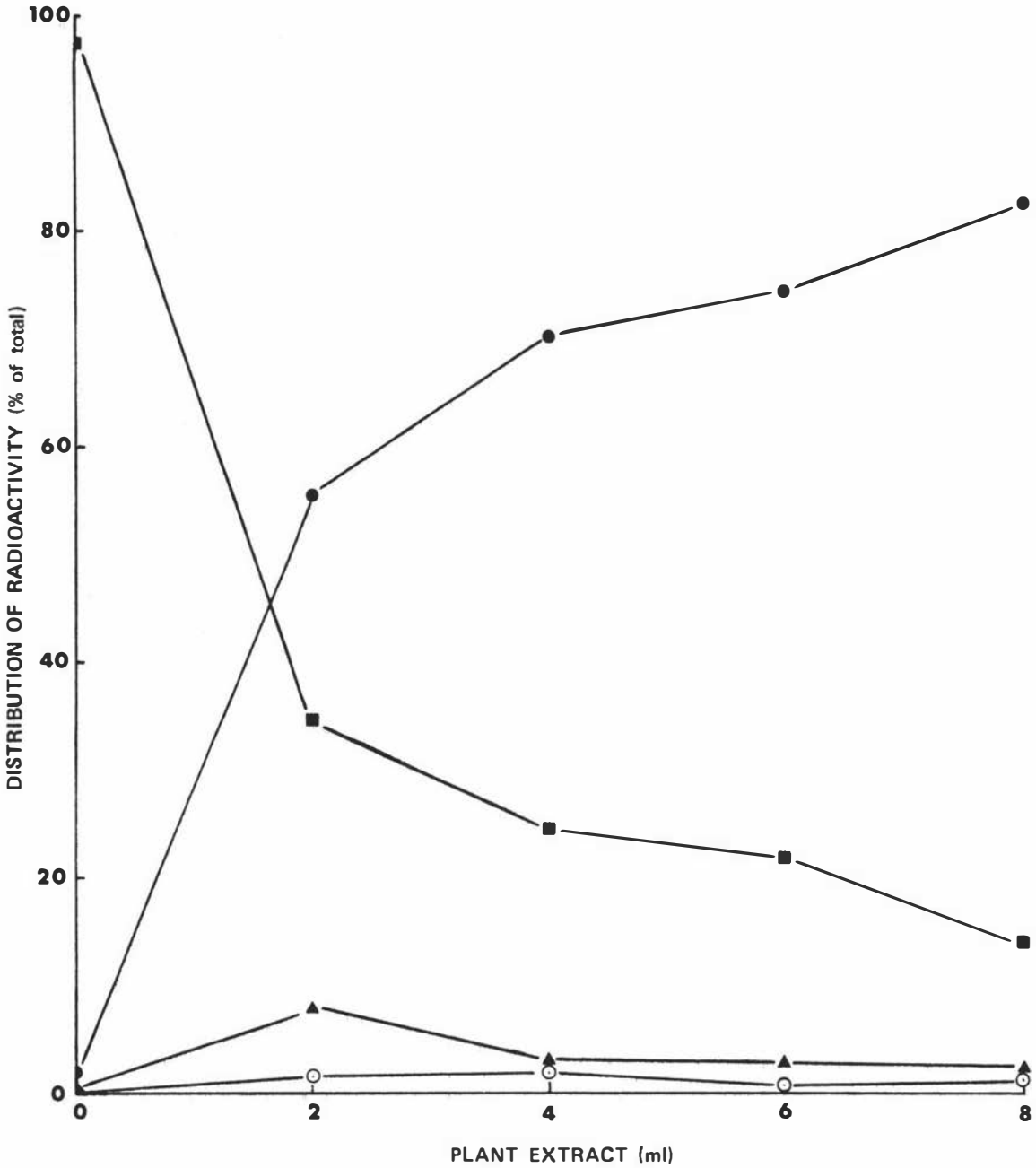


Figure 13. The relationship between the amount of ryegrass extract and lipolysis of ^{14}C-triolein in the presence of metabolising rumen microorganisms.

Conditions of lipase assay: Each individual incubation contained 0, 2*, 4, 6 and 8 ml ryegrass extract and 8, 6, 4, 2 and 0 ml autoclaved ryegrass extract respectively, 2 ml rumen fluid, 25 µl hexane containing ^{14}C-triolein (9.9 x 10^4 dpm; 1.32 nmol). Incubations were carried out for 30 min at 39°C under 10% CO_2 in N_2 with continuous shaking.

Key: Triglyceride (■ — ■); diglyceride (▲ — ▲); monoglyceride (○ — ○); free fatty acid (● — ●).

* equivalent to 0.71g fresh leaf tissue.

Further investigations were carried out to demonstrate the activity of plant lipases in the presence of rumen microorganisms. Enzymatically active and autoclaved ryegrass extracts were preincubated with rumen fluid for 0.5h followed by incubation with ^{14}C-triolein for further 0.5h (Table 2). Rumen fluid in the presence of autoclaved plant extracts showed a very low level of lipase activity — only 12.2% of the ^{14}C was released as FFA and this slower rate of lipolysis of ^{14}C-triglyceride was accompanied by increased accumulation of ^{14}C-diglyceride (8.4%). In contrast, when autoclaved ryegrass extract was replaced by active ryegrass extract, a much higher lipase activity was observed — 87% of the radioactivity was recovered in the FFA fraction (Table 2).

TABLE 2

The lipolytic activity of an extract of ryegrass leaves after incubation with rumen fluid compared with lipolytic activity of rumen microorganisms.

Conditions of incubation: Each 50ml flask contained autoclaved or unautoclaved ryegrass extract (5ml*) and rumen fluid (5ml); incubations were carried out for 30min at 39°C under a mixture of 10% CO_2 in N_2 with continuous shaking followed by the addition of 25 µl hexane containing ^{14}C-triolein (9.0 x 10^{4}dpm; 1.2 nmol). The incubation was continued for another 30min to follow lipolysis.

Conditions	Time of incubation before lipase assay (min)	Distribution of radioactivity (% of total)			
		Tri-glyceride	Di-glyceride	Mono-glyceride	Free fatty Acid
Rumen fluid + ryegrass extract	30	8.0	3.3	1.7	87.0
Rumen fluid + autoclaved ryegrass extract	30	78.6	8.4	0.8	12.2

* equivalent to 1.25g fresh leaf tissue.

3.4 Stability of plant lipases in the presence of rumen microorganisms

(a) Total rumen fluid

A limitation on the activity of enzymes of dietary origin in the rumen is likely to be imposed by the proteolytic activity of rumen microorganisms. In order to assess the probable stability of plant lipases in the rumen, fescue extracts were preincubated with rumen fluid for 1.5-5h followed by the measurement of residual lipase activity towards ^{14}C-triolein over a 1h period of incubation (Table 3 and Fig. 14). During the course of these experiments, the pH of the incubation mixtures after different times of incubation ranged from 7.1 - 6.5. The lipase activity of rumen fluid measured after preincubation periods of 1.5 to 3h in the presence of autoclaved fescue extracts was very low. However, the activity increased when the preincubation period was extended to 5h — 31.5% of the total radioactivity was recovered in the FFA (Fig. 14). This increased lipolytic activity was attributed to lipases of microbial origin. In contrast, when autoclaved fescue extract was replaced by enzymatically active fescue extract, about 50% of the radioactivity in ^{14}C-triglyceride appeared as FFA after preincubation times of 1.5 to 5h. Lipase activity was lower after 1.5h preincubation than without preincubation. The slight increase in activity with increased period of preincubation could be attributed to the activity of microbial lipases referred to above superimposed on the activity of plant extracts. During lipolysis of ^{14}C-triolein by rumen fluid in the presence of both active and autoclaved fescue extracts at different times of preincubation, there was a small but variable accumulation of mono- and diglyceride (Table 3).

In order to obtain a relationship between the extent of lipolysis

TABLE 3

The lipolytic activity of fescue leaf extract after incubation with strained rumen fluid compared with the lipolytic activity of rumen microorganisms at different levels of metabolic activity*.

Conditions of incubation: Each 50ml flask contained autoclaved or unautoclaved fescue extract (6.6ml†) and rumen fluid (3.3ml); incubations were performed at 39°C under 10% CO_2 in N_2 with continuous shaking. At the end of each incubation 25 µl hexane containing ^{14}C-triolein (9.9×10^4dpm; 1.32 nmol) was added and the incubation was continued for a further 1h to follow lipolysis.

Conditions	Time of incubation before lipase assay (h)	Distribution of radioactivity (% of total)			
		Tri-glyceride	Di-glyceride	Mono-glyceride	Free fatty acid
Rumen fluid + autoclaved fescue extract	0	87.7	2.6	0.3	9.4
	1.5	89.1	1.8	0.3	8.8
	3.0	85.3	2.4	0.3	12.0
	5.0	63.1	5.0	0.4	31.5
Rumen fluid + fescue extract	0	16.2	1.7	0.8	81.3
	1.5	46.6	3.4	0.3	49.7
	3.0	46.7	1.8	0.3	51.2
	5.0	42.0	2.7	0.2	55.1

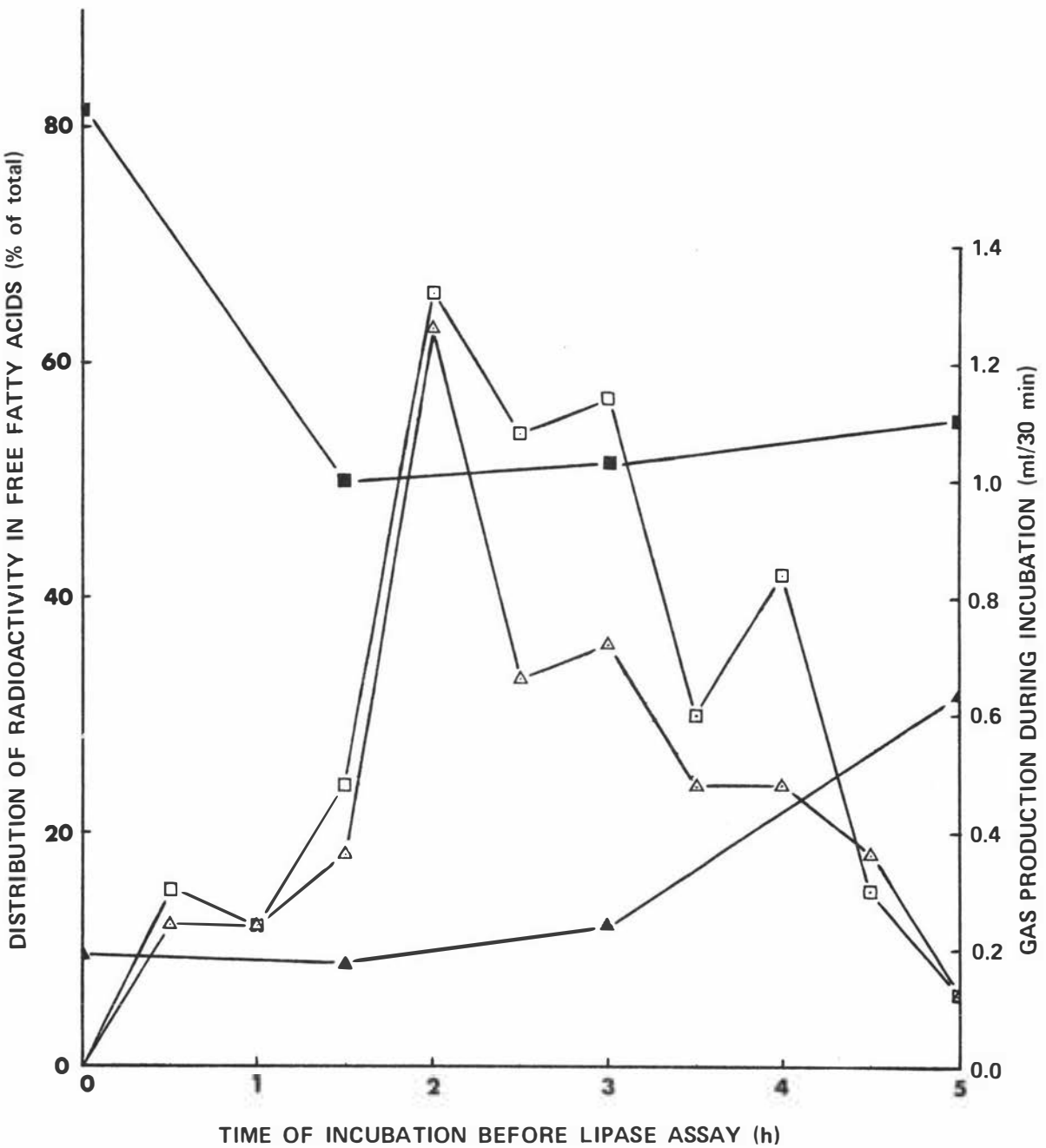
* as indicated by gas production in Fig. 14.

† equivalent to 2.2g fresh leaf tissue.

Figure 14. The lipolytic activity of fescue leaf extract after incubation with strained rumen fluid compared with the lipolytic activity of rumen microorganisms at different levels of metabolic activity.

See Table 3 for conditions of incubation.

Key: FFA's released by (a) rumen fluid + autoclaved fescue extract (▲—▲), (b) rumen fluid + fescue extract (■ — ■); gas produced by (a) rumen fluid + autoclaved fescue extract (□ — □), (b) rumen fluid + fescue extract (△ — △).


DISTRIBUTION OF RADIOACTIVITY IN FREE FATTY ACIDS (% of total)
GAS PRODUCTION DURING INCUBATION (ml/30 min)
TIME OF INCUBATION BEFORE LIPASE ASSAY (h)
0
20
40
60
80
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1
2
3
4
5

and growth of the microorganisms, microbial fermentation of the grass extracts was followed by measuring the rate of gas production per 30min incubation independently. The pattern of microbial fermentation of both active and autoclaved fescue extracts appeared to be similar. There were substantial increases in the rate of gas production during incubations of up to 2h which indicated a high metabolic activity resulting from the addition of plant extracts (Fig. 14). A similar increase in the rate of gas production was observed when plant extracts were replaced by sucrose, glucose and galactose. After 2h, the rate of gas production declined steadily. The period of increased activity of microbial lipases corresponded to times when the overall metabolic activity was declining, probably due to depletion of substrate (Fig. 14).

A more detailed examination of the relationship between the stimulation of lipase activity of microbial origin and the addition of the nutrients in plant extracts confirmed the above observations. Autoclaved ryegrass extract gave rise to an immediate substantial increase in the rate of gas production which continued for about 2h before returning to the initial level after 6h (Fig. 15). On the other hand, levels of FFA's arising from the incubations with ^{14}C-triolein did not increase until preincubation of the rumen fluid with autoclaved plant extract had continued for 3h. At this stage, the overall metabolic activity of the microorganisms was declining (Fig. 15). After 4 and 5h preincubation, 18 and 26% respectively of the added label was isolated as FFA and the latter level was maintained after a 6h preincubation. Only low levels of radioactivity were associated with mono- and diglyceride (Table 4).

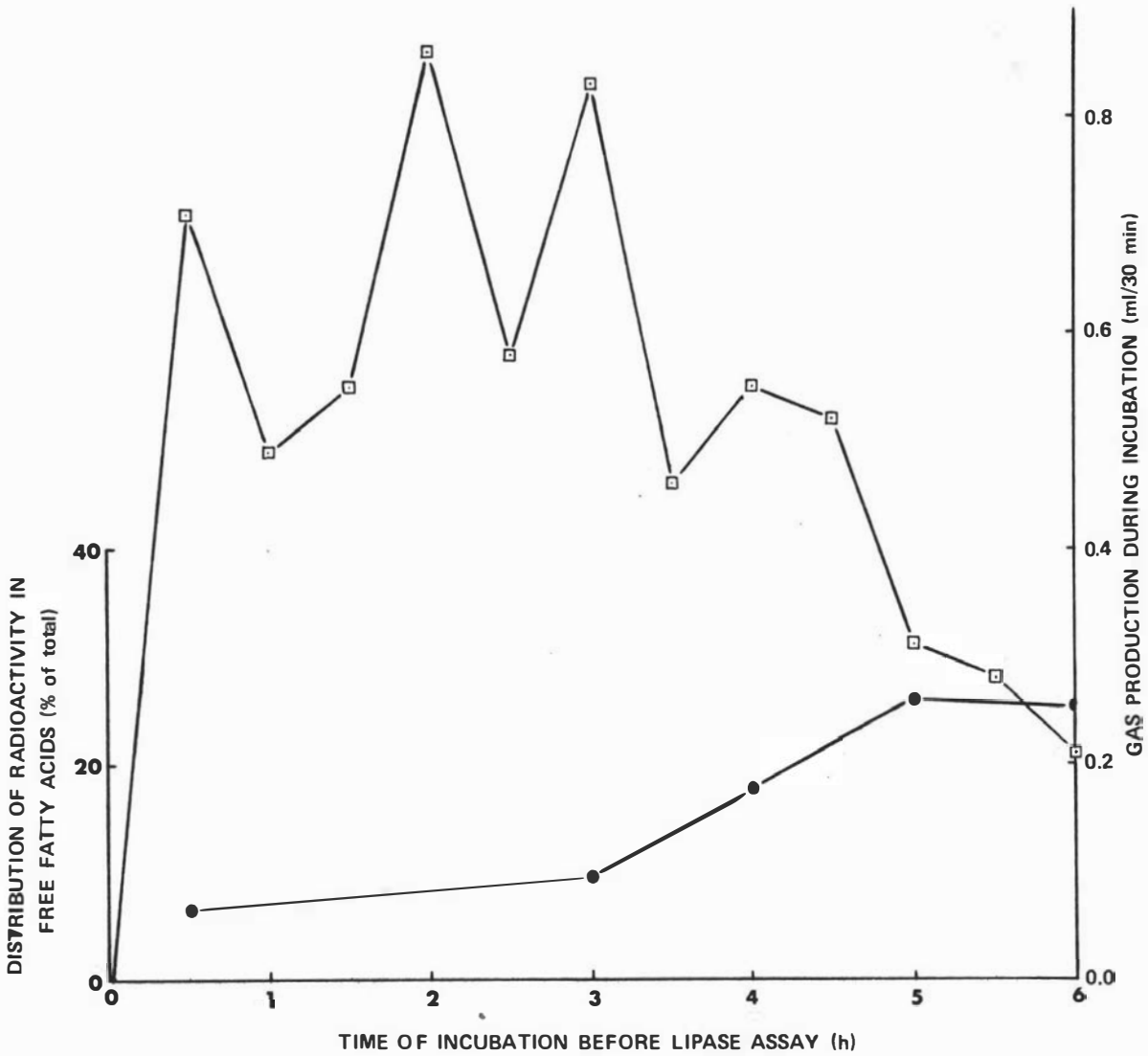


Figure 15. The lipolytic activity of rumen microorganisms at different levels of metabolic activity.

See Table 4 for conditions of incubation.

Key: Free fatty acid (● — ●); gas production (□ — □).

TABLE 4

The lipolytic activity of rumen microorganisms at different levels of metabolic activity*.

Conditions of incubation: Each 50ml flask contained autocalved ryegrass extract (6.6ml†) and rumen fluid (3.3ml); incubations were performed at 39°C under 10% CO_2 in N_2 with continuous shaking; at the end of each incubation 25 µl hexane containing ^{14}C-triolein (9.9×10^4 dpm; 1.32 nmol) was added and the incubation was continued for a further 1h to follow lipolysis.

Conditions	Time of incubation before lipase assay (h)	Distribution of radioactivity (% of total)			
		Tri-glyceride	Di-glyceride	Mono-glyceride	Free fatty acid
Rumen fluid + autoclaved ryegrass extract	0.5	91.5	1.8	0.2	6.5
	3.0	87.4	2.6	0.3	9.7
	4.0	78.6	3.3	0.2	17.9
	5.0	71.3	2.5	0.2	26.0
	6.0	70.4	4.1	0.2	25.3

* as indicated by gas production in Fig. 15.
† equivalent to 1.86g fresh leaf tissue.

(b) Protozoa-free rumen fluid

In an attempt to assess the stability of plant lipases in the presence of mixed rumen bacteria, fescue extracts were preincubated with protozoa-free rumen fluid for 1-8h followed by the measurement of lipase activity towards ^{14}C-triolein over a 2h period of incubation (Table 5 and Fig. 16). Protozoa-free rumen fluid showed essentially the same pattern of behaviour as total rumen fluid. Lipase activity of protozoa-free rumen fluid measured after a preincubation period of 1-4h with autoclaved fescue extracts was very low. The lipolytic activity increased to some extent after 8h preincubation — 24% of the total radioactivity was recovered in the FFA fraction (Fig. 16). By comparison, preincubation of active fescue extract with protozoa-free

TABLE 5

The lipolytic activity of fescue leaf extract after incubation with protozoa-free rumen fluid compared with the lipolytic activity of the same rumen fluid after incubation with autoclaved fescue extract.

Conditions of incubation: Each 50ml flask contained autoclaved or unautoclaved fescue extract (6.6ml*) and protozoa-free rumen fluid or autoclaved rumen fluid (3.3ml); incubations were carried out at 39°C in an atmosphere of 10% CO_2 in N_2 with continuous shaking; at the end of each incubation 15 µl hexane containing ^{14}C-triolein (1.37×10^5dpm; 1.83 nmol) was added and the incubation was continued for another 2h to follow lipolysis.

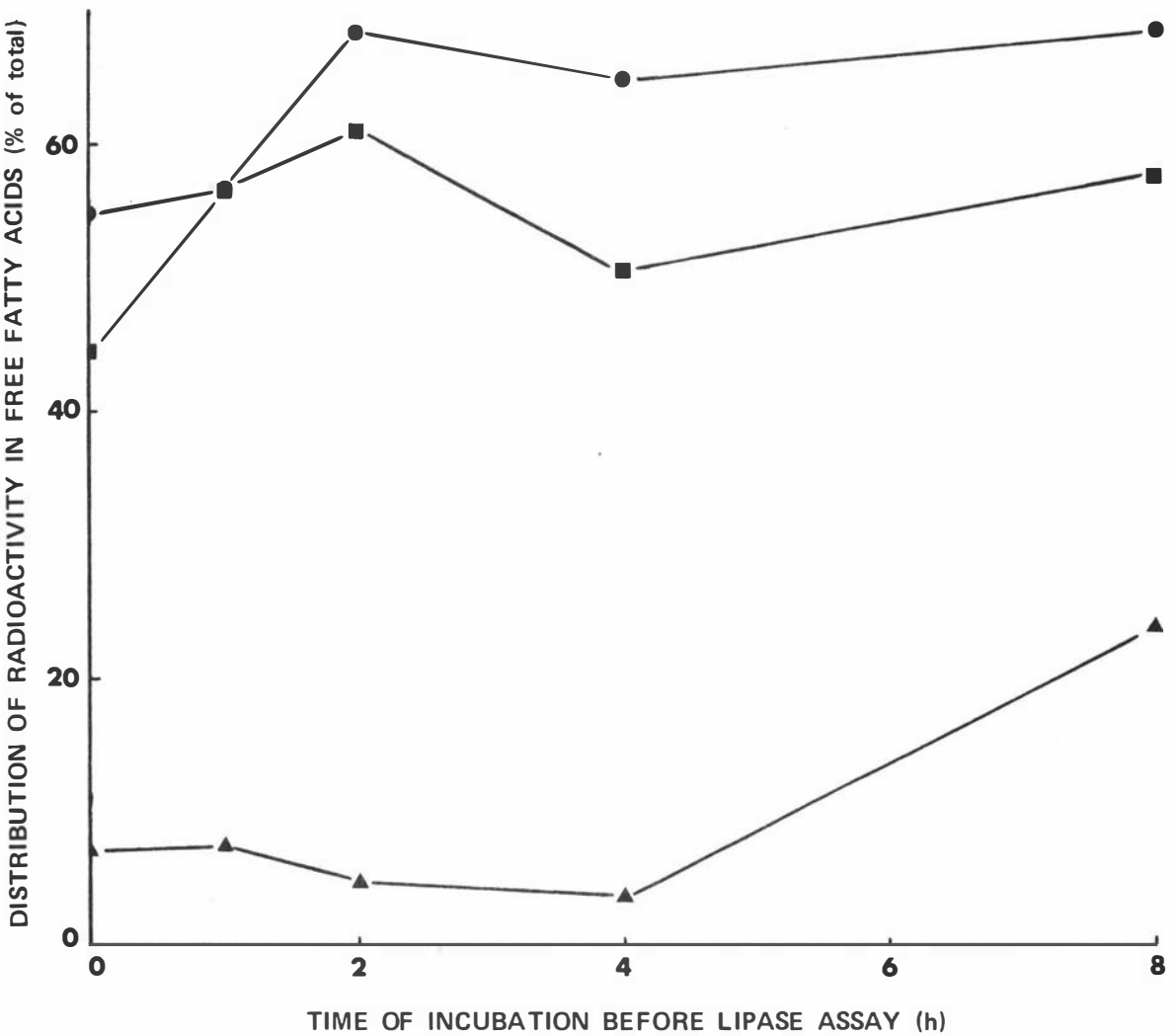
Conditions	Time of incubation before lipase assay (h)	Distribution of radioactivity (% of total)			
		Tri-glyceride	Di-glyceride	Mono-glyceride	Free fatty acid
Protozoa-free rumen fluid + autoclaved fescue extract	0	89.1	3.0	0.8	7.1
	1	87.5	4.4	0.6	7.5
	2	92.8	2.2	0.4	4.6
	4	94.2	1.8	0.5	3.5
	8	68.2	7.1	0.7	24.0
Protozoa-free rumen fluid + fescue extract	0	51.5	3.0	1.1	44.4
	1	39.6	2.9	1.1	56.4
	2	34.9	3.4	0.9	60.8
	4	44.5	4.1	0.9	50.5
	8	36.9	4.9	0.6	57.6
Autoclaved rumen fluid + fescue extract	0	40.4	3.7	1.1	54.8
	1	36.8	4.7	1.8	56.7
	2	26.5	3.1	2.1	68.3
	4	30.2	3.3	1.6	64.9
	8	22.9	5.0	3.8	68.3

* equivalent to 2.42g fresh leaf tissue.

Figure 16. The lipolytic activity of fescue leaf extract after incubation with protozoa-free rumen fluid compared with the lipolytic activity of the same rumen fluid after incubation with autoclaved fescue extract.

See Table 5 for conditions of incubation.

Key: FFA's released by (a) rumen fluid + autoclaved fescue extract (▲—▲), (b) rumen fluid + fescue extract (■—■) and (c) autoclaved rumen fluid + fescue extract (●—●).


DISTRIBUTION OF RADIOACTIVITY IN FREE FATTY ACIDS (% of total)
0
20
40
60
0
2
4
6
8
TIME OF INCUBATION BEFORE LIPASE ASSAY (h)

rumen fluid resulted in consistently high lipase activity for up to 8h — about 45% of the radioactivity from ^{14}C-triglyceride appeared as FFA when preincubation was omitted. Lipase activity was greater after 2h preincubation; although there were some fluctuations, the activity remained high for up to 8h preincubation. This indicates that plant lipases remained active in the presence of mixed rumen bacteria for up to 8h. It is also apparent from the results presented in Fig.16 that after preincubation with autoclaved rumen fluid, fescue extracts showed the highest level of lipase activity. Lipases removed 54.8% of the radioactivity from the ^{14}C-triglyceride into FFA when preincubation was omitted. The lipase activity increased after 2h preincubation and the level remained almost constant over the periods of 2-8h preincubation.

During hydrolysis of ^{14}C-triglyceride by lipases from plant and protozoa-free rumen fluid, low levels of mono- and diglycerides were formed. The proportions of radioactivity in these intermediate compounds and in residual triglyceride were shown in Table 5.

3.5 Lipase activity of rumen fluid obtained before and after feeding on ryegrass pasture

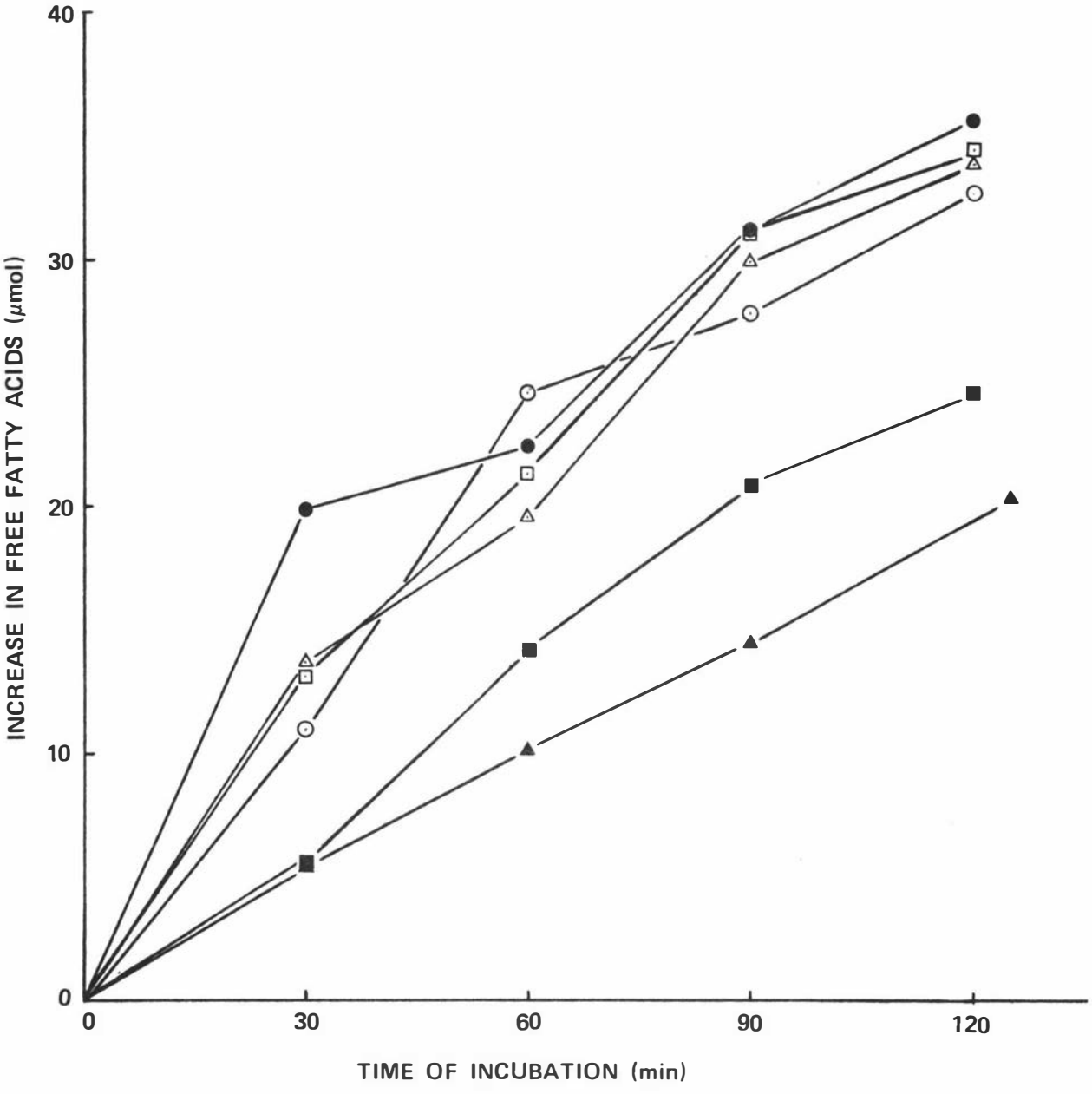
In order to assess the contribution of plant lipases to lipolytic activity under *in vivo* conditions, a fistulated cow which had been fasted for 18h, was fed *ad lib* with ryegrass for 1.5h. The cow was then held in a yard with no access to food for the period of sampling. Samples of rumen digesta were removed at regular intervals. For the measurement of lipase activity, rumen fluid samples were incubated with emulsified safflower oil and autoclaved plant extract under the conditions described in Fig. 17 immediately after removal from the

Figure 17. Release of fatty acids from emulsified safflower oil and endogenous ryegrass lipids by rumen fluid obtained from a 18h-fasted cow and after feeding on ryegrass.

Conditions of incubation: Assays were performed by mixing 5 ml aliquots from each rumen fluid sample with 5 ml* autoclaved ryegrass extract and 0.5 ml safflower oil emulsion (equivalent to 18.43 µmol esterified fatty acids) and incubating at 39°C in the presence of 10% CO_2 in N_2 with continuous shaking.

Key: Pre-feeding (18h - fasted) (▲—▲).
Post-Feeding: 0.5h (●—●); 1.45h (○—○); 3.0h (△—△); 5h (□—□); 8h (■—■).

* equivalent to 2.0g fresh leaf tissue which contained 35.35 µmol esterified fatty acids.


40
30
20
10
0
INCREASE IN FREE FATTY ACIDS (μmol)
0
30
60
90
120
TIME OF INCUBATION (min)

rumen. The release of FFA's was determined colorimetrically. Variable levels over a range of 5.36-31.07 µmol of endogenous FFA's were present in the reaction mixture of all rumen samples at zero time. FFA values formed after different times of incubation were corrected for the zero time value (Fig. 17). Rumen fluid obtained 0.5h after feeding possessed the highest level of lipase activity; after 30min, about 20 µmol FFA's were released from the substrate. Thereafter, the rate of release was linear reaching 35.6 µmol after 120min; this was about twice the amount of FFA released by rumen fluid obtained from a fasted cow during 120min incubation. Rumen fluid obtained 1.45, 3.0 and 5h post-feeding possessed lipolytic activity which was intermediate between pre-feeding and 0.5h post-feeding levels. The levels of lipase activity decreased when the time of sampling increased up to 8h after feeding (Fig. 17).

3.6 The incorporation of acetate into long-chain fatty acids by rumen microorganisms

During incubation of rumen fluid with emulsified safflower oil and autoclaved plant extract, there were increases in FFA's with the time of incubation (Fig. 17). In order to check whether these increases were due to de novo synthesis of FFA's by rumen microorganisms, investigations on the incorporation of acetate by rumen fluid into FFA's were carried out under the conditions similar to those described in Fig. 17.

5ml rumen fluid was incubated with ^{14}C-acetate in the presence of 4.8ml autoclaved plant extract and 0.5ml safflower oil emulsion (Fig. 18). There was a net incorporation of 29.5 nmol acetate into FFA's after 30min incubation (Fig. 18) and the rate of utilization of

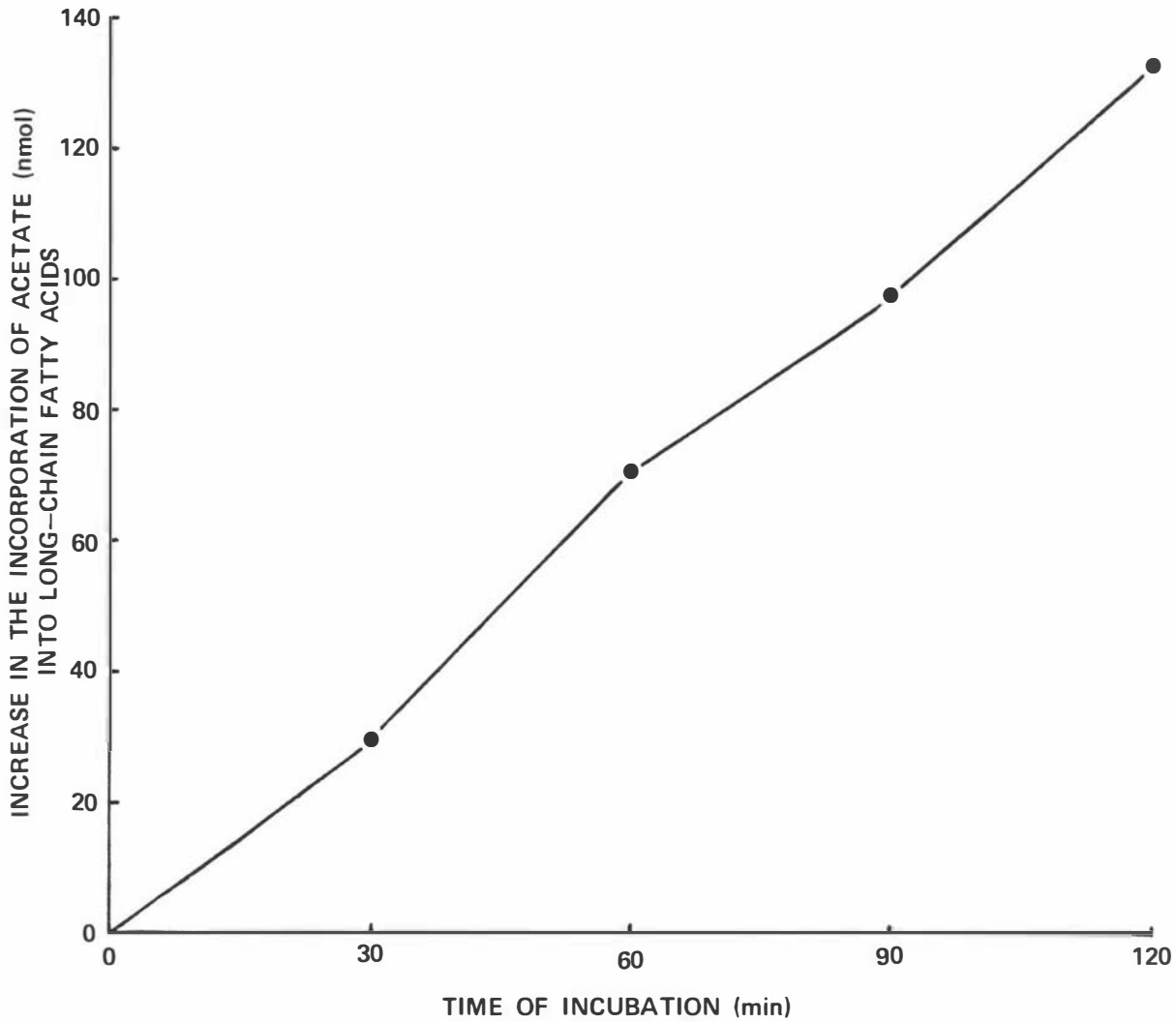


Figure 18. The incorporation of acetate into long-chain fatty acids by rumen microorganisms in the presence of autoclaved ryegrass extract.

Reaction mixture: 5 ml rumen fluid, 4.8 ml* autoclaved ryegrass extract, 0.2 ml aqueous solution of ^{14}C-acetate (2.2×10^7 dpm; 0.18 µmol) and 0.5 ml safflower oil emulsion (equivalent to 18.43 µmol esterified fatty acids). See section 2.13. for details of the conditions of incubation.

* equivalent to 1.92g fresh leaf tissue.

acetate increased with the time of incubation; and reached a value of only 132.5 nmol after 120min. Zero time value was 24.5 nmol. Consequently it was concluded that increases in FFA's with the time of incubation as observed in Fig. 17 were unlikely to be due to de novo synthesis of FFA's by rumen microorganisms.

3.7 Lipolytic activity of clarified rumen fluid prepared from a fasted and a fresh ryegrass-fed ruminant

For the comparison of the lipase activity of clarified rumen fluids (CRF) obtained from fasted and fed ruminants, a pair of monozygotic twin cows was withheld from feeding for about 18h. One of the twins was allowed to graze ryegrass for 1.5h and then withheld from feeding for 0.5h before sampling; while the other cow continued to be fasted. CRF was prepared (section 2.10) from the rumen samples of both cows immediately after sampling. To follow lipolysis, samples of CRF were incubated with emulsified safflower oil and autoclaved plant extract under the conditions described in Fig. 19. Without prior removal of VFA, total fatty acids were estimated colorimetrically (subsection 2.12.5). Since CRF is likely to be almost free from microorganisms (Blackburn and Hobson, 1960a; Dawson et al., 1964), it can be assumed that no VFA will be produced over the reaction time. At zero time, 38.52 µmol and 77.16 µmol fatty acids were present in the reaction mixture of rumen samples from the 20h-fasted and fed animals respectively. Fatty acids formed at different times of incubation were corrected for zero time values.

CRF prepared from the twin cow which was fasted for 20h showed very little lipolytic activity during 0.5h incubation; but the enzyme

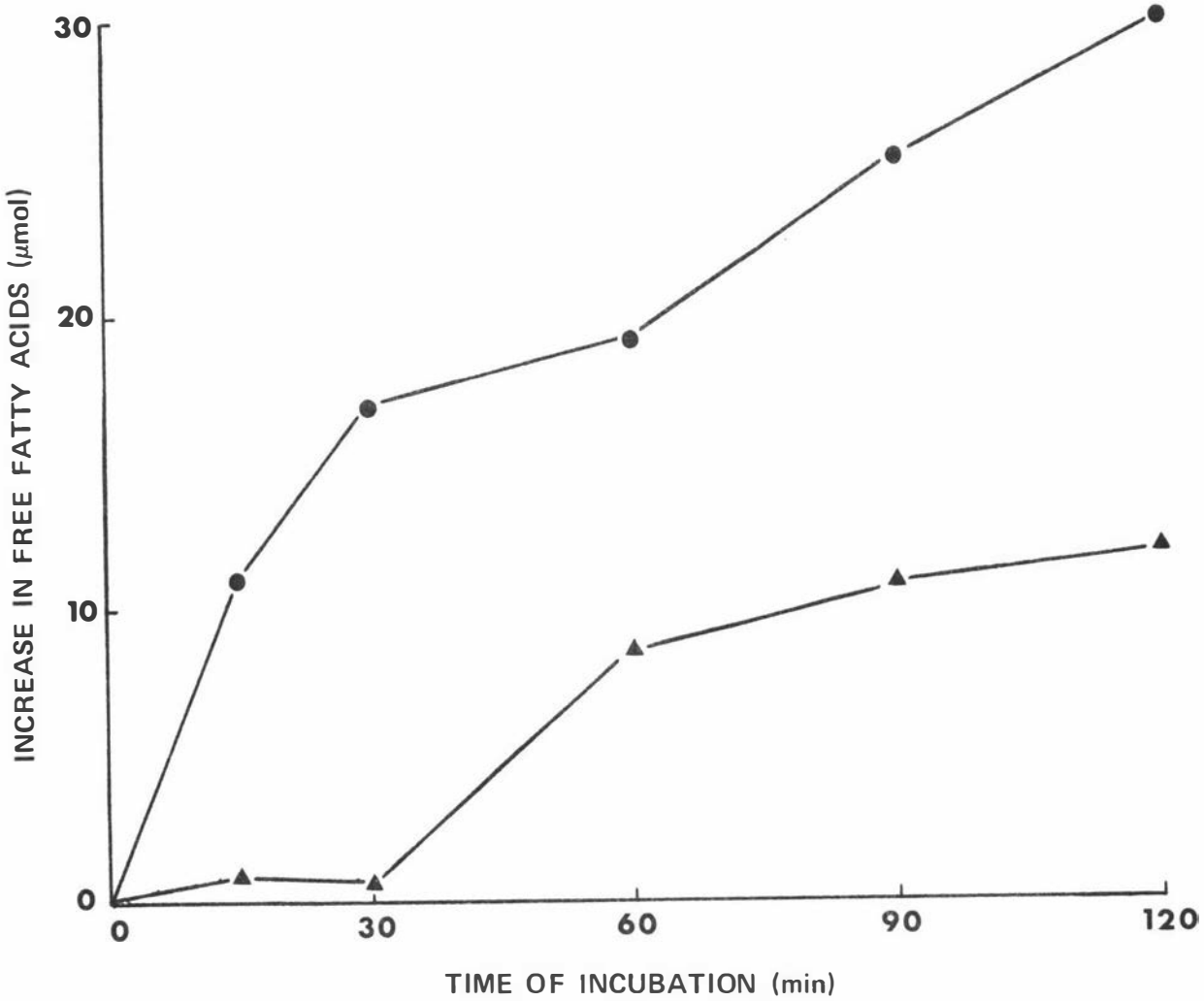


Figure 19. Release of fatty acids from emulsified safflower oil and endogenous ryegrass lipids by clarified rumen fluid from fasted and fed identical twin cows.

Conditions of incubation: Lipase assays were performed by mixing 5ml aliquots from clarified rumen fluid samples with 5ml* autoclaved ryegrass extract and 0.5ml safflower oil emulsion (equivalent to 18.43 µmol esterified fatty acids) and incubating at 39°C under 10% CO_2 in N_2 with continuous shaking.

Key: 20h-fasted (▲—▲); 0.5h after feeding ryegrass (●—●).

* equivalent to 1.8g fresh leaf tissue which contained 49.55 µmol esterified fatty acids.

activity increased after 1h when 8.6 µmol FFA's were liberated (Fig. 19). Further increase in the time of incubation resulted in only a slow increase in FFA's to reach a maximum value of 12 µmol after 2h. The corresponding incubation of substrates with CRF prepared from the twin cow 0.5h after feeding ryegrass showed a much higher lipolytic activity (Fig. 19): 10.77 µmol FFA's were released after only 15min incubation; a linear increase in the release of FFA's was observed with the time of incubation reaching a maximum of 29.91 µmol after 2h.

The higher lipase activity in CRF from a fed compared with a fasted cow was confirmed using ^{14}C-triolein as substrate, when 53% of the label was recovered as FFA compared with 38% in the fasted animal over a 30min incubation.

3.8 Lipase activity of protozoa-free rumen fluid obtained from cows fed on hay and on fresh pasture

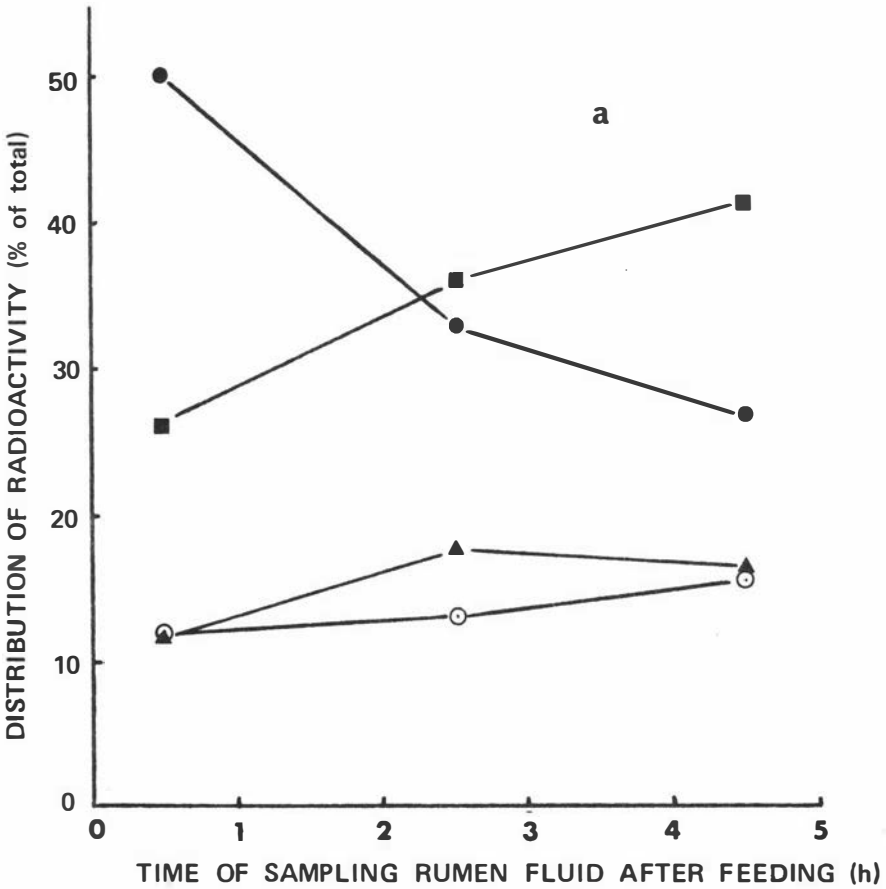
For the comparison between the lipase activity of protozoa-free rumen fluid from a hay-fed and a fresh pasture-fed animal, the cows were fasted for 18h and then allowed to feed their respective diets for 45min. They were then held in a yard with no access to food for the period of sampling. Each rumen sample was immediately freed from protozoa and plant debris according to the procedure described in section 2.10. To follow lipolysis, 10ml aliquots of each rumen fluid was incubated with ^{14}C-triolein under the conditions described in Fig. 20.

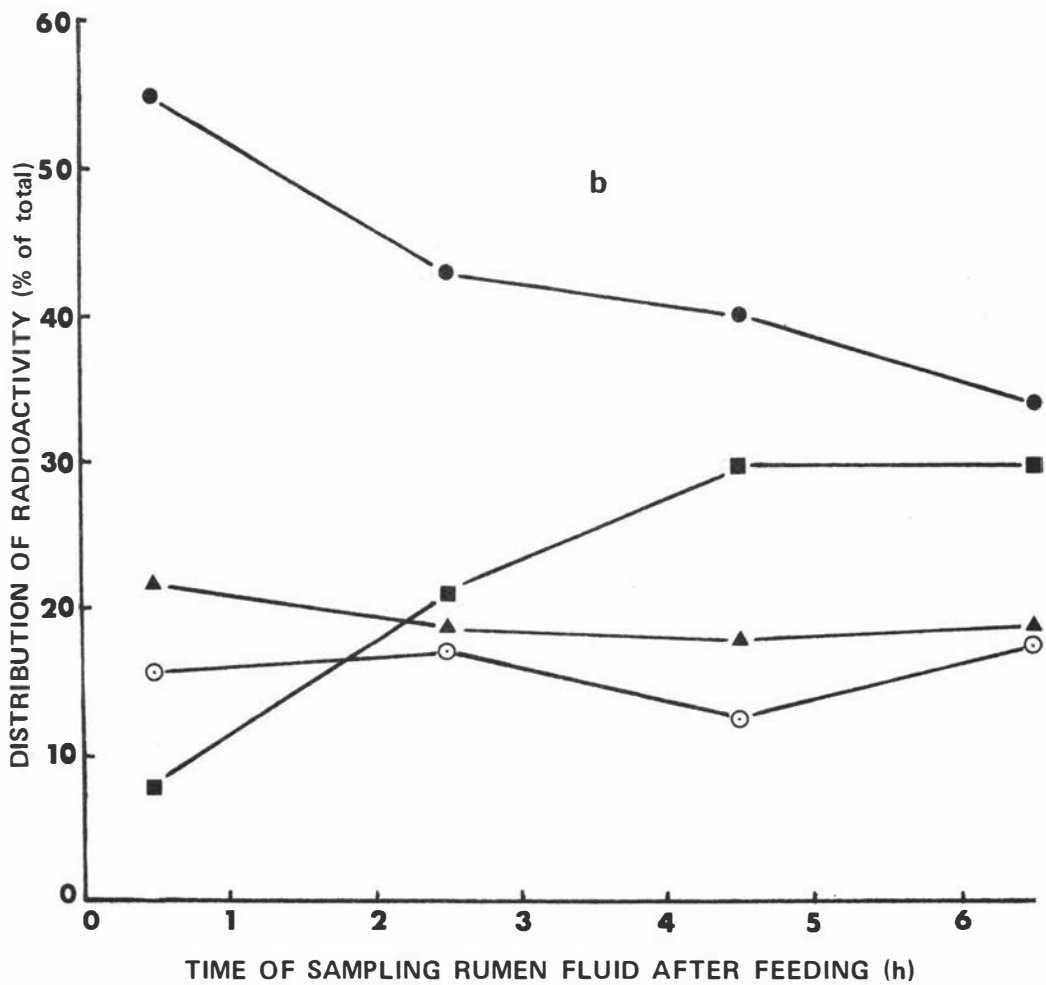
Rumen fluid prepared from the fresh pasture-fed animal 0.5h after feeding showed the highest level of lipase activity since 55.1%

Figure 20. Lipolysis of ^{14}C-triolein by protozoa-free rumen fluid obtained from cows fed on hay (a) and on fresh pasture (b) at different times after feeding.

Conditions of lipase assay: 10 ml protozoa-free rumen fluid from each rumen sample; ^{14}C-triolein (2.1×10^5 dpm; 2.84 nmol) in 20 µl hexane; 80 min incubation at 39°C in the presence of 10% CO_2 in N_2 with constant shaking.

Key: Triglyceride (■ — ■); diglyceride (▲ — ▲); monoglyceride (○ — ○); free fatty acid (● — ●).


a
DISTRIBUTION OF RADIOACTIVITY (% of total)
0
10
20
30
40
50
0
1
2
3
4
5
TIME OF SAMPLING RUMEN FLUID AFTER FEEDING (h)


b
DISTRIBUTION OF RADIOACTIVITY (% of total)
0
10
20
30
40
50
60
0
1
2
3
4
5
6
TIME OF SAMPLING RUMEN FLUID AFTER FEEDING (h)

of the ^{14}C from the triglyceride appeared as FFA's and due to an increased accumulation of mono- and diglycerides, only 7.7% of the ^{14}C remained in the unhydrolysed triglyceride (Fig. 20b). Lipase activity of rumen fluid decreased with the time after feeding. The enzymes released 34.1% of the total ^{14}C into FFA's 6.5h after the last feed (Fig. 20b). By comparison, rumen fluid preparations obtained from the animal fed on hay possessed less lipase activity. Rumen fluid obtained 0.5, 2.5 and 4.5h after feeding transferred 50.2%, 33.0% and 26.9% of the ^{14}C from the triglyceride to the FFA fractions respectively (Fig. 20a). It is also evident from Fig. 20a and 20b that during lipolysis of ^{14}C-triolein by both sources of rumen fluid, considerable levels of radio-activity were detected in both mono- and diglyceride fractions.

3.9 Persistence of soluble plant hydrolytic enzymes in the rumen

Since the above experiments point to lipases in the rumen arising from plant sources, at least in the early stages of ruminal digestion, attempts were made to obtain additional evidence for this and to measure their stability using polyacrylamide gel electrophoresis. To do so, a cow was withheld from feeding for approximately 18h and then allowed to feed ryegrass for 2.5h. The cow was held in a yard with no access to food for the period of sampling. Proteins were isolated (section 2.11.2) from each sample of rumen fluid obtained at different stages after feeding and separated by electrophoresis on polyacrylamide gel according to the procedures described in section 2.5.2. Lipase, esterase and phosphatase activity was tested in the gel according to the procedures described in sub-section 2.5.3.

(a) Lipases

Neither stearate or oleate esters of naphthol in combination with diazonium dye nor the Tween method gave evidence for lipase activity in the gels. No lipase activity was detected with these substrates in polyacrylamide gels even with pancreatic lipase.

(b) Esterases and phosphatases

One esterase band was visible in the rumen fluid of a cow fasted for 18h (Fig. 21a) when 1-naphthylacetate was used as the substrate. Rumen samples removed 0.5, 2.25, 4.5 and 6.5h after feeding on ryegrass contained 7, 4, 3 and 2 esterase bands respectively. The proteins of ryegrass extracts were separated into 9 esterase bands under the same electrophoretic conditions; of these, 6 were identifiable in the rumen fluid removed 0.5h after the end of the feeding period. These were designated as bands 1, 2, 4, 7, 8 and 10 (Fig. 21a). Bands 1, 2, 4 and 10, bands 1, 4 and 10 and bands 1 and 4 were present in rumen fluid obtained from the cow 2.25, 4.5 and 6.5h after feeding. Band 1 was detected in the pre-feeding rumen fluid (Fig. 21a).

Using 2-naphthylacetate as the substrate, a similar study of the esterase patterns of ryegrass and rumen fluid samples was made. Enzyme patterns were virtually the same as in Fig. 21a except that hydrolysis of 2-naphthylacetate was less rapid than 1-naphthylacetate as judged by the intensity of staining the esterase bands.

The appearance of phosphatase bands in leaf extracts was similar to those obtained for esterases. One phosphatase band was detected in the rumen fluid of a cow fasted for 18h. There were at least two

Figure 21. Gel electrophoresis of esterases and proteins from leaf extracts and from rumen fluid.

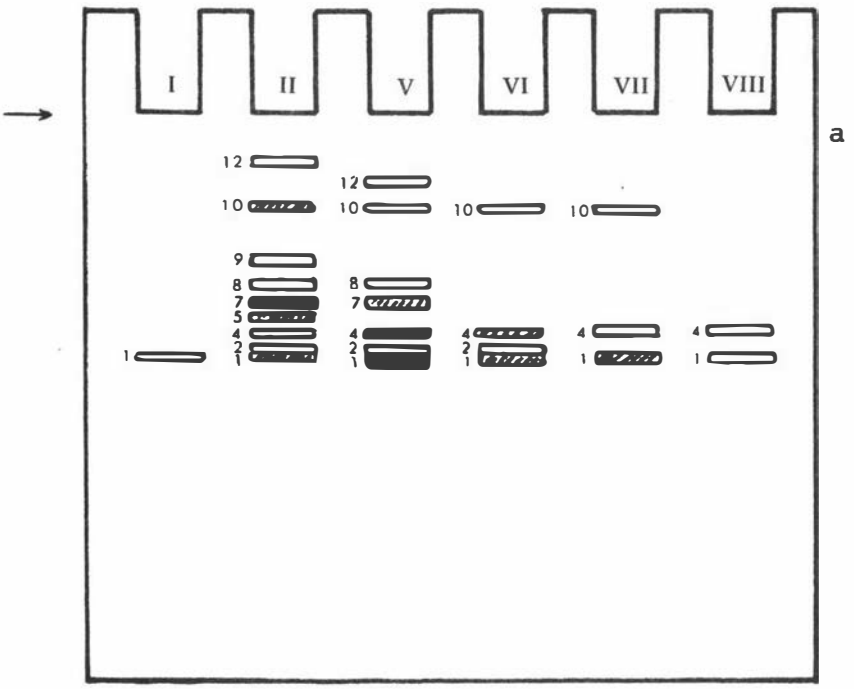
Electrophoretic conditions: µg protein applied on the gel: (a) 1, 45; 11, 50; V, 50; VI, 48; VII, 38; VIII, 50. (b) I, 152; II, 98; III, 65; VI, 119. (c) I, 82; II, 74; III, 76; VI, 95.

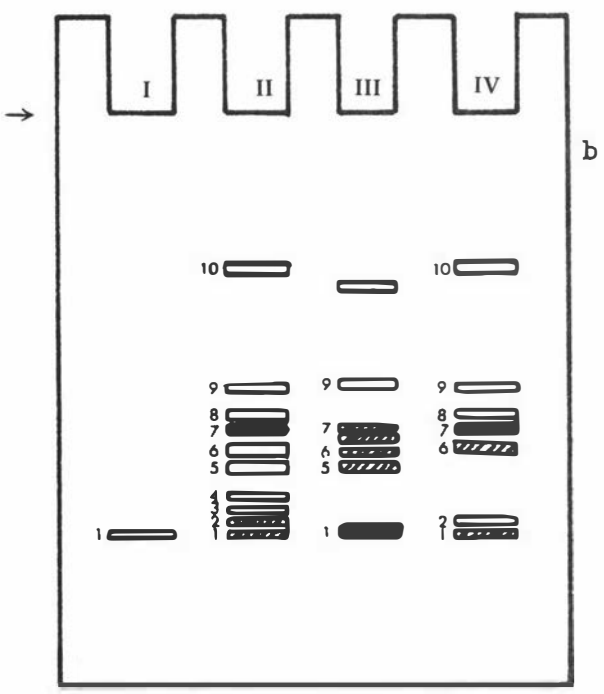
The electrophoresis was performed as described in sub-section 2.5.2. The starting point was indicated by the arrow. Migration was from the cathode (top) to anode (bottom). Electrophoretic components were identified by staining for esterase activity (a and b) and for protein (c).

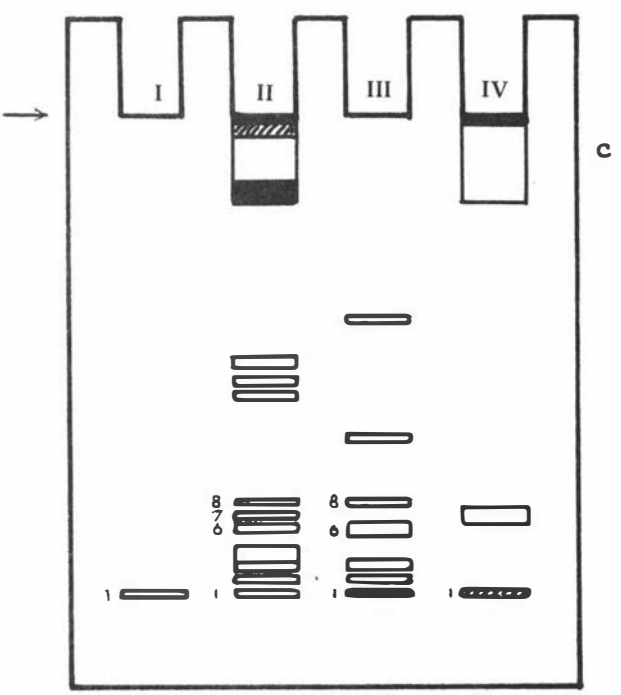
Depth of staining: ▭, weakest; ▨, medium; ▬, strongest.

Key: Rumen fluid, pre-feeding: 1, 18h-fasted. Rumen fluid, post-feeding: V, 0.5h; VI, 2.25h; VII, 4.5h: VIII, 6.5h; 111, 3.0h. Leaf tissue: 11, fresh ryegrass; IV, dried ryegrass.

A number is assigned to each esterase band in fresh ryegrass extract and to corresponding bands which persist in the rumen and in dried ryegrass. Protein bands which correspond to esterase bands are also numbered. (b) and (c) relates to the same experiment.


I
II
V
VI
VII
VIII
a


I
II
III
IV
b


I
II
III
IV
c

phosphatase bands present in samples of rumen fluid obtained 0.5, 2.25, 4.5 and 6.5h after feeding. These corresponded to phosphatase bands in leaf extracts.

Soluble extracts prepared from fresh and dried ryegrass following electrophoresis, gave intense slow-moving protein bands when stained with Coomassie Blue (Fig. 21c) but did not show any esterase activity (Fig. 21b). Faster-moving protein bands were stained weakly with Coomassie Blue and some of these bands corresponded to the esterase bands.

Despite the persistence of esterase bands, the major plant proteins had disappeared from the rumen fluid 3h after feeding (Fig. 21b and 21c). It was evident from Fig. 21b that almost all the major esterase bands in fresh ryegrass remained active after sun-drying.

CHAPTER 4

DISCUSSION

4.1 Lipolytic activity of leaf extracts of pasture plants

Known enzymes with hydrolytic activity towards endogenous lipids in leaf tissues are galactolipases (Sastry and Kates, 1964b), phospholipases (Kates, 1970) and sulpholipases (Yagi and Benson, 1962). In the present study, extracts prepared from a wide variety of pasture species have been found to release FFA's, mono- and diglyceride from ^{14}C-triglyceride at neutral pH in the presence of autoclaved rumen fluid. Furthermore, on incubation of fescue extract with ^{14}C-triolein or ^{14}C-monogalactolipid, the release of FFA's increased linearly with the time of incubation or with the increase in the concentration of plant extract. Incubation of ryegrass extract with emulsified safflower oil also showed a linear increase of FFA's with time (section 3.1 and section 3.2).

Therefore, the results of the present investigation confirm the presence in the leaf extracts from a variety of pasture plants of enzymes which catalyse the hydrolysis of natural and synthetic lipids under the conditions similar to those present in the rumen and show that endogenous substances in autoclaved rumen fluid do not inhibit these lipases.

Enzymes of plant origin are not only confined to the lipolysis of lipids containing long-chain fatty acids. Results presented in Fig. 12 show that extracts prepared from ryegrass also actively hydrolysed triglycerides containing short-chain fatty acids. It is also evident that lipases from ryegrass hydrolysed tributyrin more rapidly than triacetin whereas the rate of hydrolysis of the two triglycerides

by the lipase preparation from lucerne occurred in the reverse order (MacDonnell et al., 1950). In this connection it could be mentioned that enzyme preparations from various plants have been found to hydrolyse triacetin, tripropionin and tributyrin (MacDonnell et al., 1950; Schwartz et al., 1964).

In an investigation on the lipolysis of ^{14}C-monogalactosyldiglyceride and ^{14}C-triolein by extracts of fescue leaves, it was found that the release of FFA's from ^{14}C-monogalactolipid was slower under conditions which were similar to those used with ^{14}C-triolein as substrate (Fig. 7 and Fig. 9). Although in nature monogalactolipid is likely to be dispersed in fluid membranes such as within chloroplasts (Hawke, 1973), on isolation it is a solid of high melting point and is more difficult to disperse in aqueous media than triolein. An adequate dispersion of the added substrate to the incubation mixture is important in obtaining a reproducible rate of lipolysis. Sastry and Kates (1964b) used methanol as a media for the dispersion of galactolipid in the incubation mixture. In the present work, hexane was used for the dispersion of ^{14}C-triolein (Clarke and Hawke, 1970) and ^{14}C-monogalactolipid, because methanol was found to be inhibitory to the action of lipolytic enzymes. Under these conditions, triolein would be expected to undergo a better dispersion than monogalactolipid since the latter has a much higher melting point. Consequently, for this reason, the difference in behaviour towards the two substrates by enzyme preparations from leaf tissue is not regarded as resulting from the existence of two separate lipolytic enzymes without other supporting evidence.

Sastry and Kates (1964b) partially purified galactolipases from

runner-bean leaf tissue. Since the hydrolysis of mono- and digalactolipid by the enzyme preparation had different pH optima and Michaelis-Menten constants and the enzyme had differing stabilities on storage at 4°C, the authors suggested that the two galactolipids were hydrolysed by different lipases. However, Helmsing (1969) purified galactolipases from runner-bean leaves and obtained an electrophoretically homogeneous protein containing both the galactolipase activities. Since at each step of purification, the relative specific activities of the enzyme toward both mono- and digalactolipid increased by the same factor and gel electrophoresis of purified enzyme under different conditions resulted in a single protein, the author concluded that one enzyme catalyses the hydrolysis of monogalactolipid at pH 7.0 and digalactolipid at pH 5.6. In this connection, it could be mentioned that an enzyme preparation with mono- and digalactolipase activity has been partially purified from potato tubers (Galliard, 1971b) and it has been suggested that a single lipolytic enzyme was responsible for the deacylation of several classes of lipid.

As in runner-bean (Sastry and Kates, 1964b), lipolytic enzymes in extracts prepared from ryegrass leaves were found to be associated with the chloroplastic particles and were also present in soluble form in the cytoplasm.

4.2 Lipase activity of ryegrass extracts in the presence of metabolising rumen microorganisms

Despite the high activity of plant lipases in the presence of autoclaved rumen fluid prepared from pasture-fed animals, the crucial question was whether the plant lipases persist in the presence of

metabolising rumen microorganisms, many of which have proteolytic activity (Blackburn and Hobson, 1960a), sufficiently long to allow these enzymes to make a significant contribution to the release of FFA's from dietary lipids.

In many of the experiments described, attempts have been made to differentiate between the microbial and plant lipolytic activity by a choice of suitable control in each series of experiments. Results from such an experiment (Fig. 13) showed that lipolytic activity of rumen fluid, in the absence of active plant enzyme was very low. The addition of only small quantities of plant extracts to the rumen fluid resulted in the higher lipolytic activity which indicated the active role of plant lipases in the presence of metabolising rumen micro-organisms. This was further confirmed by varying the amount of active plant extract in rumen fluid. The release of FFA's increased with the increased enzyme concentration.

4.3 The in vitro stability of plant lipases in rumen fluid

A somewhat novel feature of the experiments carried out in the later stages of this investigation was the assay of lipase activity by the addition of radioactive substrate under the standardized conditions after the lipases from the plant and microbial sources had been incubated for varying lengths of time under conditions found in the rumen (section 3.4). Combined with the appropriate controls, this appeared to be a useful way to compare the behaviour of each lipase source separately and together. In this way, the extent of the limitations imposed on the enzymes of dietary origin by the activity of proteolytic rumen microorganisms were obtained and it was possible to make an

assessment of the stability of lipases from pasture plants in rumen fluid and to isolate the potential activity of two lipase systems.

Rumen fluid was obtained from an animal which had been withheld from food for about 18h in order to reduce the possibility of residual lipase activity from plant material. Although the microbial innoculum per unit volume of rumen fluid is large under these conditions (Bryant and Robinson, 1961), it could be argued that the microbial population has ceased to be in the growth phase and is consequently metabolically less active due to decreased available substrate (Hungate, 1966). When rumen fluid was incubated with unautoclaved or autoclaved plant extract, measurements of gas production indicated that there was a considerable increase in the metabolic activity of microbial population for 3 to 4h (Fig. 14 and Fig. 15). This response was similar to that obtained by adding substrates in the form of soluble sugars (Robertson and Hawke, 1964).

The addition of enzymatically active plant extracts to rumen fluid resulted in a lipase activity of the combined mixture which was similar to that of the plant extract itself under similar conditions (Fig. 14 and Fig. 16). Furthermore, it is evident that plant lipases remain active even after preincubation for several hours with rumen fluid. In contrast, the addition of autoclaved plant extract to rumen fluid gave a mixture with very low lipolytic activity. Using gas production as an index of the metabolic activity, the period of low lipase activity would appear to coincide with the period of high metabolic activity of the rumen microorganisms. The increase in lipolytic activity of rumen fluid containing autoclaved plant extract after

extended periods of incubation did not coincide with increases in gas production but occurred when the metabolic activity of the microorganisms were decreasing. This increase in lipolytic activity may have been due to bacterial lysis and release of intracellular enzymes (Jarvis, 1968; Hoogenraad and Hird, 1970). Alternatively, this increase in the release of ^{14}C-labelled FFA's from the added triolein may have been due to a reduction in the amount of endogenous lipids in the preincubations of longer duration and thus resulting in higher specific activities of added radioactive substrate. Whatever the explanation for this increase, the maximum lipolytic activity of rumen fluid or protozoa-free rumen fluid containing autoclaved plant extract over a period of 0-8h was considerably less than when active plant extract was present.

4.4 The in vivo stability of plant lipases in the rumen

A role for plant lipases in hydrolysing dietary lipids in the rumen during the post-ingestion period was further indicated from measurements of lipolytic activity in rumen contents obtained at different times after the ingestion of fresh pasture (section 3.5). Lipolytic activity of rumen fluid obtained 0.5h after feeding on ryegrass was approximately twice that of the rumen fluid obtained after the cow had been fasted for 18h. The higher level of lipolytic activity in the rumen fluid of the post-feeding animal further indicates that active plant lipases arising in the rumen at the early stage of ruminal digestion play a significant role in the release of FFA's from the dietary lipids. If microbial lipases were solely responsible it would be expected that a decrease in activity would arise from the large dilution effect arising from the ingestion of grass. Rumen fluid obtained 1.45, 3.0 and 5.0h post-feeding possessed lipase activity which was

intermediate between pre-feeding and 0.5h post-feeding levels. These observations gave a measure of the stability of the lipolytic enzymes of plant origin in the rumen. Inactivation of the enzymes of dietary origin is most likely to arise from the proteolytic activity of the rumen microorganisms. It is also possible that movement of the digesta containing unfermented dietary protein (Ulyatt et al., 1974) with lipolytic activity from the rumen into the abomasum could further contribute to the decrease of lipase activity of rumen fluid in vivo. However, under the conditions of the in vivo experiment performed with the pasture-fed animal having a limited grazing period, it appears that lipolytic enzymes of plant origin are stable in the rumen at least up to 5.0h after feeding. Thereafter, microbial and possibly some residual soluble or insoluble dietary lipases still present in rumen fluid cause lipolysis in the rumen. This conclusion is supported by the observation that rumen fluid obtained during pre-feeding and 8h post-feeding period showed almost similar levels of lipase activity.

The rate of fermentation of glucose by pre-feed rumen ingesta was considerably greater than that of the ingesta obtained after feeding (McAnally, 1943). This was possibly caused by the presence of higher concentration of microorganisms in the pre-feeding rumen fluid. In subsequent studies, it was shown that the bacterial count per unit volume of rumen fluid is much higher before feeding than after feeding fresh pasture (Christian and Williams, 1957). During feeding fresh pasture to animals, the bacterial count per unit volume of rumen fluid diminishes (Christian and Williams, 1957) because of dilution by feed and saliva. From these observations regarding the fluctuations of the number of bacteria in the rumen before and after feeding, it appears

that had the bacteria been entirely responsible for the lipolytic activity, the activity would have been higher before feeding than after feeding. Since, the lipase activity is lower before feeding than after feeding, as reported in the present work, a good portion of the total lipolysis occurring in the rumen could be ascribed to the enzymes of plant origin.

Proteolytic activity of rumen contents was high before feeding and changed little immediately after feeding (Blackburn and Hobson, 1960b, c) or with diet (Blackburn and Hobson, 1960a, 1962). In contrast to the proteolytic activity, the lipolytic activity of rumen contents from an overnight fasted cow was found to be low but increased to a large extent 0.5h after feeding fresh pasture (section 3.5) which indicates the possible role of lipolytic enzymes arising from the pasture in the rumen.

It is possible to make some assessment of the overall activity of plant lipases in the rumen of normally grazing ruminants from the observations of lipase activity in the rumen of fasted and re-fed animals and grazing behaviour. The average times occupied by cattle in grazing, ruminating and idling are 7.9, 7.8 and 9.3h respectively (Hughes and Reid, 1951). The time spent on grazing depends on the quality of pasture and observations on the grazing behaviour of dairy cows on six different pastures indicated that cows on good pasture spent less than half their time on grazing but as the feeding value of the pasture decreased, the time of grazing increased (Atkeson et al., 1942). From a study of 56 cows, the same authors found that the cows showed a tendency to graze during four primary periods of the day (a) just after

dawn (b) during mid-morning (c) in the early afternoon (d) during the evening until dusk. Furthermore, two grazing periods occur during the night. These were less sharply defined because the cattle tended to act more as individuals than as a herd at this time compared to their daylight grazing. It was also reported, that of the total grazing time in 24h, 60% occurs during the day while 40% occurs at night (Johnstone-Wallace and Kennedy, 1944). It would appear that initial mastication of leaf tissue followed by rumination would result in a continuous release of fresh dietary protein in the rumen and the average lipase activity in the rumen of normally grazing animals would approximate that observed 1.45 to 3.0h post-feeding (Fig. 17).

4.5 Lipase activity of clarified rumen fluid

The presence of lipase activity in the clarified rumen fluid is of particular interest since it indicates the persistence of soluble lipases in the rumen (section 3.7). Earlier attempts (Garton et al., 1961) to demonstrate lipase activity in the microorganism-free rumen fluid which was prepared from sheep rumen contents 3-4h after feeding hay and concentrates, were not successful. In the present study, rumen fluid was obtained from a cow 0.5h after feeding fresh ryegrass and from a 20h-fasted cow. To avoid further microbial degradation of soluble plant protein, features of the experiment were the preparations of clarified rumen fluid by centrifugation of rumen fluid at 0°C within 45min of collection of the rumen samples.

The amount of soluble lipases in the rumen fluid varied considerably between the fasted and the ryegrass-fed animal. For example, clarified rumen fluid prepared from the 20h-fasted twin pair showed

very little lipolytic activity during 0.5h incubations with the lipid substrates although the activity was detected in extended periods of incubation. In contrast, clarified rumen fluid prepared from the twin pair 0.5h after it had been grazing on ryegrass showed much higher lipolytic activity which further suggests that soluble lipases in the rumen arise from plant sources.

4.6 Release and disappearance of soluble plant hydrolytic enzymes in the rumen

Esterases and phosphatases exist in multiple form in leaf extracts (Rudolph and Stahmann, 1966). The multiple form of these and other hydrolytic enzymes change with age of tissue, nutrition and disease (Furness, 1961). The presence of these hydrolytic enzymes in multiple form in pasture plants has been confirmed in the present study (section 3.9). In an attempt to investigate the stability of these hydrolases in the rumen of a pasture-fed cow, the distribution of these enzymes was followed in the rumen. Only one esterase band was detected in rumen fluid obtained from an overnight-fasted cow but the several esterases detected in rumen fluid obtained 0.5h after feeding ryegrass corresponded to the esterase bands in plant extracts. Apart from demonstrating the stability of these esterases in the rumen, it provides evidence for the rapid release of plant protein including enzymes of hydrolytic nature in the rumen. The number of esterases decreased with the time after feeding and returned almost to the pre-feeding level 6.5h after feeding. Phosphatase bands showed similar behaviour as well as demonstrating the release of a further hydrolytic enzyme of plant origin in the rumen.

Since plant and microbial enzymes exist in multiple forms in the rumen, many of the difficulties of interpretation mentioned above in assigning lipase activity to plant or microbial sources could have been resolved if changes in the lipase patterns of proteins isolated from the rumen could be followed in polyacrylamide gel. Consequently, attempts were made to obtain direct evidence of the stability of soluble lipases arising from plant in the rumen using polyacrylamide gel electrophoresis. Unfortunately, as found by other workers (Rudolph and Stahmann, 1966), it was not possible to detect lipase bands from plant extracts and rumen fluid in polyacrylamide gels. Moreover, no lipase band was detected in the portion of the gel which contained pancreatic lipase. So, it was not possible to assign lipase activity, as distinct from esterase activity, to either plant or microbial sources using this technique.

The disappearance of soluble protein nitrogen from the rumen of a sheep fed on a diet containing straw, soluble casein, minerals and carbohydrates has been estimated by Blackburn and Hobson (1960b). It was observed that the nitrogen content of the soluble protein of the rumen fluid immediately after feeding was very low; it reached a maximum 1h after feeding and then returned almost to the initial level 2h after feeding. This level was maintained up to 7h after feeding. However, Hemsley (1968) found that soluble forage proteins were more resistant to rumen microbial attack than other proteins such as casein.

4.7 Effect of diet on the lipase activity of rumen fluid

Several earlier reports have emphasized the variable nature of lipase activity in the rumen. Rumen fluid from animals grazing on lucerne pasture possessed several times more lipase activity than ingesta

from dry feeds (Hill et al., 1960). Wright (1961) found that the extent of hydrolysis of linseed oil by rumen fluid obtained from a cow 30min after feeding fresh clover was twice that of a hay-fed animal but could not decide whether this was due to animal or dietary differences. In the present study, there was appreciable lipase activity in the protozoa-free rumen fluid obtained from the hay-fed as well as the pasture-fed animal although activities were higher in the latter (section 3.8). In both treatments the highest levels of lipase activity were present in the rumen samples removed 0.5h after feeding and there was a steady decline in activity over the 4.5h and 6.5h sampling periods. On the basis of the findings of the present and previous studies (Hill et al., 1960; Wright, 1961), it is suggested that higher levels of lipase activity in the rumen fluid of fresh pasture-fed ruminants is due to the activity of lipolytic enzymes arising from fresh pasture and that variability in lipase activity in rumen fluid may arise from both the nature of the diet and the time of sampling in relation to the time of feeding.

The fatty acid composition of fresh pasture remains essentially the same after ensilaging (Ward and Allen, 1957), after artificial drying (Garton, 1960) and after being made into hay (Ward et al., 1964). In the present study, substantial lipase activity was present in extracts prepared from dried ryegrass indicating the stability of lipases under the conditions of drying used in fodder conservation. However, when a direct comparison between the lipase activity of extracts prepared from fresh and dried grass was made, it was found that fresh grass contained more lipase activity than its dried product (Table 1).

Soluble extracts prepared from fresh and dried ryegrass following electrophoresis on polyacrylamide gel, showed an almost similar pattern of esterase bands indicating that these hydrolytic enzymes also resist denaturation during drying. Extracts from fresh ryegrass gave very slow-moving protein bands which did not show any esterase activity but the corresponding bands in the extracts from dried ryegrass were less intense (Fig. 21). It is possible that the loss of nutrients from the grass during the process of drying (Morrison, 1959) resulted in the extraction of less soluble protein by the extraction buffer used.

4.8 Lipolysis of dietary lipids by the enzymes of dietary origin in a pasture-fed ruminant during the process of ingestion and rumination

As discussed above, the presence of lipolytic enzymes in a wide variety of pasture plants has been confirmed in the present study. Previous workers have studied a number of highly active lipolytic enzymes which are released from leaf tissues on masceration and homogenisation and rapidly degrade the normal cellular lipids into their constituent parts. These include galactolipases (Sastry and Kates, 1964b), sulpholipase (Yagi and Benson, 1962) and phospholipases (Kates, 1970) which catalyse the hydrolysis of endogenous galactolipids, sulpholipids and phospholipids respectively. These enzymes are so active that principal runner-bean leaf lipids cannot be detected in water homogenates and chloroplasts preparation of leaves although a high concentration of lipids is present in intact leaves (Sastry and Kates, 1963, 1964a, b). The released enzymes from the disrupted cells begin to act on the appropriate substrates as soon as the cells are broken and possibly this may occur during ingestion and rechewing of food by the pasture-fed

ruminants. The effect of chewing on the release of cell constituent from leaf tissue has been investigated (Reid et al., 1962). Due to the grinding and crushing action of ruminants' jaws, a large proportion of the cells in leaf tissue are effected; in some cases a complete rupture of the cell wall takes place and as a result the entire cell constituents are released. The rate of release of the cell constituents depends on the nature of the leaf tissues and the duration and vigour of chewing by the animal. Cells of fresh grass are ruptured more easily than those of flaccid leaves. The release of soluble protein and chloroplastic material from the chewing of ingested red clover has been estimated to be 55-65% and 26-28% respectively (Reid et al., 1962). Further breakdown of the unfragmented plant cells occur during rumination. There is incomplete mastication of plant material during feeding but mastication is more thorough during rumination. The actual amount of chewing during rumination depends on the individual animal and the nature of the feed (Downie, 1954; Gordon, 1958). Digesta containing coarse food material is rechewed more than that from less resistant food (Gordon, 1958).

Thus, under conditions of ingestion and rumination, some lipolysis of the dietary lipids by the enzymes released from leaf tissue in a pasture-fed ruminant is likely to occur before the bolus reaches the rumen. Large quantities of saliva are secreted during these processes of ingestion of food material by the ruminants but lipolytic enzymes are absent from saliva of the adult ruminants (Garton et al., 1958) although lipases exist in the saliva of young ruminant calves (Grosskopf, 1965). Consequently, contributions to lipid hydrolysis are unlikely from this source, at least in the adult animal.

4.9 Positional specificity of the lipolytic enzymes of rumen contents

The pathway of enzymatic degradation of triglyceride in rumen contents has not been investigated to any great extent. In early studies, Garton et al. (1961) could not detect mono- and diglyceride as partial products of triglyceride hydrolysis by rumen contents but in subsequent in vivo experiments, the formation of mono- and diglyceride was demonstrated in the rumen contents of pasture-fed animal after infusion of linseed oil (Hawke and Robertson, 1964). The accumulation of these intermediate compounds during in vitro hydrolysis of triglyceride by bovine rumen contents was further confirmed by Hawke and Silcock (1970), Clarke and Hawke (1970) and Faruque et al. (1974). In the present study, the formation of mono- and diglyceride during lipolysis of ^{14}C-triolein by plant extracts in the presence or absence of rumen fluid and by rumen fluid was apparent; but from the low levels observed, it would appear that they have a transitory existence only. Furthermore, diglycerides were found in rumen contents obtained from a cow fed on fresh pasture (Hawke and Robertson, 1964); galactolipids, the most abundant lipid in leaf tissue, have been suggested to be the most likely precursors of these diglycerides (Hawke and Robertson, 1964). However, unlike pancreatic lipase (Savary and Desnuelle, 1956), castor bean lipase (Ory et al., 1969) and several microbial lipases (Alford et al., 1964), lipolytic enzymes in the rumen have been considered to be non-specific for positions in the triglyceride (Garton et al., 1961; Clarke and Hawke, 1970). These experimental findings would not be unexpected because lipases in rumen contents are likely to be derived from dietary origin and from many different bacterial sources. Whether or not there are specific lipases present, cannot be determined until the characteristics of the lipases arising from the different sources can be examined separately.

SUMMARY

1. The lipolytic activity in the leaf extracts of a wide range of pasture plants have been studied under conditions similar to those found in the rumen. Safflower oil, ^{14}C-triolein and ^{14}C-monogalactolipid were used as substrates for the lipolytic enzymes. The release of fatty acids from these substrates by different plant extracts was linear either with the time of incubation or with the increase in concentration of plant extract. Lipases were found to be present in the leaf extracts of all pasture plants examined and also in dried grass. Triglycerides containing short-chain fatty acids were also hydrolysed. Endogenous substances present in autoclaved rumen fluid did not inhibit these lipases.

2. Lipolytic enzymes were associated with the chloroplastic particles and were also present in soluble form in the cytoplasm.

3. *In vitro* experiments in which plant and microbial lipolytic activity were differentiated showed that lipolytic activity of rumen fluid in the absence of active plant enzyme was very low since only 2.0% of the radioactivity from the ^{14}C-triglyceride appeared in FFA. The addition of an equal volume of enzymatically active plant extract to rumen fluid resulted in the release of 55.6% of the radioactivity as FFA. The release of FFA increased with the increased addition of plant extract, indicating the active role of plant lipases in the presence of rumen microorganisms.

4. Further *in vitro* experiments in which plant extracts were incubated with rumen fluid before assaying for lipase activity showed that

plant lipases remained highly active even after incubation with actively metabolising rumen microorganisms for at least 5h. When plant extracts were replaced by autoclaved plant extracts the lipolytic activity of actively metabolising rumen microorganisms in rumen fluid was found to be very low. A slight increase in lipolytic activity attributable to rumen microorganisms occurred after about 4h incubation with autoclaved plant extract at which stage the metabolic activity of rumen micro-organisms had passed its peak.

5. Essentially the same pattern of behaviour of microbial lipases was obtained when rumen fluid was replaced by protozoa-free rumen fluid. Plant lipases remained highly active during incubation with protozoa-free rumen fluid for at least 8h.

6. Measurements of lipase activity in rumen contents obtained at different times after ingestion of fresh pasture showed that rumen fluid obtained 0.5h after feeding contained approximately twice the activity of rumen fluid obtained after overnight fasting of the animal. This high activity was maintained for about 5h. The results suggested that lipases in the rumen arising from plant sources remained active up to 5h after ingestion of fresh pasture.

7. Clarified rumen fluid prepared from a ryegrass-fed twin 0.5h after feeding showed much higher lipolytic activity when compared with the 20h-fasted twin. This further indicates that soluble lipases in the rumen arise from plant sources.

8. Multiple forms of esterases and phosphatases arising from plant

sources in the soluble fraction of rumen fluid obtained several hours after feeding fresh pasture were detected using polyacrylamide gel electrophoresis. This indicated the stability of hydrolytic enzymes from plant sources in the rumen but attempts to detect lipase activity in polyacrylamide gel were unsuccessful.

9. Paired-feeding experiments showed that lipase activity was higher in the protozoa-free rumen fluid obtained from the fresh-pasture-fed than from the hay-fed animal. In both treatments, lipase activity was highest immediately after feeding and declined with time after feeding. It was concluded that the variability in lipase activity in rumen fluid may arise from both the nature of the diet and the time of sampling in relation to the time of feeding.

10. Experiments with ^{14}C-labelled acetate showed that rumen micro-organisms did not incorporate short-chain fatty acids into long-chain fatty acids to any significant extent.

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