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# AN INVESTIGATION OF LIPOLYSIS

# IN THE BOVINE RUMEN

A thesis presented in partial fulfilment of
the requirements for the degree of
Master of Agricultural Science
in Animal Science

at

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New Zealand.

David Graeme CLARKE
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# Chapter 1

## INTRODUCTION

### 1.1. Lipases

#### 1.1.1. Terminology

Lipolytic enzymes may be considered as a special class of carboxyl esterases, as they catalyse the hydrolysis of ester linkages in lipids with the formation of alcohol and fatty acid moieties. In mammalian systems lipolytic enzymes are generally subdivided into three classes; those acting on fats (lipases); those acting on fats in the form of lipoprotein (lipoprotein lipases); and those acting on the ester bonds in phospholipids (phospholipases). However this classification based on substrate specifity is of limited value only, as many of the enzymes that hydrolyse carboxyl esters, exhibit a very wide substrate specificity.

Consequently a review of lipases is complicated by the general confusion centred around the exact meaning of the term 'lipase'. With a natural triglyceride, e.g. triolein, specificity of the enzyme may be referred to the alcohol glycerol, so that enzymes hydrolysing fatty acids from glycerol are lipases. Alternatively specificity may be referred to the long chain fatty acid, and enzymes hydrolysing long-chain fatty acids from esters of several different alcohols may be regarded as lipases (Balls and Matlack, 1938).

Thus, an enzyme hydrolysing tributyrin would be classed as a lipase by the first definition but not by the second and the reverse would be the case for an enzyme hydrolysing benzyl stearate.

An alternative definition for lipases is that based on the work of Sarda and Desnuelle (1958) who showed that true lipases will only act in an heterogenous medium, and do not act, or act very slowly, on water soluble substrates. Fortunately, if a lipase is defined as an enzyme hydrolysing triglyceride esters, or as an enzyme hydrolysing esters in a heterogenous system, no serious conflict arises because, of the common triglycerides, only triacetin is appreciably water soluble. However when a heterogenous system is provided, lipases will hydrolyse glyceryl esters more rapidly than esters of other alcohols (Sarda and Desnuelle, 1958).

The International Union of Biochemistry (1961) accordingly defined a lipase as a "glycerol ester hydrolase" (E.C. 3.1.1.3), and further recommended that ester emulsions be used as substrates. This definition will be used in the present review, but it must be emphasized that there is no evidence to suggest that esterase activity with soluble substrates, and lipase activity with insoluble substrates, refer to different catalytic mechanisms.

### 1.1.2. Historical

Lipase activity in the pancreas was demonstrated as early as 1846 by Claude Bernard, and gastric lipase by Marcet in 1858 (Wills, 1965). Despite rapid advances in enzymology in the last 40 years very little progress was made on the purification and properties of lipase, and it was not until the past decade that comprehensive information became available in this field, mainly as a result of the work of Desnuelle and his colleagues. Pancreatic lipase has been the most extensively studied, but lipases in other digestive juices, animal tissues, plants and microorganisms have received minor

attention, with the possible exception of the recently discovered lipoprotein lipase.

It should be emphasized that much of the present knowledge of the properties of lipase, has been obtained with impure substrate and enzyme preparations, and consequently this has led to conflicting reports in the literature. However, with the preparation of pure naturally occurring and synthetic substrates, and the development of new fractionation techniques this question has largely been resolved.

For detailed reviews on earlier work with lipases, the reader is referred to Ammon and Jaarma (1950), Desnuelle (1951), Bergström and Borgström (1955, 1956), and Kates (1960). Some informative reviews which have been published recently are Oosterbaan and Jansz (1965), Wills (1965) and Lawrence (1967), the latter being mainly concerned with microbial lipases.

1.1.3. Methods of detection

Lipases specifically hydrolyse glycerol esters as defined by the International Union of Biochemistry (1961), and in the majority of cases, the hydrolysis of a triglyceride follows the pathway outlined in Fig. 1.

Obviously, the rates of lipase reaction can be measured by determining either the rate of disappearance of the triglyceride or the rate of production of the fatty acids. Determination of diglyceride, monoglyceride or glycerol formation is possible, but experimentally very difficult.

(a) Measurement of rate of disappearance of triglyceride

The rate of disappearance of triglyceride can be measured by

following the rate of clarification of the emulsion. As hydrolysis of triglyceride proceeds, the products become increasingly water soluble and the clarification of the turbid emulsion can be measured (Rottem and Razin, 1964). A quantitative and sensitive diffusion assay, which uses a thin layer of agar, containing a low concentration (0.1% v/v) of triglyceride has been developed by Lawrence, Fryer and Reiter (1967), for use in the detection of microbial lipases.

(b) Measurement of rate of fatty acid production

Most of the methods for estimating lipase activity reported in the literature, are based on the determination of free fatty acids liberated from triglycerides.

Pure cultures of microorganisms have been grown in the presence of fat or triglyceride substrate (Hobson and Mann, 1961; Vadehra and Harmon, 1965). The whole culture is acidified and extracted with ether and the total acidity determined. This method can be criticised on the grounds that many of the acids that arise from protein and carbohydrate metabolism are also ether soluble. Washed cell suspensions have been incubated with triglyceride (Hugo and Beveridge, 1962) as well as cell walls and cell free supernatants of many bacteria. The liberated fatty acids are normally extracted and the acidity determined by titration with alcoholic NaOH (Alford and Pierce, 1963).

Continuous automatic titration of the fatty acid liberated from triglyceride substrate in a pH stat (Shahani, Sarda, Desnuelle and Azoulay, 1964; Shah and Wilson, 1965; Downey and Andrews, 1965) has the advantage over direct titration methods (Dole and Meinertz, 1960) in that the initial reaction velocity can be measured in short

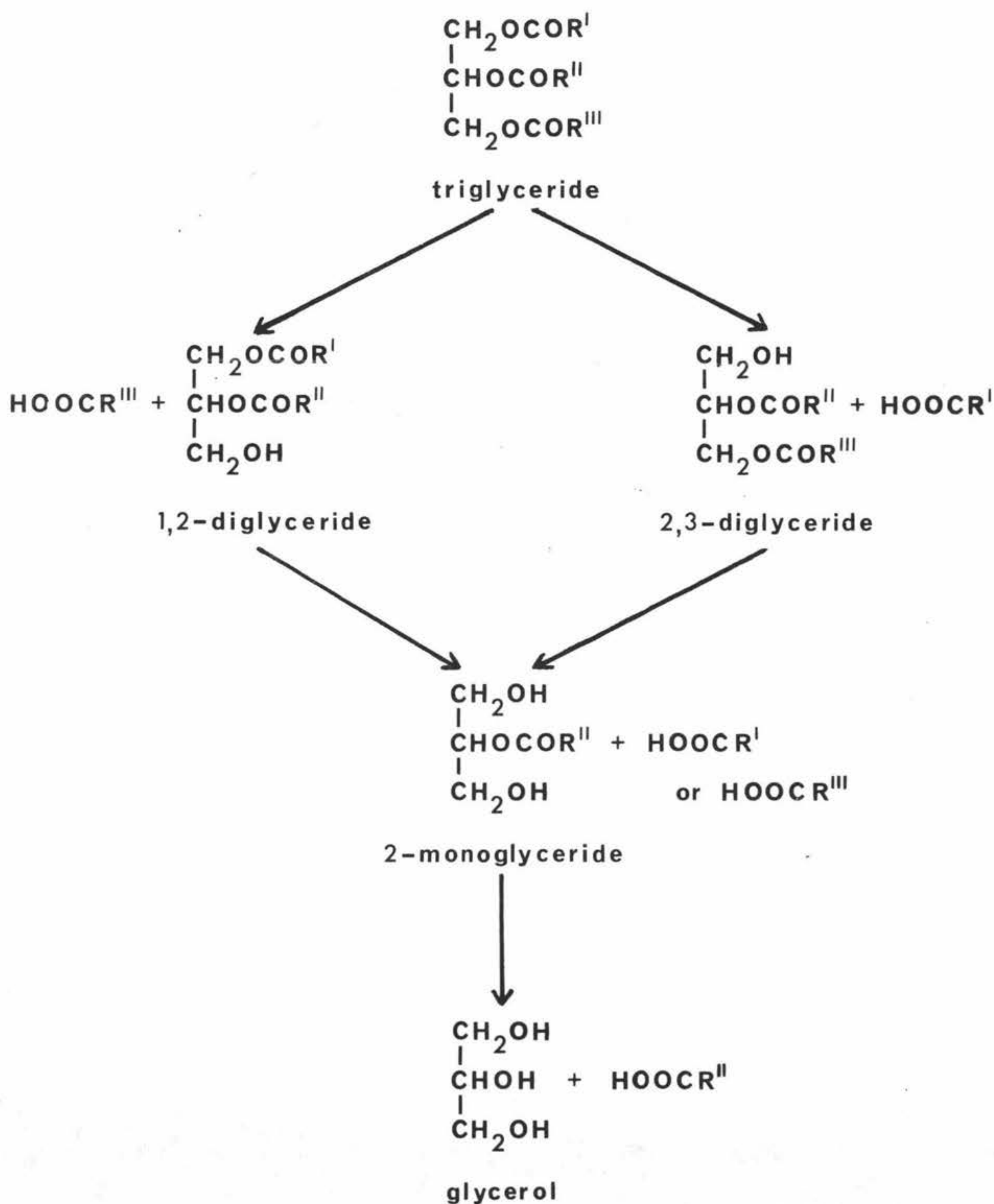


Figure 1. The major pathway for the hydrolysis of triglyceride by lipase. (R', R'', R''' represent fatty acids of the same or different structure).

incubation times. However the former method suffers from the fact that it is difficult to measure lipase activity at pH 7.0, probably due to incomplete titration of fatty acids. A silica gel method has also been used to estimate fatty acids without prior solvent extraction (Khan, Chandan, Dill, and Shahani, 1964; Niki, Yoshioka, and Ahiko, 1966).

The rate of acid production may also be measured manometrically by determining the rate of liberation of $CO_2$ from a bicarbonate buffer (Wills, 1961).

Colorimetric determination of the liberated fatty acids has been used extensively. Original methods involved the use of a special substrate designed to give a coloured end-product after hydrolysis, or one that could be easily converted to a coloured product (Seligman, and Nachlas, 1950). Other methods have been based on the selective transfer of copper or cobalt soaps into chloroform (Ayres, 1956). Sensitivity is increased when these soaps are combined with triethanolamine buffer (Iwayama, 1959) and diethyldithiocarbamate for copper detection (Duncombe, 1963), the useful range for the latter method being 0.05 - 0.5 $\mu$ moles of fatty acid.

The use of radioactive substrates and the subsequent analysis of radioactive products of hydrolysis has been a technique used in only recent years. Chiso and Gilbert (1965) described a sensitive radiochemical assay which uses a mixture of $^{14}$C-labelled triolein and unlabelled carrier as substrate. The hydrolysis products are isolated by column chromatography and the radioactivity measured in the column effluent. Lipid extracts of serum enzyme digests of $^{14}$C-labelled lipid preparations have been chromatographed on thin

layer silica gel, to observe the localisation and appearance of reaction products by Kelley (1966). The same author has developed a more rapid and efficient assay for the detection of labelled-fatty acids, released from labelled-triglyceride by lipolysis (Kelley, 1968). The fatty acids in lipid mixtures are adsorbed on dehydrated hydroxy-charged ion exchange resin, the other lipids are removed by washing with solvent and the adsorbed fatty acids are released with quaternary ammonium base for counting. This method has the advantage that all manipulations can be carried out directly in scintillation vials.

1.1.4. Purification

Most of the work on lipase purification has been with the mammalian pancreas as the enzyme source. Early attempts involved the use of adsorption techniques, and it was not until Borgström (1955) used zone electrophoresis that any great advance was made in its preparation. A lipase fraction was isolated, containing 2% of the protein in the original pancreatic juice and had a specific activity 50 times that of the original juice.

Since this initial breakthrough the Marseilles' group have made remarkable advances in the preparation of hog pancreatic lipase. The technique developed, involved an aqueous extraction of solvent-dried pancreas powder, two selective precipitations of the extract by ammonium sulphate and acetone, two selective adsorptions on calcium phosphate and aluminium hydroxide and finally a high-voltage electrophoresis on starch at pH 5.25 (Sarda, Marchis-Mouren, Constantin and Desnuelle, 1957; Marchis-Mouren, Sarda and Desnuelle, 1959, 1960). The product obtained gave a 20% yield and was electro-

phoretically and chromatographically homogeneous. Benzonana, Entressangles, Marchis-Mouren, Pasero, Sarda and Desnuelle (1964) improved the purification technique by using lyophilised supernatants of pancreas as starting material. This was followed by two selective precipitations with ammonium sulphate and acetone. The acetone precipitate was eluted on a DEAE - cellulose column with phosphate buffer, pH 8.0 of increasing molarity, and lipase emerges as a sharp peak together with 18% of the total proteins when the molarity of the buffer reaches 0.10. Further purification was achieved by subjecting this material to a Sephadex G-200 chromatographic column. Although all remaining nucleotides were removed by this step the specific activity was not increased over that obtained from the DEAE - cellulose column, due to inactivation. This final product was estimated to contain 60-65% active lipase.

1.1.5. Factors affecting velocity of hydrolysis

It is recommended by the International Union of Biochemistry (1961) that lipid emulsions be used as substrate for lipase studies. However if the substrate is not emulsified, or if the emulsification is not complete, then the extent of shaking the incubation medium becomes an important factor. Wills (1961) showed that if shaking was rapid, as in a Warburg apparatus, an almost optimal hydrolysis rate is possible without emulsification. Other factors which may influence the velocity of hydrolysis are pH and temperature.

(a) Effect of emulsification of substrate

The work of Desnuelle and his collaborators has clearly established that pancreatic lipase acts preferentially at the oil-water interface in heterogenous systems (Sarda and Desnuelle, 1958;

Desnuelle, 1961). Although true solutions of methylbutyrate, tributyrin or triolein are not completely resistant to lipase action, the rate of hydrolysis increases very sharply as the concentration of these compounds is increased to form a heterogenous system. As shown in Fig. 2 triacetin in solution is slowly hydrolysed, but the velocity increases if the compound is present in the emulsified form (Desnuelle, 1961).

It can also be seen from Fig. 2 that the rate of lipolysis plotted against the interfacial area of the substrate gives a similar curve to that obtained by plotting velocity against substrate concentration for a typical enzyme in a homogenous system. Wills (1965) concludes that it is the adsorption of the enzyme at the interface that is important, in addition to the normal enzyme/substrate adsorption. Desnuelle (1961) found that when triolein is emulsified to give different interfacial areas (small and large globules), the rate of hydrolysis was fastest when the interfacial area was greatest. It should be pointed out that these experiments are limited due to the difficulty in evaluating the interfacial area, but it can be stated that the rate of hydrolysis is greatest when the interfacial area is greatest, i.e., when the emulsion is finely dispersed. The mechanism of this phenomenon is not understood at present.

Although emulsification and the subsequent increase of the interfacial area of the triglyceride/aqueous phase can increase the rate of lipase hydrolysis, the effect is complex and appears to be dependent on the exact chemical nature of the emulsifying agent. Emulsifying agents used in the determination of lipase activity in-

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clude bile salts, egg albumin, gum arabic, soaps and synthetic detergents. Some emulsifying agents activate lipase while others inhibit hydrolysis. Bile salts have been the most extensively studied but the situation is not clear whether they increase the rate of triglyceride hydrolysis because they are surface-active agents, or because they have a specific activating effect on lipase itself. Wills (1965) concludes that "it seems likely that the exact alignment of enzyme molecules in the interfacial layer is important, and that bile salts may promote this alignment".

(b) Effect of pH

In general, the optimum pH for lipases is around neutrality or on the alkaline side of neutrality. However the effect of pH on the rate of hydrolysis is the result of its combined effects on the enzyme itself, on the emulsified substrate and on the properties of the substrate/aqueous phase interface (Wills, 1965).

(c) Effect of temperature

Most lipases are at optimum activity in the temperature range $30^{\circ}$-$40^{\circ}$. Several studies have been made of lipase stability at different temperatures. Wills (1960) demonstrated that pancreatic lipase lost 36% of its activity after 10 minutes at $50^{\circ}$. This author also showed that the stability of pancreatic lipase to heat is dependent on the presence of calcium ions - removal by chelation renders the enzyme far more susceptible to inactivation at elevated temperatures. Lipase becomes more temperature dependent on the addition of bile salts to the incubation (Desnuelle, 1961).

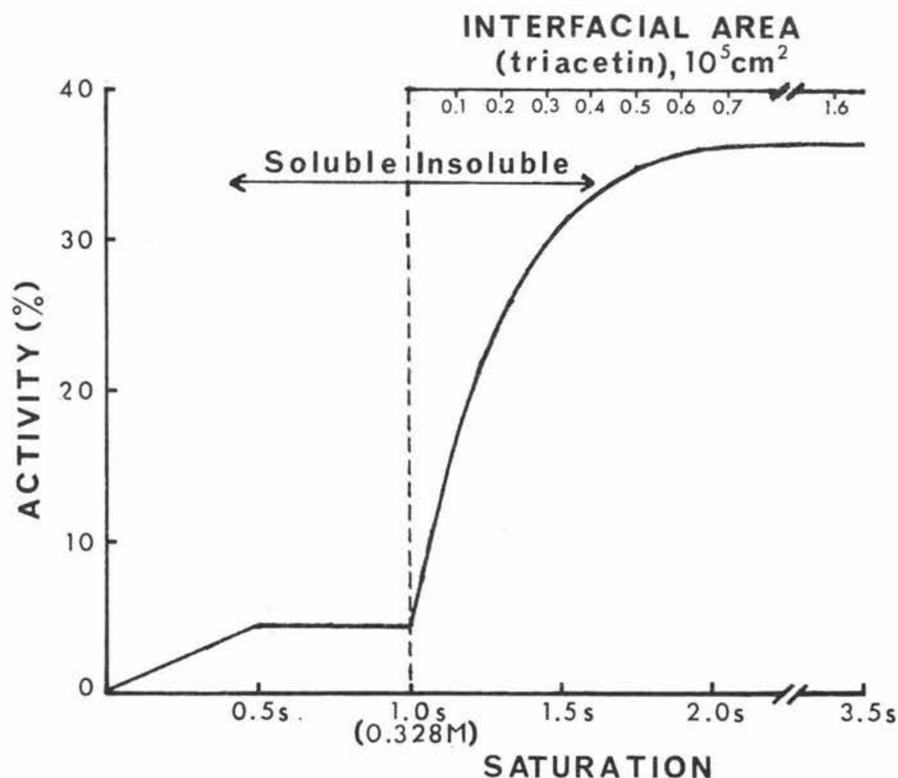



Figure 2. The influence of the saturation concentration on the rate of hydrolysis of triacetin by pancreatic lipase. The substrate concentration is expressed as a fraction of the saturation concentration. (from Sarda and Desnuelle, 1958).

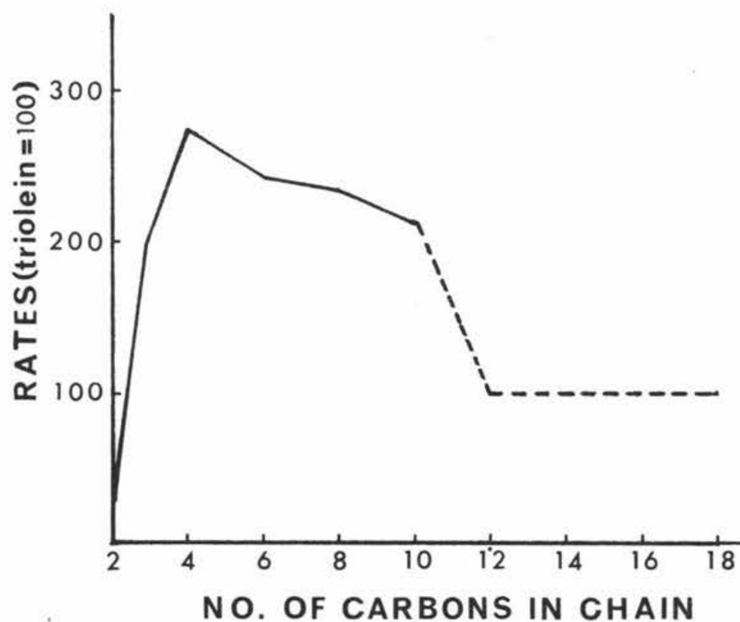



Figure 3. Variation of the rate of hydrolysis of simple glycerides with the chain length of the fatty acid. Hydrolysis rates are expressed as a percentage of the rate of hydrolysis of triolein. (from Entressangles et al, 1961).

### 1.1.6. Substrate specificity

The hydrolytic activity of a lipase depends on the structure of both the fatty acid and alcohol moieties of the substrate. The specificity of many lipases appears to be related to the position of the fatty acid moiety on the triglyceride, and in other cases, to the fatty acid itself (chain length and degree of saturation).

#### (a) Alcohol moiety

The specificity of lipases towards the alcohol moiety of its ester substrates has not been investigated in great detail. Sarda and Desnuelle (1958) showed that methyl butyrate was hydrolysed by pure pancreatic lipase provided the ester was present in an emulsion, but hyrolysis was very much slower than that of tributyrin. Also methyl oleate was hydrolysed by pancreatic lipase at one thirtieth the rate of triolein.

#### (b) Fatty acid moiety

The relative rates of hydrolysis of fat substrates by lipase has been investigated using different acyl side chains. The two main factors thought to influence hydrolysis, are the extent of saturation and the chain length of the constituent fatty acids. A minor controversy has arisen over the effect of unsaturation of a fatty acid on its hydrolysis rate. Ono (1940) cited by Wills (1965) found that unsaturated acids were preferentially hydrolysed by pancreatic lipase, but other workers found the situation reversed (Clement and Clement-Champougny, 1954). Savary and Desnuelle (1956) observed the hydrolysis of palmityldiolein and oleyldipalmitin and found that the fatty acids in the 1- or 3- positions were readily hydrolysed. Oleic acid in the 1-position was hydrolysed at a

slightly faster rate than palmitic acid in the 1-position. Desnuelle (1961) has concluded, that for the $C_{18}$ fatty acid series, saturation has little if no effect on the hydrolysis rate.

Several studies have been made on the effect of fatty acid chain length on the rate of hydrolysis of the ester linkage. The most extensive study in this field was carried out by Entressangles, Pasero, Savary, Sarda and Desnuelle (1961). These workers tested a series of triglycerides, and found that tributyrin was hydrolysed far more rapidly than triolein. Fig. 3 shows the effect of chain length of the fatty acid side chains, on hydrolysis rate (Triolein = 100).

When the same workers hydrolysed 1-palmityl-3-butyryl glycerol to the extent of 10%, the free fatty acids were in the ratio 18/7 (butyric acid/palmitic acid). As lipase cannot distinguish between the 1- and 3-positions on the triglyceride molecule, this clearly shows the high affinity of the enzyme for shorter chain fatty acids. This same specificity has been observed in Ricinus lipase (Ory, St. Angelo, and Altschul, 1962).

(c) Positional specificity of lipases

The special affinity of pancreatic lipase for the esters of primary alcohol groups has been known for some time. Mattson and Beck (1956) used a number of synthetic triglycerides with different acyl moieties, and after their hydrolysis by pancreatic lipase, found that the 2-monoglyceride was the main product in each case. A small percentage of 1-monoglyceride was formed and they concluded that this may have been due to bond migration. Savary and Desnuelle (1956) supported these findings and calculated that 10-20% of the

isomers were 1-monoglycerides.

The likelihood of isomerisation was investigated by Mattson and Volpenhein (1962). These workers found that when 2-monoglycerides were dispersed in slightly alkaline aqueous buffer at 40° there was a rapid isomerisation to the 1-monoglyceride. Previous to this, Savary, Constantin and Desnuelle (1961) observed that most inner fatty acid chains of dietary triglycerides were found in the same position in the chylomicron triglyceride. This finding led to a belief that the 2-monoglyceride must be stablised in some manner during lipase attack. Benzonana et al (1964) found that when 2-monoolein was dispersed in buffer alone isomerisation proceeded until the $\alpha/\beta$ ratio was 40/60. However when free fatty acids were mixed with the 2-monoglyceride as would be the case in in vivo, the isomerisation ratio reached an equilibrium at 60/40. The presence of free fatty acids appears to have limited the isomerisation of oleic acid from an inner to an outer position on the triglyceride molecule. This effect is important biologically, in the resynthesis of the chylomicron triglycerides.

Tattrie, Bailey and Kates (1958) studied the hydrolysis of the D and L isomers of 1,2-dipalmityl-3-oleyl glycerol, and found that the rates of hydrolysis of the two isomers and the DL mixture were identical. Thus pancreatic lipase does not appear to exhibit any stereospecificity.

Milk lipase was also found to be specific for the hydrolysis of primary alcohol esters (Jensen, Duthie, Gander and Morgan, 1960), but Ricinus lipase (Savary, Flanzy and Desnuelle, 1958) and lipoprotein lipase (Korn, 1961) do not exert this specificity.

## 1.2. Dietary Lipids of Ruminants

The major diet of domestic ruminants consists of leaves of grasses and leguminous plants. The ether-extractible portion of the leaves of grass generally constitutes between 2 - 8% of the total dry matter. Glycerides account for up to 70% of this total lipid fraction, mainly in the form of galactosyl glyceryl esters, together with small amounts of phospholipid and sulpholipid. The major galactolipids are monogalactosyl diglyceride and digalactosyl diglyceride (Weenink, 1959, 1961; Shorland, 1961). The remaining components of the lipid fraction consist of sterols, sterol esters, waxes, hydrocarbons and free fatty acids (Hilditch, 1956; Weenink, 1962).

It has been observed that for other dietary foodstuffs viz, silage (Ward and Allen, 1957), artificially dried grass, (Garton, 1960) and hay (Ward, Scott and Dawson, 1964), the lipid content and fatty acid composition remains essentially the same as that of fresh pasture.

The fatty acids present in the mono- and diglactosyl diglycerides of pasture contain a very high proportion of unsaturated components (approximately 80%) as shown in Table 1.

Until recently it was thought that all the unsaturated fatty acids had the <u>cis</u> configuration, and that the <u>trans</u> acid did not occur in fatty acids of plant origin. However, Weenink and Shorland (1964) have reported the occurrence of hexadec-<u>trans</u>-3-enoic acid at low levels in pasture leaves.

The lipid content of the concentrate feeds such as maize meal, linseed meal and cereals again, consists largely of triglycerides

characterised by a relatively high concentration of unsaturated $C_{18}$ acids. In these cases however linoleic acid is the predominant unsaturated acid (Hilditch, 1956).

An assessment of the total lipid intake per day for a non-lactating dairy cow (based on a daily consumption of 20 lb grass, on a dry weight basis) would be in the region of 450g. (ether-extractible portion of 5%). This could be as high as 1 Kg for a growing, pregnant or lactating cow. The daily fatty acid intake, which represents approximately half of this value, would be in the region of 225g to 500g (Hawke, 1963).

### 1.3. Modification of Dietary Lipids by Rumen Microorganisms

Studies during the last decade have shown that rumen microorganisms effect extensive changes in the dietary lipid. The earlier observations which made it apparent that there were differences between the lipid metabolism of ruminants and other mammals were:
(a) the depot fats of ruminants contained a high proportion of stearic acid (Hilditch, 1956),
(b) the depot fats of ruminants contained a high proportion of *trans*-acids (Swern, Knight and Eddy, 1952 and Hartman, Shorland and McDonald, 1954),
(c) dietary unsaturated fatty acids known to be readily assimilated into the depot fats of non-ruminants did not appear to be present in the ruminant tissue lipids (Garton, 1964),
(d) depot fats and milk fats of ruminants contained a complex mixture of unsaturated fatty acids (especially isomeric forms of octadecenoic and octadecadienoic acids) not found in the fats of other herbivores (Hilditch, 1956).

TABLE 1

Fatty acid composition of pasture lipids
(% by weight of total fatty acids)

| Fatty Acid | Clover-Rich Pasture (1) | Mixed Pasture Grasses (2) |
|---|---|---|
| **Saturated:** | | |
| $C_{14}$ (Myristic) | - | 1.1 |
| $C_{16}$ (Palmitic) | 8.9 | 15.9 |
| $C_{18}$ (Stearic) | 2.8 | 2.0 |
| Others | 3.9 | 0.5 |
| **Unsaturated:** | | |
| $C_{16}$ (Palmitoleic) | 7.9 | 2.5 |
| $C_{18}$ (Oleic) | 9.5 | 3.4 |
| $C_{18}$ (Linoleic) | 8.1 | 13.2 |
| $C_{18}$ (Linolenic) | 58.9 | 61.3 |

(1) Shorland *et al*, 1955

(2) Garton, 1960

These observations indicated that the peculiar features of the ruminant lipids were associated with the assimilation of the products of microbial modification of dietary lipids in the rumen.

The microorganisms of the rumen modify the dietary lipid in three main ways:

(a) hydrolytic release of esterified fatty acids,

(b) reductive modification of unsaturated fatty acids (i.e. hydrogenation).

(c) fermentation of free glycerol liberated during hydrolysis, and of galactose released from galactolipids.

1.3.1. Hydrolysis of dietary lipids in the rumen

Hydrolysis of dietary lipids includes the release of fatty acids from ester combination with triglycerides, and the release of galactose from galactolipids (the principal form of lipid in green leaves).

That rumen microorganisms can bring about lipolysis of triglycerides was first reported by Garton, Hobson and Lough (1958). When linseed oil was incubated with sheep rumen contents *in vitro* a considerable part of the esterified fatty acid residues were liberated as free fatty acids. No lipolytic activity was shown by boiled rumen contents nor by sheep saliva, and it was concluded that microorganisms were responsible for this action. The same workers examined the lipid content of the rumen, abomasum and upper intestine of a sheep at slaughter, 7 hrs after the last feed, and found that 80-90% of the lipid was present as free fatty acid. The experimental diet included 40 g of linseed oil per day.

In subsequent studies (Garton, Lough and Vioque, 1959; 1961)

the effects of incubating linseed oil and a number of other lipid substrates were studied in more detail. Garton *et al* (1961) incubated three naturally occurring triglycerides which differed in the degree of saturation of the fatty acids, and also in the fatty acid composition, in an attempt to show if the enzyme exhibited any specificity. The results suggested that there was some selectivity towards triglycerides containing high proportions of unsaturated fatty acids, especially linolenic acid, although the authors pointed out that the more saturated triglycerides were more difficult to emulsify.

In an attempt to examine this effect more thoroughly Garton *et al* (1961) carried out a partial hydrolysis of linseed oil by rumen contents. Changes were noted in the fatty acid composition of the free fatty acids, and the residual glycerides after hydrolysis was allowed to proceed to the extent of 32%. These changes are shown in Table 2.

From these figures it would appear that linolenic acid has been selectively hydrolysed from the triglyceride. However it must be remembered that hydrogenation is also taking place concurrently and interpretation is difficult. The results of Garton *et al* (1961) suggest that hydrogenation of fatty acids free in the rumen, proceeds at a faster rate than if the fatty acids were still esterified. This suggestion was confirmed by Hawke and Robertson (1964). In more precise studies, Hawke and Silcock (1969) incubated 1-palmityl-2- 1-$^{14}$C -linolenyl-3-oleyl glycerol with rumen contents of a cow *in vitro*. Analysis of free fatty acids and fatty acids of mono-, di-, and triglycerides, after incubation,

TABLE 2

Composition of free fatty acids and the fatty acids of neutral lipids following 32% hydrolysis of linseed oil by rumen contents

| Fatty Acid | Free fatty acid | Fatty acid residual glycerides | Composition (weight %) of linseed oil |
|---|---|---|---|
| palmitic acid | 5.7 | 5.3 | 5.7 |
| stearic acid | 4.0 | 5.2 | 4.2 |
| oleic acid | 24.4 | 18.8 | 15.6 |
| linoleic acid | 38.4 | 21.6 | 13.2 |
| linolenic acid | 26.8 | 49.1 | 61.3 |

showed that hydrogenation had only taken place when the fatty acids had been removed from ester combination.

The pathway of hydrolysis of triglycerides by rumen contents has not been studied to any great extent. In the *in vitro* studies of Garton *et al* (1961) no diglyceride or monoglyceride intermediates could be shown. Free glycerol was only found in trace amounts, although this was probably further fermented by the rumen microorganisms. Hawke and Robertson (1964) obtained chromatographic evidence for the presence of mono- and diglycerides in the rumen contents of cows after infusion of linseed oil, and it was suggested that these intermediates had a transitory existence only. The *in vitro* studies of Hawke and Silcock (1969) confirmed the presence of mono- and diglyceride intermediates in the hydrolysis of triglycerides by bovine rumen contents.

Silcock (1968) incubated radioactive triglyceride specifically labelled in the 2-position with the rumen contents of a cow *in vitro* and the results suggested a specificity of hydrolysis for fatty acids esterified at the 1- or 3- positions on the triglyceride molecule. The results did not indicate any stereospecificity between these two positions.

Studies on the lipolytic activity of rumen contents has not been confined to triglycerides and galactolipids. Sheep rumen contents have been shown to hydrolyse lecithin and lysolecithin giving rise to free fatty acids and glyceryl phosphorylcholine (Dawson, 1959). Other workers have shown that fatty acids can be liberated from ester combination with sterol esters and methyl esters by sheep rumen contents (Garton, unpublished), Tween 80 (Wright, 1961), and ethyl esters by ox rumen contents (Hill, Saylor, Allen and Jacobson, 1960).

Attempts to obtain cell-free lipolytic enzyme preparations from rumen bacteria have not been very successful. However Dawson (1959) isolated a lecithinase from rumen contents which was capable of hydrolysing lecithin via glyceryl phosphorylcholine and glycerophosphoric acid.

Hobson and Mann (1961) isolated a pure bacterial culture on both a saliva-based medium and a rumen fluid-based medium which was able to hydrolyse linseed oil. The bacteria were obtained from sheep rumen contents and appeared as Gram-negative, curved rods which were strictly anaerobic. Although morphologically similar to many types of rumen bacteria they differed from all known species in their limited fermentation reactions. The authors

pointed out that these bacteria were probably not the only ones which hydrolyse glycerides in the rumen, but because of the large number present (approximately $10^8$/ml) they probably play a large part in this action. Hobson and Mann (1961) also found that these bacteria did not metabolise the free fatty acids released, a finding that is in agreement with Garton et al (1961). More recently Wood, Bell, Grainger and Teekel (1963) introduced 1-$^{14}$C linoleic acid into the rumen of a sheep and found that less than 1% of radioactivity subsequently appeared in the steam volatile fraction of rumen contents. Garton (1964) found no radioactivity in carbon dioxide and fatty acids of chain length less than $C_{18}$, following the incubation of stearodiolein (containing $^{14}$C-stearic acid) with rumen contents.

The release of galactose from galactolipids is catalysed by $\alpha$ and $\beta$ galactosidases. Conchie and Levvy (1957) showed that mixed microorganisms of sheep rumen contents possessed both $\alpha$ and $\beta$ galactosidase activity and Bailey (1962, 1964) prepared cell-free extracts of bovine rumen bacteria which hydrolysed mono- and digalactosyl glycerol. These fractions however could not release galactose if the remaining alcohol moieties of glycerol were esterified, leading to the conclusion that hydrolysis of esterified fatty acids must precede galactosidase activity. However it has been shown that $\alpha$ and $\beta$ galactosidases of a number of protozoal species can hydrolyse galactose from galactoglycerides, whether the remaining alcohol moieties are esterified with fatty acid or not (Howard, 1963; Bailey and Howard, 1963).

### 1.3.2. Hydrogenation of unsaturated fatty acids by rumen fluid

Reiser (1951) first demonstrated that rumen fluid possessed

the ability to hydrogenate unsaturated fatty acids. On incubation of sheep rumen fluid with linseed oil, the linolenic acid content of the oil decreased from 30% to approximately 5% with a corresponding increase in the level of linoleic acid. Boiled rumen contents failed to give this effect as did rumen fluid from which bacteria had been removed by centrifugation, thus it was concluded that rumen microorganisms were responsible for this process. Willey, Riggs, Colby, Butler and Reiser (1952) fed steers on a ration containing 5% cottonseed oil and concluded that the higher stearic acid content in their fat depots, compared with that in control animals, resulted from the assimilation of stearic acid produced by hydrogenation of the unsaturated $C_{18}$ acids of the oil, by rumen microorganisms.

Reiser and Reddy (1956) provided the first direct evidence for hydrogenation taking place in the rumen. They fed goats a diet containing 10% linseed oil, and measured the fatty acids present in the rumen after slaughter, 6 hrs after feeding. The results confirmed that hydrogenation had taken place in the rumen.

The mechanisms involved in hydrogenation of unsaturated fatty acids of the diet was first investigated by Shorland, Weenink, Johns and McDonald (1957). These workers incubated large amounts of oleic, linoleic and linolenic acid with sheep rumen contents for 48 hrs at 37°, and found that about 20% of each unsaturated acid was converted to stearic acid. Trans-acids were formed to the extent of 17%, 48% and 67% from oleic, linoleic and linolenic acids respectively. Positional isomers were also formed, particularly from linoleic acid, which gave rise to a conjugated acid, apparently resistant to further hydrogenation.

Ward, Scott and Dawson (1964) provided useful information on the intermediates of hydrogenation by incubating radioactive $C_{18}$ fatty acids with sheep rumen contents in an artificial rumen. Incubation of $^{14}C$ - linoleic acid gave rise to a mono-unsaturated acid with a <u>trans</u> configuration, with the double bond predominantly at C-13 or C-14. Linolenic acid gave rise to two dienoic acid isomers with a <u>cis</u>-<u>cis</u>-nonconjugated configuration and with the majority of the double bonds at C-11 or C-12 and C-15 or C-16. This supports Shorland <u>et al</u> (1957) who found that the major dienoic acid in the non-conjugated fraction, arising from the hydrogenation of linolenic acid was octadeca-11, 15-dienoic acid.

It is obvious that during the hydrogenation process there is a considerable amount of bond migration. This bond migration and the formation of a <u>trans</u> structure during microbial hydrogenation closely parallels events known to take place when unsaturated fatty acids are subjected to the influence of molecular hydrogen, in the presence of finely divided metal catalyst (Markley, 1961). Polan, McNeill and Tove (1964) have discounted the possibility that molecular hydrogen participates directly in hydrogenation in the rumen and suggested that the mechanism involved a microbial hydrogenase and a redox compound. Polan <u>et al</u> (1964) also reported that boiled rumen fluid stimulated the hydrogenation of linoleic acid and oleic acid by washed-cell suspensions of mixed rumen bacteria. This was supported by the studies of Wilde and Dawson (1966).

The studies of Wilde and Dawson (1966) gave similar results to Ward <u>et al</u> (1964) with the exception that the major pathway involved the <u>cis</u>-<u>trans</u> (or <u>trans</u>-<u>cis</u>) octadecadienoic acid intermed-

iate, instead of the *cis-cis* octadecadienoic acids obtained in the 1964 studies. Wilde and Dawson (1966) also isolated the first intermediate in the biohydrogenation, as an octadecatrienoic acid with two double bonds conjugated and suggested that it could be either an octadeca-9,11,15-trienoic acid or an octadeca-9,13,15-trienoic acid. This would seem to lend support to the earlier work of Shorland *et al* (1957) where the octadeca-11,15-dienoic acid was the major intermediate formed in the hydrogenation of linolenic acid. The scheme for the hydrogenation of linolenic acid as proposed by Wilde and Dawson (1966) is shown in Fig. 4.

Kepler, Hirons, McNeill and Tove (1966) studied the intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. They found that this bacterium could hydrogenate a mixture of *cis*-9-*trans*-11-octadecadienoic acid and *trans*-10-*cis*-12-octadecadienoic acid, as readily as linoleic acid itself. However *trans*-9-*trans*-12- and *cis*-9-*trans*-12- and *trans*-9-*cis*-12-octadecadienoic acids were not hydrogenated. This led to the belief that the two former dienoic acids were intermediates in linoleic acid hydrogenation by *B. fibrisolvens* and this was shown experimentally. Kepler and Tove (1967) showed that the isomerisation of linoleic acid to octadeca-*cis*-9-*trans*-11-dienoic acid was catalysed by linoleate *cis*-12, *trans*-11-isomerase, an enzyme localised in the cell envelope.

### 1.3.3. Fermentation of glycerol and galactose

The glycerol and galactose moieties released after hydrolysis of dietary lipids in the rumen are fermented by rumen bacteria to yield volatile fatty acids.

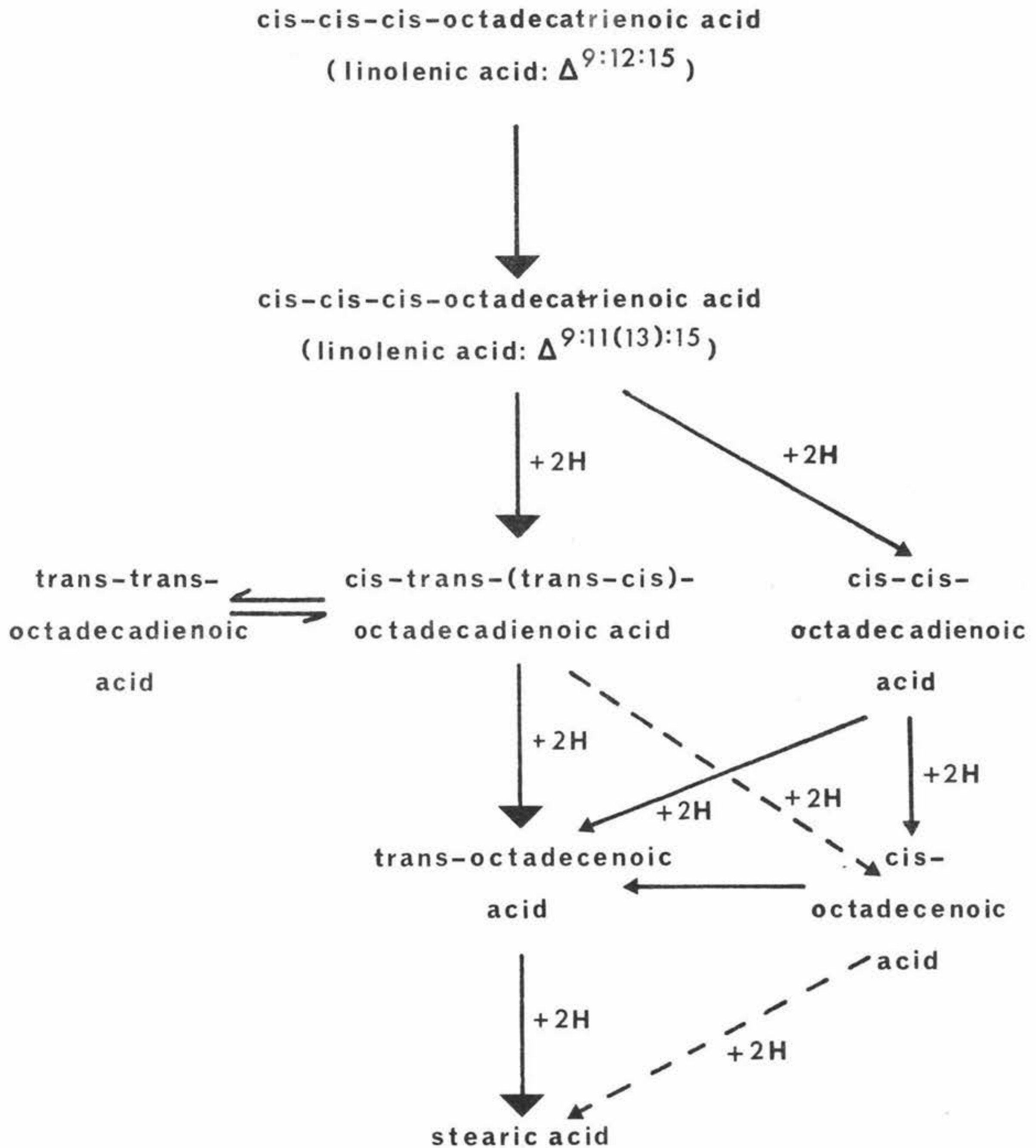



Figure 4. Pathway for the hydrogenation of linolenic acid by rumen microorganisms. (from Wilde and Dawson, 1966).

Hobson and Mann (1961) isolated _Selenomonas ruminantium_ var _lactilyticus_ from sheep rumen contents and found it to be capable of fermenting glycerol to propionic acid. It is agreed that propionic acid is the main product of glycerol fermentation (Johns, 1953; Hobson and Mann, 1961; Garton _et al_, 1961).

Galactose can be fermented to yield a mixture of acetic, propionic and butyric acids by several isolated bacterial species from the rumen. Hobson and Mann (1961) showed that _Selenomonas ruminantium_ var _lactilyticus_ could also ferment galactose, as could a _Butyrivibrio_ isolated by Hobson and Purdom (1961). Howard (1959) found that the rumen protozoan _Dasytricha ruminantium_ could also ferment galactose and it has been shown that several proteolytic bacteria also possess this ability (Blackburn and Hobson, 1962).

### 1.4. Significance of Hydrolysis in the Rumen

Although the lipid content of the diet of most ruminants is small, it has been pointed out that the actual intake of lipid per day can be quite considerable. Lipid is an important dietary constituent for all the major metabolic processes of the animal, _viz_. maintenance, body storage, lactation and pregnancy. Consequently the modification of dietary lipid into suitable metabolites for use in these processes is most important.

The majority of the dietary lipid consists of glycerides (Hilditch, 1956; Shorland, 1961), and from the foregoing discussion, it is apparent that hydrolysis is the first step in the breakdown of these dietary components. The question of interreaction of hydrolysis and hydrogenation has largely been resolved by Hawke and Silcock (1969) who found that hydrogenation in the rumen

required free fatty acid substrate i.e. fatty acids were not hydrogenated while still in ester combination with glycerol.

Thus it is apparent that the degree of saturation of the fatty acids available to the ruminant, is dependent on the activity of hydrolytic enzymes in the rumen. This could also be reflected in the degree of saturation of the fatty acids in the blood, depot tissues and milk - a slow rate of hydrolysis of dietary triglycerides in the rumen could mean less exposure of free fatty acids to hydrogenation, and the subsequent appearance of less saturated fatty acids in the body tissues and the milk.

Hydrolysis also makes available glycerol and galactose for fermentation by rumen microorganisms, providing a further energy source for the host, in the form of volatile fatty acids (Garton _et al_, 1961; Hobson and Mann, 1961).

## Chapter 2

### THE OBJECT OF THE PRESENT STUDY

Studies during the last decade have shown that rumen micro-organisms can effect extensive changes in dietary lipids. The release of free fatty acids from ester link with glycerol by rumen microorganisms was first reported by Garton et al (1958). This finding has subsequently been confirmed by Garton et al (1959), Garton et al (1961), Hawke and Robertson (1964), and Silcock (1968). These observations were mainly confined to a demonstration of hydrolytic activity by both in vivo and in vitro techniques, or to show the effect of lipid on overall metabolism.

Garton et al (1958) in their original studies, showed that it was the rumen bacteria that were responsible for this hydrolytic activity, but attempts to obtain cell-free lipolytic enzyme preparations of rumen bacteria have not been successful. However, Dawson (1959) isolated a lysolecithinase, and Hobson and Mann (1961) isolated rumen bacteria in pure culture which were capable of hydrolysing triglycerides such as linseed oil.

The object of this work was to isolate the lipolytic fraction from the bovine rumen, and if possible, prepare a cell-free lipolytic fraction capable of hydrolysing both natural and synthetic triglycerides.

# Chapter 3

## MATERIALS AND METHODS

### 3.1. Solvents and Reagents

All solvents used were purified according to Vogel (1956), and all reagents were of either "Analar" or "Technical" grade unless otherwise stated. Radioactive glyceryl tri(oleate-1-$^{14}C$) was obtained from the Radiochemical Centre, Amersham, England.

### 3.2. Assay of Lipase Activity by the Colorimetric Microdetermination of Long-chain Fatty Acids

The method employed was essentially the colorimetric method devised by Ayers (1956) and modified by Iwayama (1959) and Duncombe (1963).

This method is dependent on the formation of copper salts of free fatty acids (FFA) in chloroform solution, on the addition of a copper reagent (9vol. aq. IM-triethanolamine; 1vol. IN-acetic acid; 10vol. 6.45% (w/v) $Cu(NO_3)_2 \cdot 3H_2O$).

A 5ml aliquot of the total lipid residues from the incubations, to give FFA's in the range of 0.05 - 0.5 $\mu$ moles, was shaken by inversion with copper reagent for 2 minutes, and then centrifuged. The excess reagent at the top of the tube was drawn off with the aid of a suction pump. A 3ml aliquot of these copper salts in chloroform was added to 0.5ml of diethyldithiocarbamate reagent (0.1% (w/v) solution of sodium diethyldithiocarbamate in redistilled butan-2-ol). The solutions were then mixed by shaking, and the extinction measured at 440m$\mu$ in a 1cm light path, against

a blank solution that had been subjected to the same procedure, but with no added lipid.

The amount of FFA in the products of incubation could then be determined by comparison against a standard curve of known concentration (Fig. 1).

## 3.3. Thin Layer Chromatography

### 3.3.1. Preparation of thin layers

Thin layers of Silica Gel G (E.Merk, A-G., Darmstadt, Germany) were prepared by shaking a weighed amount of the silica gel with twice the weight of distilled water. Layers of 0.25mm thickness were spread onto glass plates using a Desaga applicator. The layers were allowed to settle at room temperature, and were then transferred to an oven at 110$^{o}$ for approximately 60 minutes for activation.

### 3.3.2. Preparation of the eluting solvents

The eluting solvents used in all separations were hexane, diethyl ether, and acetic acid mixed in the proportions 70:30:1 (v/v). With the exception of the latter the solvents were dried over anhydrous sodium sulphate.

### 3.3.3. Chromatographic procedure

Samples were taken up in hexane and applied to the layers at the origin with glass capillary tubes. All layers were developed in a previously dried and sealed chromatography tank. Following development the layers were dried, and for a visual appraisal were sprayed with a 0.1% (w/v) solution of 2,7-dichlorofluorescein in ethanol, and viewed under ultraviolet light. Components were identified by comparison with known standards chromatographed

simultaneously on the same layer.

## 3.4. Radiochemical Methods

### 3.4.1. Radioisotope counting

Radioactivity was counted in a Packard Model 3375 Liquid Scintillation Spectrometer. The scintillation fluid consisted of 0.6% (w/v) 2,5-biphenyloxazole (PPO), and 0.05% 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP), dissolved in toluene. PPO, POPOP, and scintillation grade toluene were obtained from Nuclear Enterprises Ltd., England.

A suitable aliquot of sample was placed in a counting vial using a 5 or 30 microlitre syringe (Hamilton), and evaporated to dryness under a 250W lamp. 10ml of scintillation fluid was then added to each counting vial.

The efficiency of the counting system was determined by including a standard sample of $^{14}C$-hexadecane in scintillation fluid, with each batch.

### 3.4.2. Determination of radioactive components of thin layers

The distribution of $^{14}C$-radioactivity between the various lipid components separated on thin layer plates (20cm X 5cm) was measured on a Packard Model 7200 Radiochromatogram Scanner. The most suitable conditions for preparing the radiochromatogram scan were; voltage, 300; time constant, 30sec; chart speed, 6cm/hr; gas flow, (1.3% *iso*butane, 98.7% helium) 110ml/min.

Identification of the radioactive components was achieved by spraying marker compounds on the plates with 2,7-dichlorofluorescein after scanning, and viewing under ultraviolet light.

To obtain a relative estimate of the proportions of radioact-

ivity in each lipid component, peak areas were measured with the use of a planimeter (G.Coradi A.G., Zurich). A base line was drawn through the average background, and the peak areas were measured until a constant reading was obtained.

Alternatively, radioactivity was determined after thin layer chromatography, by scraping the appropriate areas of the chromatogram directly into counting vials. Scintillation fluid was added and the samples counted as described above.

### 3.5. Rumen Sampling

Throughout this study, samples were taken from a lactating Jersey cow which had been fitted with a rumen fistula. The cow was a member of the milking herd grazing on pasture consisting essentially of ryegrass, with some clover. Rumen samples were withdrawn after the morning milking so that the animal had not eaten for three hours. Each sample was taken from a central position in the rumen i.e. below the rumen fistula in an endeavour to reduce sampling errors (Bryant, 1959).

### 3.6. Preparation of Rumen Contents for Incubation

In preliminary studies, rumen contents were strained immediately after sampling, through two layers of cheese cloth to remove the coarser food particles. The strained material was stored in a flask at 39°, under anaerobic conditions (in a nitrogen atmosphere) until required. In later experiments total rumen contents were removed and placed directly into a previously warmed thermos flask, under the same conditions.

During the fractionation of rumen contents care was taken at

all times to ensure that a temperature of 39° was maintained, and that systems were kept anaerobic, in a continuous stream of nitrogen.

When fractionation of rumen contents involved loss of material, each fraction was made up to the volume of the starting material with clarified rumen liquor (CRL), to permit valid comparisons between fractions. The preparation of CRL is described in Section 3.6.2.

### 3.6.1. Preparation of protozoal and "debris" fractions

Strained rumen contents were subjected to a centrifugation at 500g for 10 minutes in a Sorvall Model SS-3 Automatic Superspeed Centrifuge using a GSA rotor, to precipitate protozoa and fine food particles. Separation of protozoa was achieved by a sedimentation technique, based on the method of Heald, Oxford and Sugden (1952). The modified buffer system used was:

| Component | Concentration (g/1) |
|---|---|
| $K_2HPO_4$ | 1.5 |
| NaCl | 6.0 |
| $MgSO_4.7H_2O$ | 0.05 |
| $CaCl_2$+aq | 0.05 |

This was made up to a litre with distilled water and just prior to use, the following additions were made:

| | |
|---|---|
| cysteine-HCl | 0.2 |
| $NaHCO_3$ | 5.0 |

The buffer system was then added to the 500g precipitate and mixed several times by inversion, in a separating funnel. The suspension

was allowed to stand for 30 minutes at 39°, in which time a greyish-white layer consisting essentially of protozoa sedimented out at the bottom of the funnel, and the plant debris material collected as a thick green layer at the top. The layer which contained the partially purified protozoa was run off and washed several times with fresh buffer until the precipitate appeared as a clean white layer. This was then run off, and made up to the original volume, with CRL for incubation.

The plant debris material which rose to the surface in the buffer, was drawn off with the aid of a suction pump and made up to the original volume as above.

### 3.6.2. Preparation of clarified rumen liquor and bacterial fractions

The supernatant from the first 500g centrifugation was subjected to a further centrifugation at 14,000g for 30 minutes. The supernatant was decanted off and recentrifuged at 36,000g for 30 minutes in a Spinco Model L Ultracentrifuge to yield rumen liquor which was optically clear (clarified rumen liquor-CRL).

The precipitate from the first centrifugation was washed and recentrifuged several times with CRL to yield a clean bacterial pellet. This was made up to the original volume by dispersion in CRL.

### 3.6.3. Homogenisation of unstrained rumen contents

Total rumen contents were removed from the thermos flask and homogenised in a Polaris Homogeniser for 10 minutes. Where necessary CRL was added to give a suitable consistency for thorough homogenisation. Allowances were made for this addition so that com-

parisons could be made with other fractions. Following this treatment the mixture was strained through two layers of cheese cloth, and then centrifuged at 500g for 10 minutes. Supernatant and centrifugate were then treated as described in Sections 3.6.1. and 3.6.2.

## 3.7. Incubation Procedures for Following Lipolysis

During the course of this study, the incubation procedures were dependent on the method used for the assay of lipolytic activity.

(a) Colorimetric assay

When the colorimetric method was used for the assay of lipolytic activity, 200ml of strained rumen contents was used as the starting material. Prepared centrifugates were dispersed in CRL to give a final volume equal to that of the starting material. Each fraction was incubated in 250ml Erlenmyer flasks in a water bath at 39°. Flasks were attached to a shaker to ensure thorough mixing throughout the incubation, and nitrogen was continually bubbled through the incubation medium in each flask to maintain anaerobic conditions. Boiling tubes were inserted between flasks in the flow system to serve as traps in the event of the development of back-pressures.

Peanut oil (Kempthorne, Prosser and Co's) was used as the substrate for hydrolysis and was added as an emulsion which remained stable throughout the course of the experiment. The emulsion was formed by mixing equal volumes of peanut oil and water with 1% Lissapol (Imperial Chemical Industries), in an homogeniser, and sufficient emulsion was added to the incubation medium to give a

lipid concentration of 1.0% (v/v). 5ml aliquots were withdrawn from the single incubations at 30 minute intervals up to a total time of 4 hrs to determine the rate of hydrolysis.

In all experiments a control was run consisting of boiled rumen contents with added substrate, or rumen contents with no added substrate.

(b) Radiochemical assay

Radioactive glyceryl tri(oleate-1-$^{14}C$) (35.4 μc/uM) in hexane, was added together with unlabelled carrier to give a final count of 2 μc/20ml of incubation medium. Assay procedures were conducted with two slight variations.

(i) In preliminary studies, incubation conditions and experimental procedures were identical to those described above, with the exception that in two instances 0.1M phosphate buffer pH 7.0 replaced CRL, for resuspending centrifugates.

(ii) When the assay was used to follow the separation of active lipolytic fractions, radioactive substrate in hexane was added to either a 5ml or a 20ml aliquot of each fraction, with no unlabelled carrier. Centrifugates were made up to the original volume of the starting material with phosphate buffer. Incubation conditions were the same as described above with the exception that the incubation time was shortened to 1 hour and conditions were not kept anaerobic after cell-free extracts had been prepared. In this case the total incubation medium was used for the analysis of reaction products.

### 3.8. Termination of Reaction and Extraction of Lipid

The reaction was terminated by the addition of an equal volume of ethanol, and boiling the mixture for 5 minutes. The mixture was then transferred to a separating funnel and the lipid was extracted into chloroform. The chloroform layer was run off and the remaining aqueous layer washed twice with the same solvent. The combined chloroform extracts were collected in round-bottom flasks and evaporated to dryness *in vacuo* on a rotary evaporator. The lipid extracts were taken up in a known volume of chloroform (colorimetric analysis) or hexane (radiochemical analysis), and stored in the refrigerator at $4^{\circ}$ to await analysis of their lipid content.

### 3.9. Preparation of Cell-Free Bacterial Extracts

The preparation of cell-free bacterial extracts was carried out in an endeavour to release hydrolytic enzymes from the rumen bacteria. Treatment of whole cells by osmotic shock was based on the method of Nossal and Heppel (1966). An alternative method was based on high frequency sonication, using a sonic probe Type 1130A (Dawe Instruments Ltd., London).

#### 3.9.1. Osmotic shock treatment

Rumen bacterial cells were obtained as described in Section 3.6.2., and were washed three times with phosphate buffer, pH 7.0. Approximately 1g of washed cells was taken, to which was added 40ml of 40% sucrose in 0.033M Tris-HCl buffer, pH 7.0, followed by sufficient 0.1M disodium ethylenediaminotetra-acetic acid (EDTA), pH 7.0 to give a final concentration of $10^{-4}$M. This was then

shaken in a Griffen Flask Shaker for 10 minutes at room temperature and then centrifuged at 14,000g for 20 minutes in a Sorvall SS-3 centrifuge. The pellet was then dispersed in 80 ml of ice-cold 5 X $10^{-4}$M magnesium chloride and gently stirred in an ice-bath for 10 minutes. Fractions from a centrifugation of this suspension at 90,000g (Spinco Model L Ultracentrifuge), were incubated with radioactive glyceryl tri (oleate-1-$^{14}$C) for 1 hr as described in Section 3.7.

3.9.2. High frequency sonication

Approximately 1g of the washed cells was resuspended in phosphate buffer to form a thick slurry. This was placed in an ice-bath and subjected to sonication for 2 minutes at 5.0 amperes. Precipitate and supernatant fractions prepared at 90,000g were used for incubation.

# Chapter 4

## EXPERIMENTAL AND RESULTS

### 4.1. Assay of Lipolytic Activity

#### 4.1.1. Preparation of a standard curve for the colorimetric determination of free fatty acids released during lipolysis

Standard solutions of palmitic acid were made up in the range 0.05-0.5 $\mu$ moles in chloroform, to a final volume of 5 ml, and subjected to the treatment described in Section 3.2. The extinction of a range of free fatty acid (FFA) levels were recorded on a Beckman Model DU Quartz Spectrophotometer.

The relationship between extinction and FFA levels for palmitic acid in chloroform is shown in Fig. 5. This standard graph was used for all the subsequent FFA estimations using the colorimetric method. Appropriate standard solutions of palmitic acid in chloroform were included with each series of determinations.

#### 4.1.2. Formation of $^{14}C$-fatty acid from $^{14}C$-triolein as an assay of lipolytic activity

An assay was developed using pancreatic lipase, as this enzyme is possibly the best characterised of the lipolytic enzymes, and is available commercially.

Hog pancreatic lipase (Type II, Sigma Chemical Co., St. Louis, Mo., U.S.A.) was incubated with glyceryl tri(oleate-1-$^{14}C$) for 6 minutes under the following conditions of incubation; $1.4 \times 10^{-3}$ $\mu$ moles glyceryl tri(oleate-1-$^{14}C$) ($1.1 \times 10^{5}$ dpm) in 5 $\mu$l hexane; 5 $\mu$l 4% (w/v) $CaCl_2$; 0.02mg pancreatic lipase in 1ml 1M Tris-HCl

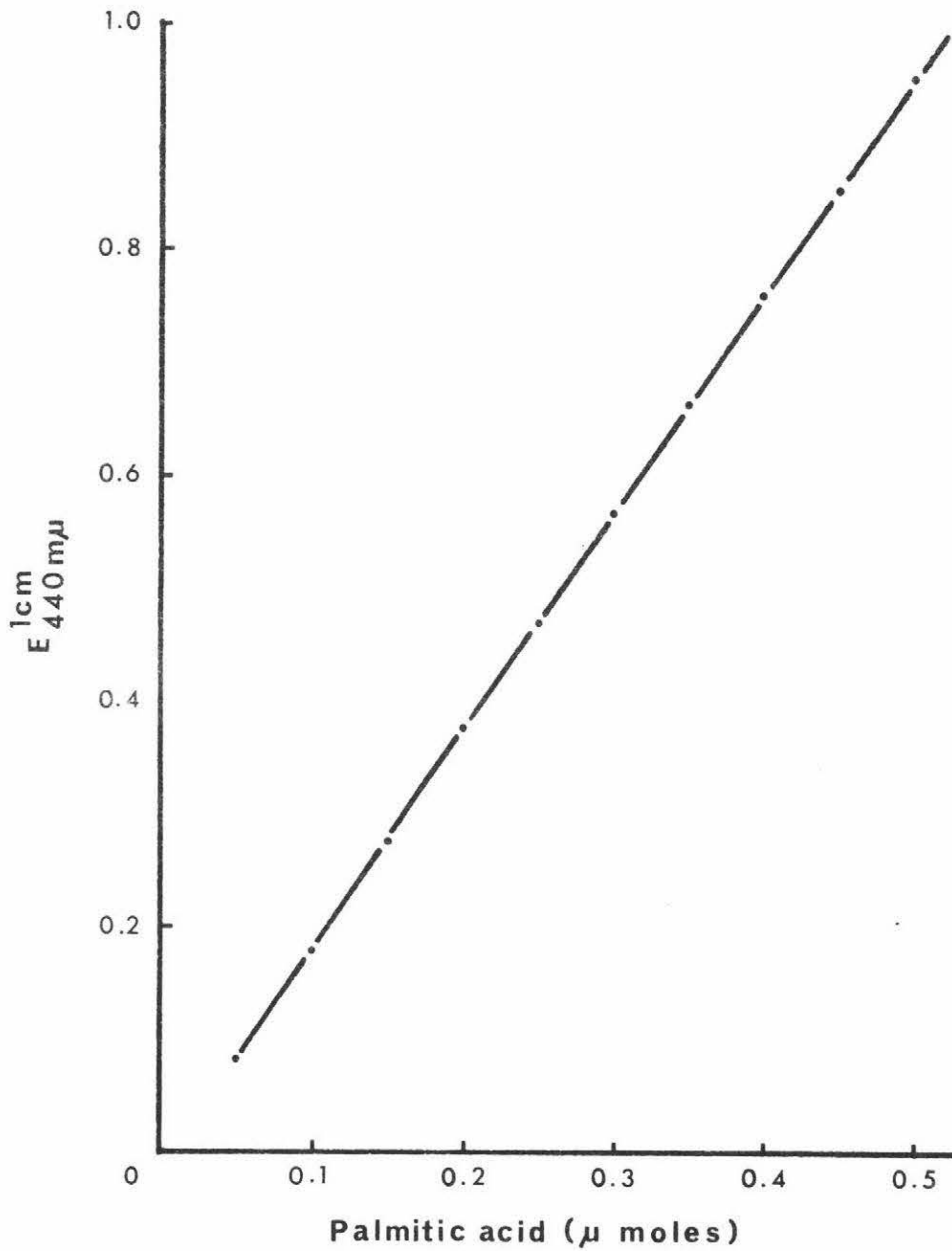



Figure 5. The relationship between extinction and free fatty acid levels, for solutions of palmitic acid in chloroform.

buffer, pH 7.5. Incubations were conducted in a water bath at 38° with constant shaking. The reaction was stopped and the lipids extracted as described in Section 3.8. Following evaporation to dryness the lipid was dissolved in a suitable volume of hexane and an aliquot of this solution (containing approximately 3,000 dpm) was applied to a thin layer of silica gel and chromatographed as described in Section 3.3.3.

The distribution of radioactivity between the products of lipolysis, after incubation of $^{14}C$-triolein with pancreatic lipase for 5 minutes, as shown by a radiochromatogram scan of a thin layer of silica gel, is illustrated in Fig. 6.

The formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein by pancreatic lipase was determined by individual incubations of 1 to 6 minutes under the conditions described above (Table 3).

Approximately 45% of the total radioactivity was recovered in the FFA fraction after a 6 minute incubation, the rate of release being almost linear over this period (Fig. 7). The formation of diglyceride reached a maximum of 24.3% after 4 minutes and then decreased. An approximately linear formation of monoglyceride was observed, reaching a level of 11.1% of the total radioactivity after an incubation time of 6 minutes.

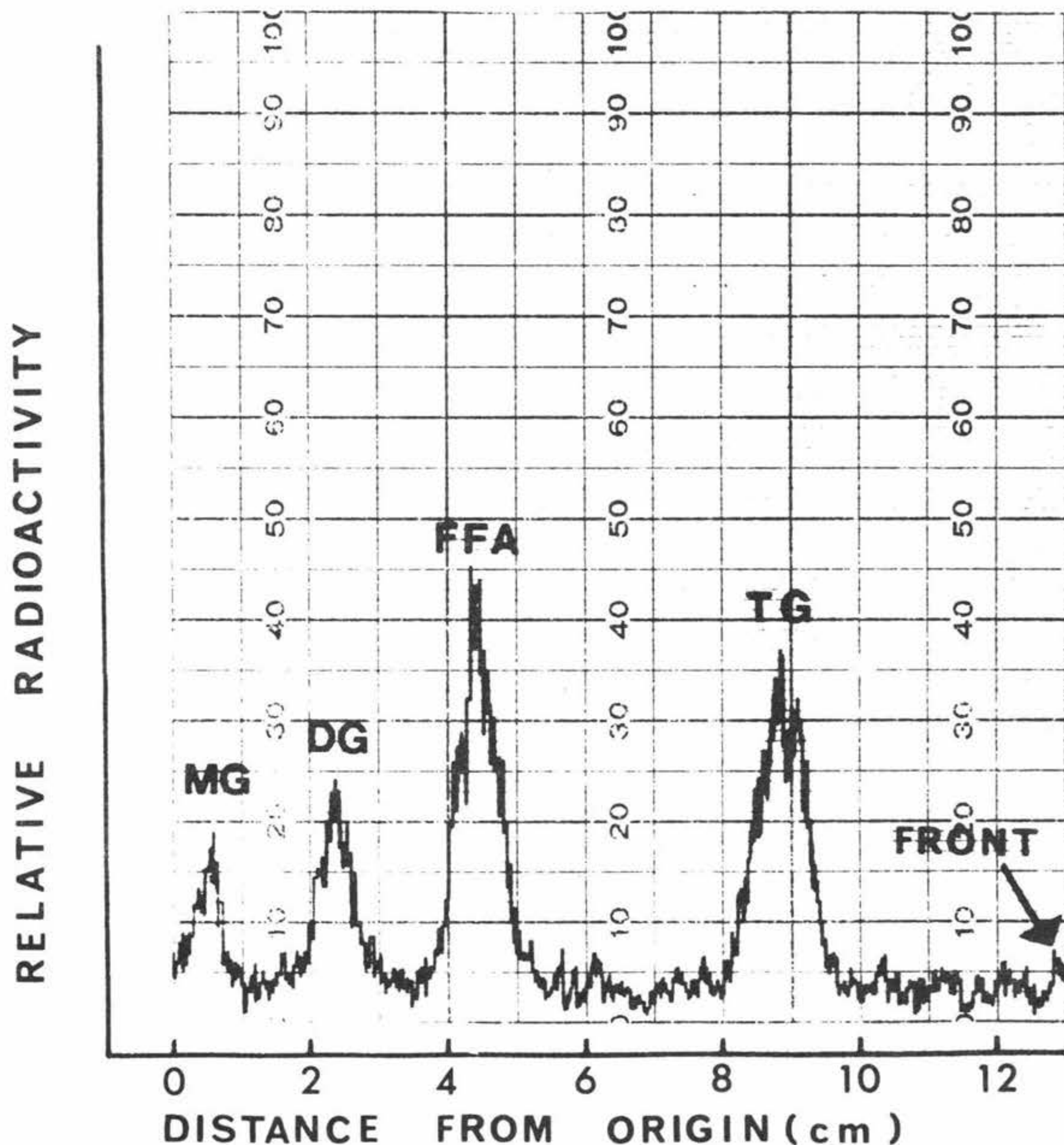



Figure 6. Radiochromatogram scan of the products of hydrolysis of $^{14}C$-triolein separated by thin layer chromatography. Conditions of incubation: 5 ul substrate containing $1.4 \times 10^{-3}$ u moles triolein ($1.1 \times 10^{5}$ dpm), pancreatic lipase (0.02mg) suspended in 1ml 1M Tris-HCl buffer (pH 7.5), 5 ul 4% CaCl , incubated for 5 min at 39°. MG = monoglyceride, DG = diglyceride, FFA = free fatty acid, TG = triglyceride. (See text for scanning conditions).

TABLE 3

Formation of $^{14}C$-labelled hydrolysis products
from $^{14}C$-triolein by pancreatic lipase
(for conditions of incubation see text)

| Time of incubation (min) | % radioactivity in each lipid fraction as a % of total radioactivity | | | |
|---|---|---|---|---|
| | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 0 | 100 | - | - | - |
| 1 | 78.7 | 9.3 | 9.7 | 2.3 |
| 2 | 64.8 | 16.4 | 15.6 | 3.1 |
| 3 | 54.3 | 21.6 | 17.3 | 6.9 |
| 4 | 43.7 | 26.2 | 24.3 | 5.8 |
| 5 | 38.1 | 37.1 | 16.5 | 8.3 |
| 6 | 28.4 | 44.4 | 16.1 | 11.1 |

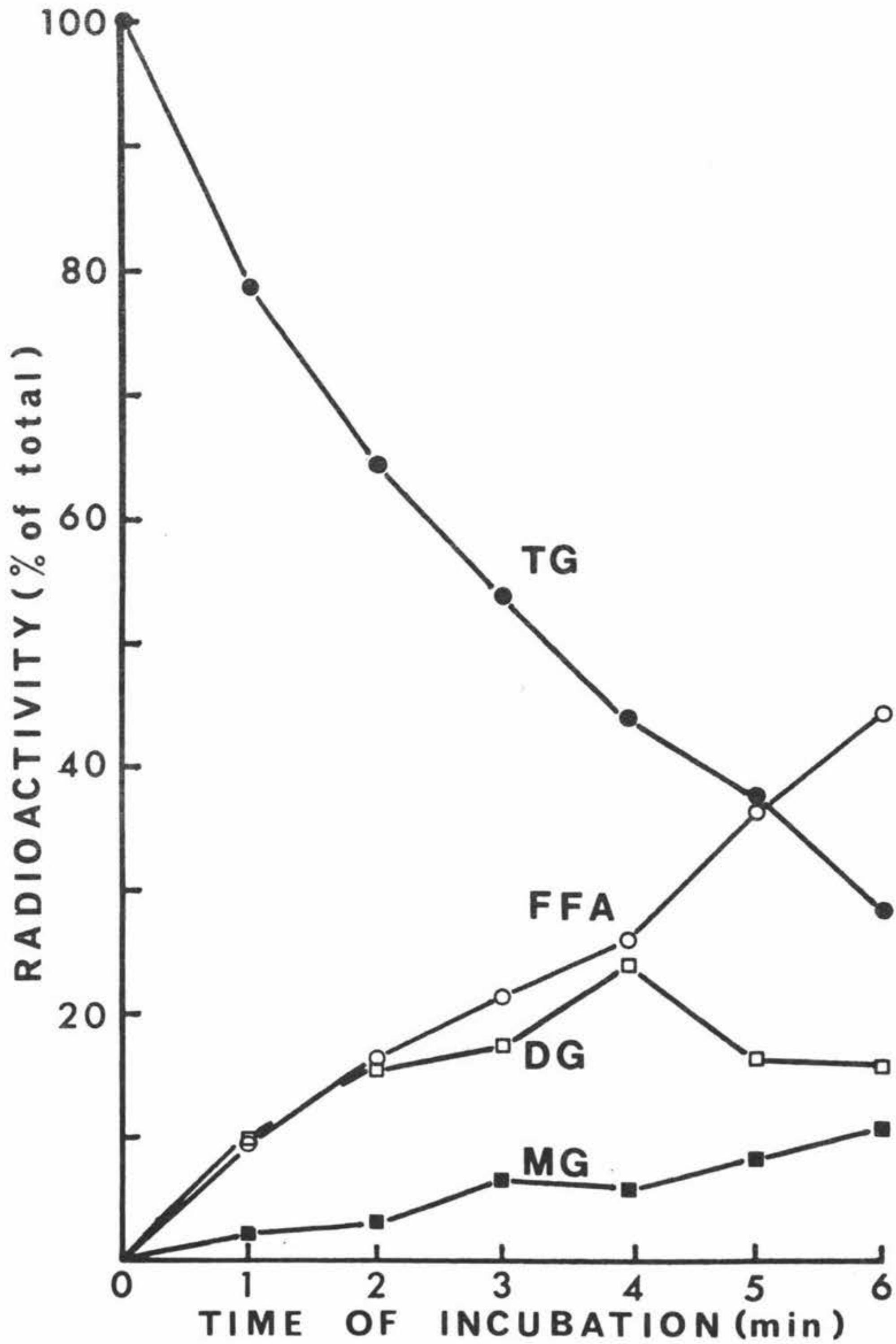



Figure 7. Changes in concentration of labelled components when $^{14}C$-triolein was incubated with pancreatic lipase. TG = triglyceride, DG = diglyceride, MG = monoglyceride, FFA = free fatty acid. (See text for incubation conditions).

4.1.3. Evaluation of radiochemical techniques

On analysis, the products of hydrolysis, and the determination of the percentage hydrolysis of the substrate involved either; (a) a radiochromatogram scan of the thin layers of silica gel and planimetric measurement of peak areas or (b) scraping off the appropriate areas of the silica gel directly into counting vials, followed by counting in a liquid scintillation spectrometer. Both techniques were used throughout this study and consequently it was necessary to compare these two analytical procedures.

20ml of strained rumen liquor was incubated with 5 $\mu$l $^{14}$C-triolein in 0.4ml of emulsified peanut oil, for different time intervals to give a range of hydrolysis rates and these were used as a source of $^{14}$C-labelled hydrolysis products. The thin layer chromatograms of the extracted lipids were first scanned by a radiochromatogram scanner and then the radioactive areas were scraped directly into counting vials and counted in a liquid scintillation spectrometer. The two alternative procedures gave similar proportions for each of the lipid components (Table 4).

TABLE 4

$^{14}C$-triolein following thin layer chromatography by: Determination of the hydrolysis products of

(a) radiochromatogram scanning and planimetric measurement of peak areas
(b) removing radioactive components and counting by liquid scintillation spectrometry

| Component | % radioactivity in each lipid component (as a % of total radioactivity) | |
|---|---|---|
| | Radiochromatogram Scan (a) | Liquid Scintillation Spectrometry (b) |
| triglyceride | 77.9 | 77.5 |
| fatty acid | 16.8 | 16.8 |
| diglyceride | 4.3 | 4.7 |
| monoglyceride | 1.0 | 1.0 |

The figures quoted in Table 4 are for one set of incubation products. A statistical analysis of the results of four incubation products gave a correlation (r) = 1.000 and a regression ($b_{scan.\ scint.}$) = 1.0049. It is clear, that within the limits of experimental error, both analytical procedures gave comparable results, and the data obtained from one procedure could equally be applied to the other procedure.

Reproducibility of the procedure of scintillation spectrometry combined with thin layer chromatography was tested on four aliquots of the same lipid extract prepared from an incubation mixture. These were separated into reaction products on thin layers of silica gel and areas corresponding to the location of each

component were scraped directly into counting vials and counted in toluene scintillation fluid. Table 5 illustrates the reproducibility of four separate determinations of $^{14}C$ components of the same incubation mixture.

TABLE 5

Reproducibility of the determination of percentage hydrolysis products, by removing $^{14}C$-labelled components from the chromatogram and counting by liquid scintillation spectrometry

| Aliquot No. | % Radioactivity in each lipid component (as a % of total radioactivity) | | | |
|---|---|---|---|---|
| | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 1 | 40.3 | 51.7 | 5.9 | 2.2 |
| 2 | 39.4 | 52.8 | 5.8 | 2.1 |
| 3 | 39.8 | 51.9 | 6.8 | 1.5 |
| 4 | 40.0 | 51.8 | 6.4 | 1.8 |

A statistical analysis on the percentage of FFA recovered in each case gave a figure of 52.03 ± 0.22 ($\bar{x}$ + $SE_{\bar{x}}$) where $\bar{x}$ is the mean and SE is the standard error.

## 4.2. Lipolytic Activity of Strained Rumen Liquor

### 4.2.1. Demonstration of lipolytic activity by qualitative thin layer chromatography

The lipolytic activity of strained rumen liquor was demonstrated by incubating 4ml of emulsified peanut oil with 200ml of strained rumen liquor. 5ml aliquots were taken from the incubation

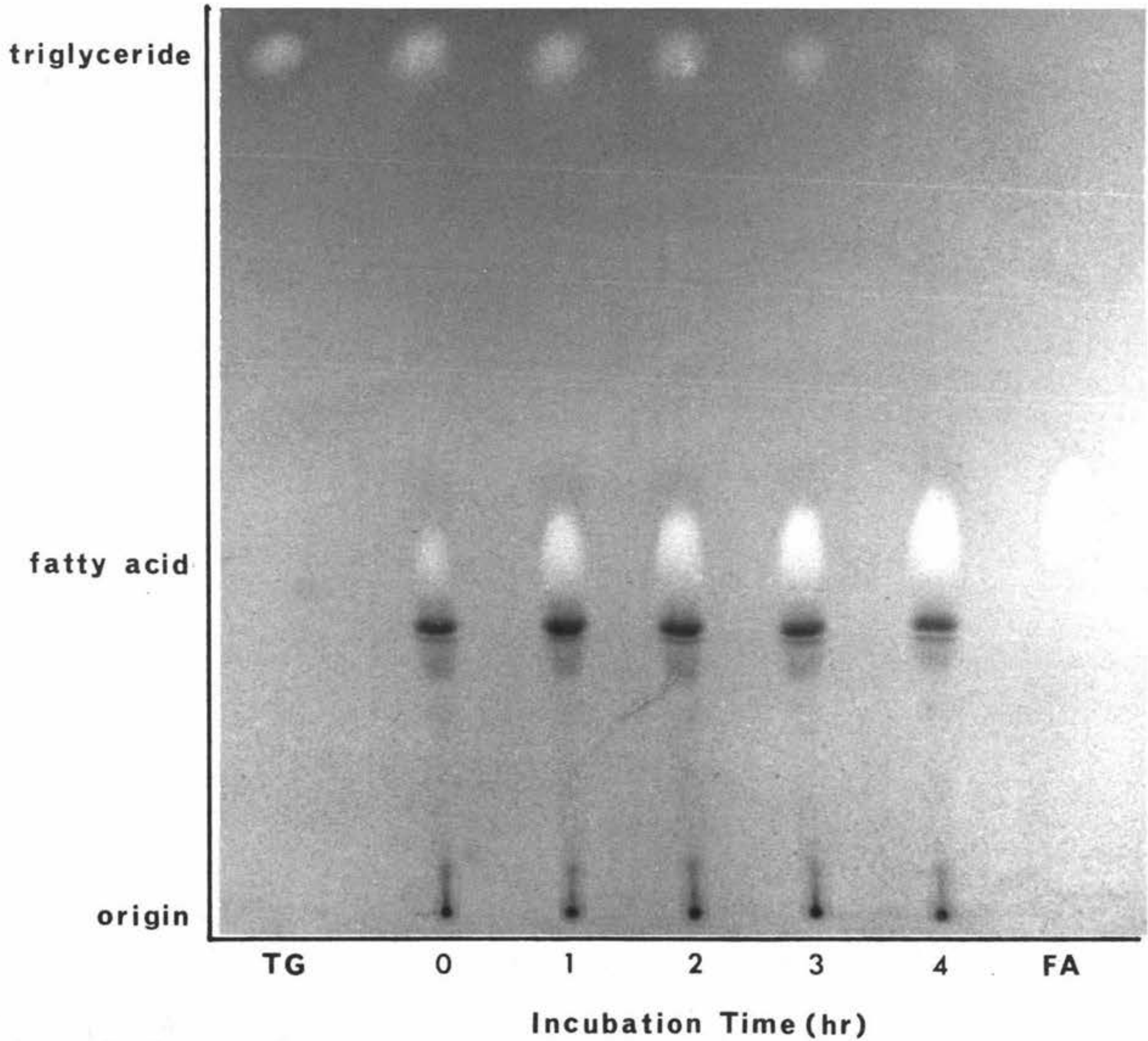



Figure 8a. Thin layer chromatographic separation of free fatty acid and triglyceride components following incubation of emulsified peanut oil with strained rumen liquor. TG = triglyceride standard, FA = fatty acid standard.

medium at 30 minute intervals for a period of 4 hr, and the lipid extracted. A qualitative measure of the formation of FFA was obtained by separating approximately equivalent amounts of each aliquot on thin layers of silica gel. It can be seen from Fig. 8a, that FFA increases and triglyceride decreases with time of incubation. After 1 hr of incubation a small quantity of FFA was present and most of the lipid was in the triglyceride fraction. However, after 4 hrs of incubation triglyceride had almost disappeared and FFA was the major component.

### 4.2.2. Quantitative measure of lipolytic activity of strained rumen liquor

#### (a) Colorimetric assay of free fatty acid released

A quantitative measure of the FFA formation illustrated in the above Section was obtained by a colorimetric determination as described in Sections 3.2. and 4.1., and the results are presented in Table 6.

TABLE 6

Formation of free fatty acid from emulsified peanut oil, by strained rumen liquor

Incubation conditions: 200ml of strained rumen liquor; 4ml of emulsified peanut oil Temperature, 39°; in nitrogen; with constant shaking

| Time of | Free Fatty Acid (μ moles) | |
|---|---|---|
| Incubation (hr) | Strained rumen liquor with substrate | Strained rumen liquor without substrate |
| 0.0 | 11.28 | 11.34 |
| 0.5 | 15.20 | 11.48 |
| 1.0 | 17.04 | 11.56 |
| 1.5 | 20.40 | 12.08 |
| 2.0 | 24.24 | 12.20 |
| 2.5 | 26.96 | 12.20 |
| 3.0 | 29.92 | 12.60 |
| 3.5 | 33.84 | 13.44 |
| 4.0 | 36.40 | 13.60 |

The control consisted of strained rumen liquor with no added substrate. A slight increase in the formation of FFA by the control, was observed (2.26 moles) indicating that some hydrolysis of endogenous substrate occurred. However the addition of lipid increased the FFA formation from 11.28 μ moles to 36.40 μ moles, the increase being linear over the 4 hr incubation period (Fig. 8b).

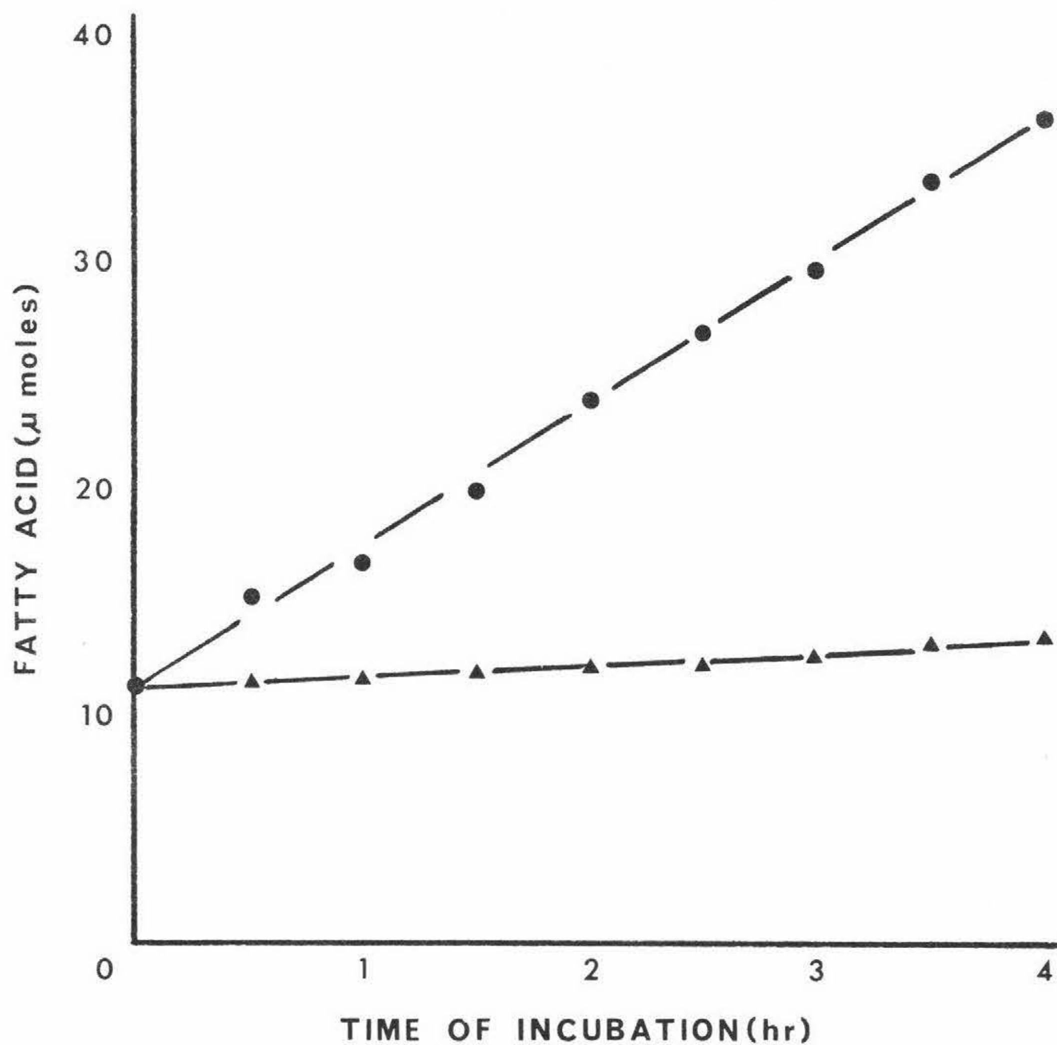



Figure 8b. Rate of formation of free fatty acid by strained rumen liquor. Incubation conditions: 200ml strained rumen liquor with or without 4ml emulsified peanut oil, at 39° in $N_2$ with constant shaking.

● with emulsified peanut oil

▲ no added substrate

(b) Radiochemical assay of hydrolysis products

$^{14}C$-triolein in 4 ml of emulsified peanut oil was incubated with 200 ml of strained rumen liquor as described in Section 3.7. 5 ml aliquots were removed from the incubation at 60 minute intervals over a 4 hr period. The incubation was conducted at 39° under an atmosphere of nitrogen, with constant shaking. Lipids were extracted from each aliquot and separated by thin layer chromatography. The products of hydrolysis determined by planimetric measurement of radiochromatogram scans are given in Table 7. Approximately 60% of the total radioactivity appeared in the FFA fraction after 4 hr of incubation, the rate of formation being linear for 3 hr (Fig. 9). Diglyceride was at a maximum level at 1 hr and subsequently decreased with increasing time of incubation. Monoglyceride was not detected in this experiment but in similar experiments a small peak corresponding to monoglyceride was observed. The low levels of diglyceride and monoglyceride suggest that rumen lipase is not specific for any position on the triglyceride molecule.

TABLE 7

Formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein by strained rumen liquor

(For incubation conditions see text)

| Time of incubation (min) | % radioactivity in each lipid component (as a % of total radioactivity) | | | |
|---|---|---|---|---|
| | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 0 | 100 | - | - | - |
| 60 | 82.4 | 14.4 | 3.3 | - |
| 120 | 64.2 | 32.7 | 3.2 | - |
| 180 | 46.9 | 51.0 | 2.1 | - |
| 240 | 39.8 | 59.1 | 1.1 | - |

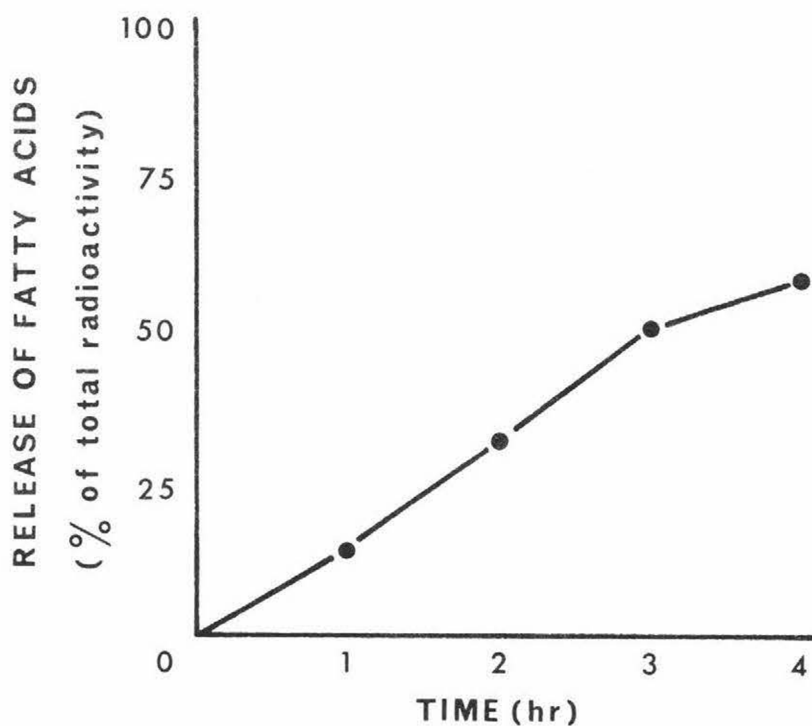



Figure 9. Effect of time of incubation on the release of $^{14}C$-fatty acid from $^{14}C$-triolein by strained rumen liquor. (See text for incubation conditions).

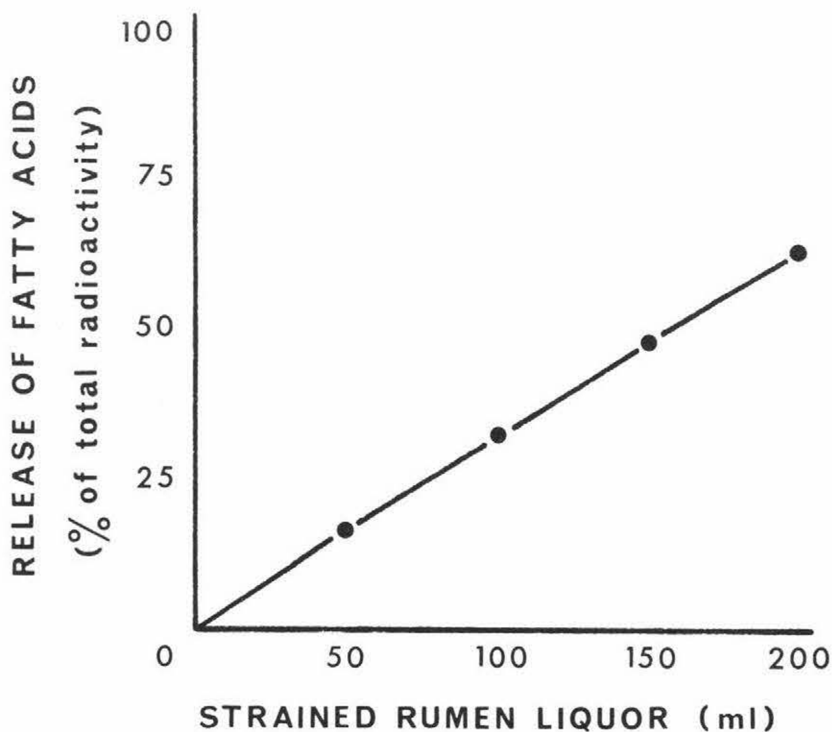



Figure 10. Effect of amount of strained rumen liquor on the formation of $^{14}C$-fatty acid from $^{14}C$-triolein. (See text for incubation conditions).

### 4.2.3. Effect of concentration of strained rumen liquor on rate of hydrolysis

50, 100, 150 and 200 ml of strained rumen liquor (made up to 200 ml with clarified rumen liquor) were incubated with $^{14}C$-triolein in 4 ml of emulsified peanut oil as described in Section 3.7., for four hours. A known aliquot of the extracted lipids was chromatogramed on thin layers of silica gel and the separated lipid components were measured on the chromatogram scanner. The effects of the strained rumen liquor concentration on the rate of formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein are shown in Table 8 and Fig. 10.

Over a 4 hr incubation period, a linear relationship exists between the amount of strained rumen liquor and the formation of FFA.

### 4.2.4. Effect of substrate concentration on the rate of hydrolysis by strained rumen liquor

The effect of substrate concentration on the rate of hydrolysis of $^{14}C$-triolein by strained rumen liquor was investigated by varying the amount of unlabelled carrier in the incubation. 200 mls of strained rumen liquor was incubated with $^{14}C$-triolein in 50, 100, 150 and 200 mg of emulsified peanut oil. The formation of products of hydrolysis as measured by thin layer chromatogram scans are given in Table 9.

TABLE 8

Effect of concentration of strained rumen liquor on the formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein

(See text for incubation conditions)

| Strained rumen liquor (ml) | Clarified rumen liquor (ml) | % radioactivity in each lipid component (as % of total radioactivity) | | | |
|---|---|---|---|---|---|
| | | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 0 | 200 | 100 | - | - | - |
| 50 | 150 | 77.9 | 16.8 | 4.3 | 1.0 |
| 100 | 100 | 58.9 | 32.6 | 5.7 | 2.8 |
| 150 | 50 | 49.5 | 47.5 | 5.0 | - |
| 200 | 0 | 28.6 | 63.6 | 2.2 | 1.6 |

TABLE 9

Effect of substrate concentration on the formation of $^{14}C$-labelled products of hydrolysis of $^{14}C$-triolein by strained rumen liquor

(for incubation conditions see text)

| Substrate Concentration (gm substrate /200 ml strained rumen liquor) | % radioactivity in each lipid component (as % of total radioactivity) | | | |
|---|---|---|---|---|
| | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 0.05 | 32.7 | 61.4 | 3.3 | 2.4 |
| 0.10 | 9.5 | 85.4 | 2.2 | 3.0 |
| 0.20 | 25.8 | 68.6 | 3.7 | 2.0 |
| 0.30 | 38.5 | 55.2 | 4.4 | 2.0 |
| 0.40 | 49.3 | 43.3 | 6.1 | 1.3 |

Conversion of the percentage of FFA as a percentage of the total radioactivity to the equivalent amount of FFA in mgm enables a direct comparison to be made with the initial substrate concentration (Fig. 11).

It is apparent that at a substrate concentration of 200 mg triglyceride /100 ml strained rumen liquor, the enzyme is almost saturated, and an appropriate substrate concentration for assay of enzyme activity is in the range 50-100 mgm triglyceride/100 ml strained rumen liquor.

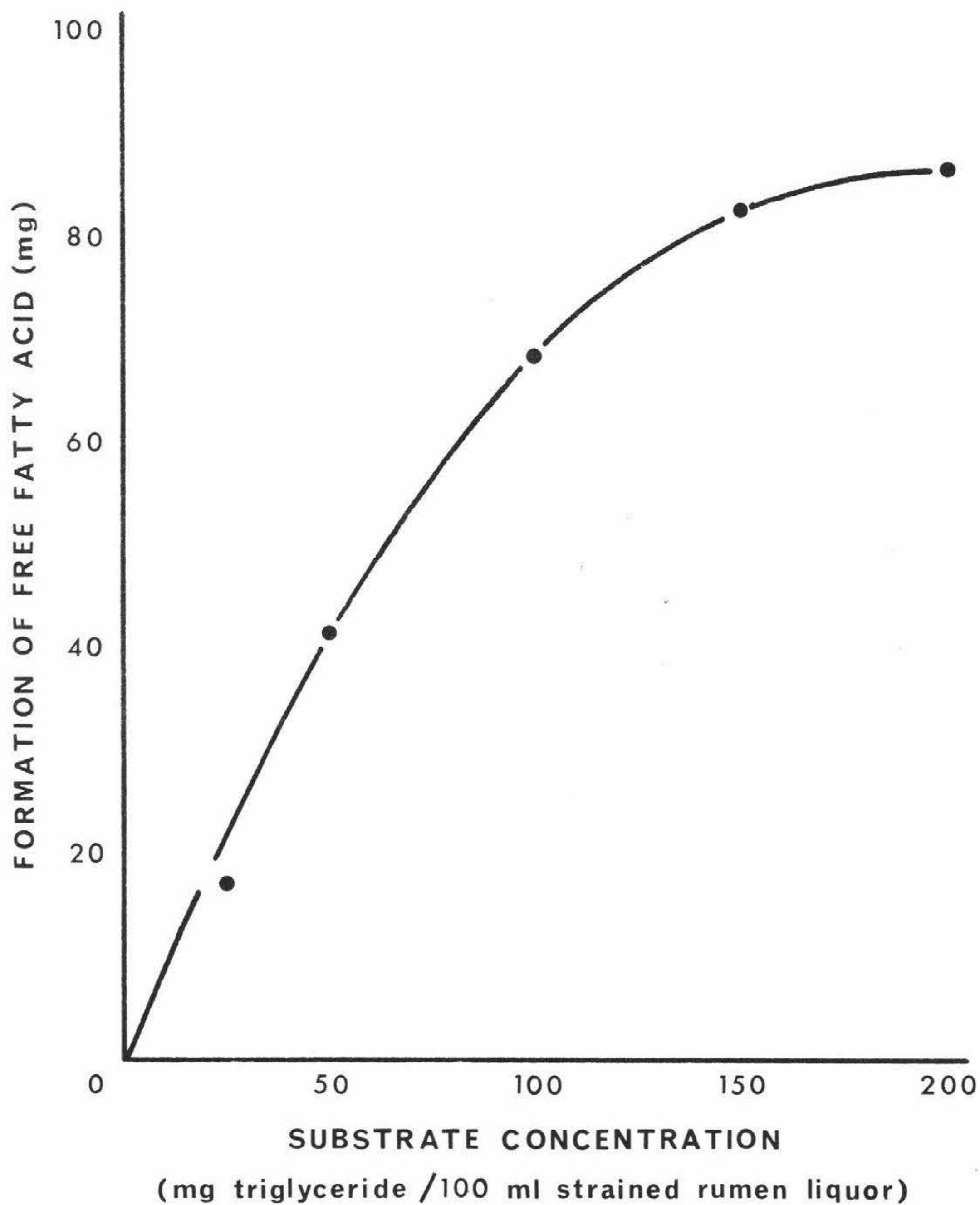



Figure 11. Effect of substrate concentration on the formation of free fatty acid by strained rumen liquor. (See text for incubation conditions).

## 4.3. Isolation of Rumen Lipase

### 4.3.1. Lipolytic activity of fractions of strained rumen liquor

Fractions from a sample of strained rumen liquor were prepared as described in Section 3.6., and made up to a final volume of 200 ml with clarified rumen liquor. Each fraction was incubated with 4 ml of emulsified peanut oil at 39°, under an atmosphere of nitrogen and with constant shaking. 5 ml aliquots were withdrawn from each incubation at 30 minute intervals over a 4 hr period for the colorimetric determination of FFA (Table 10 and Fig. 12).

The variable levels of FFA between fractions at zero time (Table 10) indicates the way in which the total endogenous FFA in the rumen is distributed. The rate of formation of FFA was greatest in the fraction precipitated by centrifuging strained rumen contents at 500g for 10 minutes, and this increase from 11.52 $\mu$ moles to 33.76 $\mu$ moles of FFA was linear during the 4 hour incubation. The removal of protozoa by sedimentation of this 500g centrifugate did not appreciably affect the rate of FFA formation in that 20.84 $\mu$ moles of FFA was released in the incubation period as compared with 22.24 $\mu$ moles by the original 500g centrifugate. The protozoal fraction and the 14,000g supernatant did not exhibit any lipolytic activity and the small increase from 11.44 $\mu$ moles to 13.60 $\mu$ moles exhibited by strained rumen contents with no added substrate, indicates some lipolytic activity of the endogenous substrate.

Similar trends were observed in a number of experiments, although absolute values varied slightly with different rumen samples. Appreciable variation occurred in the formation of FFA by the 14,000g centrifugate prepared from the 500g supernatant from the

TABLE 10

Formation of free fatty acid from emulsified peanut oil by fractions prepared from strained rumen liquor

(See text for incubation conditions)

| Time of incubation (hr) | Free fatty acid ($\mu$ moles) | | | | | |
|---|---|---|---|---|---|---|
| | Strained rumen liquor with no added substrate | 500g centrifugate | | | 500g supernatant | |
| | | Total | Total less protozoa | Protozoa | 14,000g centrifugate | 14,000g supernatant |
| 0.0 | 11.44 | 11.52 | 8.60 | 1.13 | 4.96 | 0.15 |
| 0.5 | 11.48 | 14.28 | 11.24 | 1.13 | 5.84 | 0.15 |
| 1.0 | 11.56 | 17.12 | 14.00 | 1.12 | 7.12 | 0.15 |
| 1.5 | 12.08 | 20.00 | 14.42 | 1.13 | 7.04 | 0.15 |
| 2.0 | 12.20 | 22.40 | 19.20 | 1.13 | 8.32 | 0.15 |
| 2.5 | 12.20 | 24.96 | 21.92 | 1.12 | 9.04 | 0.15 |
| 3.0 | 12.60 | 28.56 | 24.32 | 1.13 | 9.20 | 0.15 |
| 3.5 | 13.44 | 30.96 | 26.88 | 1.13 | 10.88 | 0.15 |
| 4.0 | 13.60 | 33.76 | 29.44 | 1.13 | 11.40 | 0.15 |

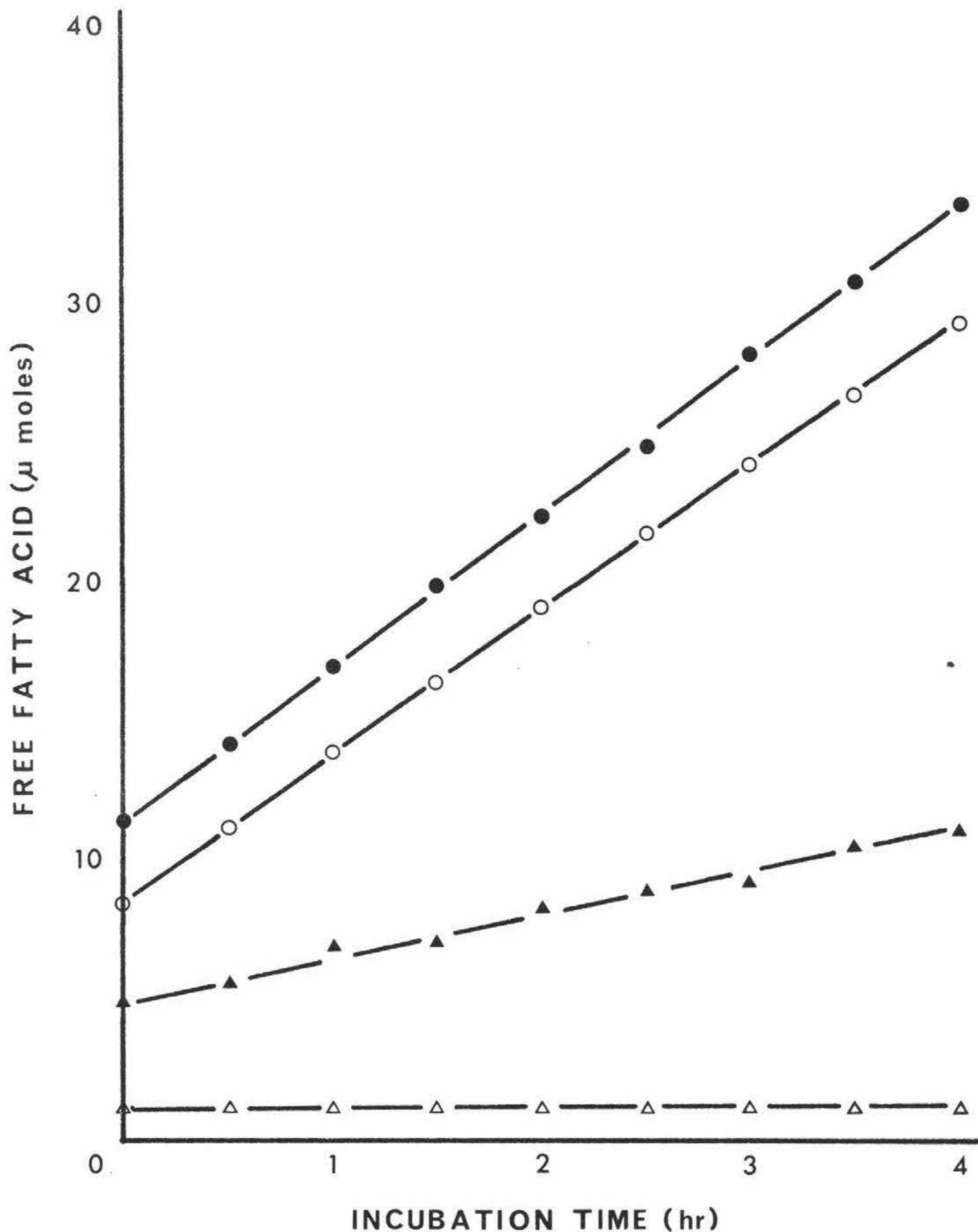



Figure 12. Formation of free fatty acid from emulsified peanut oil by fractions prepared from strained rumen liquor. Incubation conditions: 200ml of each fraction incubated with 4ml of emulsified peanut oil at 39°, under nitrogen, with constant shaking, for 4hrs. ● 500g centrifugate, ○ 500g centrifugate less protozoa, △ protozoa, ▲ 14,000g centrifugate

first centrifugation. In the experiment cited, FFA formation by this fraction increased from 4.96 $\mu$ moles to 11.40 $\mu$ moles, a rise of 6.44 $\mu$ moles indicating considerable lipolytic activity. However in several other instances this rise was not more than 2.00 $\mu$ moles.

Microscopic examination of the incubation medium after the 4 hour incubation showed that the protozoa remained viable in the control and 500g centrifugate but viability had declined to almost zero in the pure protozoal fraction.

4.3.2. <u>The effect of homogenisation of unstrained rumen contents on lipolytic activity</u>

Since it was apparent that most of the lipolytic activity in rumen contents was associated with the particulate material (i.e. 500g centrifugate less the protozoa) and the 14,000g precipitate (containing mainly bacteria), prepared from the original 500g supernatant, it seemed likely that most of the lipolytic organisms were closely adhered to the partly digested plant material in the rumen. Consequently the total rumen contents, including the solid ingesta were subjected to homogenisation, as described in Section 3.6.3. in an attempt to detach the lipolytic organisms.

To examine the effect of homogenisation, fractions separated from strained rumen liquor and from total rumen contents which had been homogenised were compared. All fractions were made up to 200 ml with clarified rumen liquor and incubated with 4 ml of emulsified peanut oil, at 39$^{\circ}$, in nitrogen, with constant shaking. The results (Table 11, and Fig. 13) show a much higher level of FFA at zero time than was observed in the experiment summarised in Table 10. This

TABLE 11

Effect of homogenisation of total rumen contents on the formation of free fatty acid by a 500g supernatant and a 500g centrifugate fraction

(See text for incubation conditions)

| Time of incubation (hr) | Free fatty acid (μ moles) | | | | | |
|---|---|---|---|---|---|---|
| | Strained rumen liquor | | | | Homogenised rumen contents | |
| | Control (no added substrate) | Total | 500g centrifugate | 500g supernatant | 500g centrifugate | 500g supernatant |
| 0.0 | 24.00 | 24.00 | 22.96 | 2.50 | 17.84 | 6.40 |
| 0.5 | 24.24 | 28.16 | 26.40 | 2.54 | 19.68 | 7.72 |
| 1.0 | 24.56 | 31.44 | 29.92 | 2.60 | 22.00 | 9.60 |
| 1.5 | 24.96 | 34.64 | 31.20 | 3.10 | 23.84 | 10.96 |
| 2.0 | 25.36 | 39.04 | 36.56 | 2.94 | 25.60 | 12.40 |
| 2.5 | 25.60 | 42.24 | 40.08 | 3.50 | 27.52 | 13.92 |
| 3.0 | 25.92 | 45.92 | 43.68 | 3.50 | 29.28 | 15.12 |
| 3.5 | 26.16 | 49.12 | 47.52 | 4.34 | 31.36 | 17.36 |
| 4.0 | 26.72 | 52.80 | 50.88 | 4.80 | 33.36 | 18.72 |

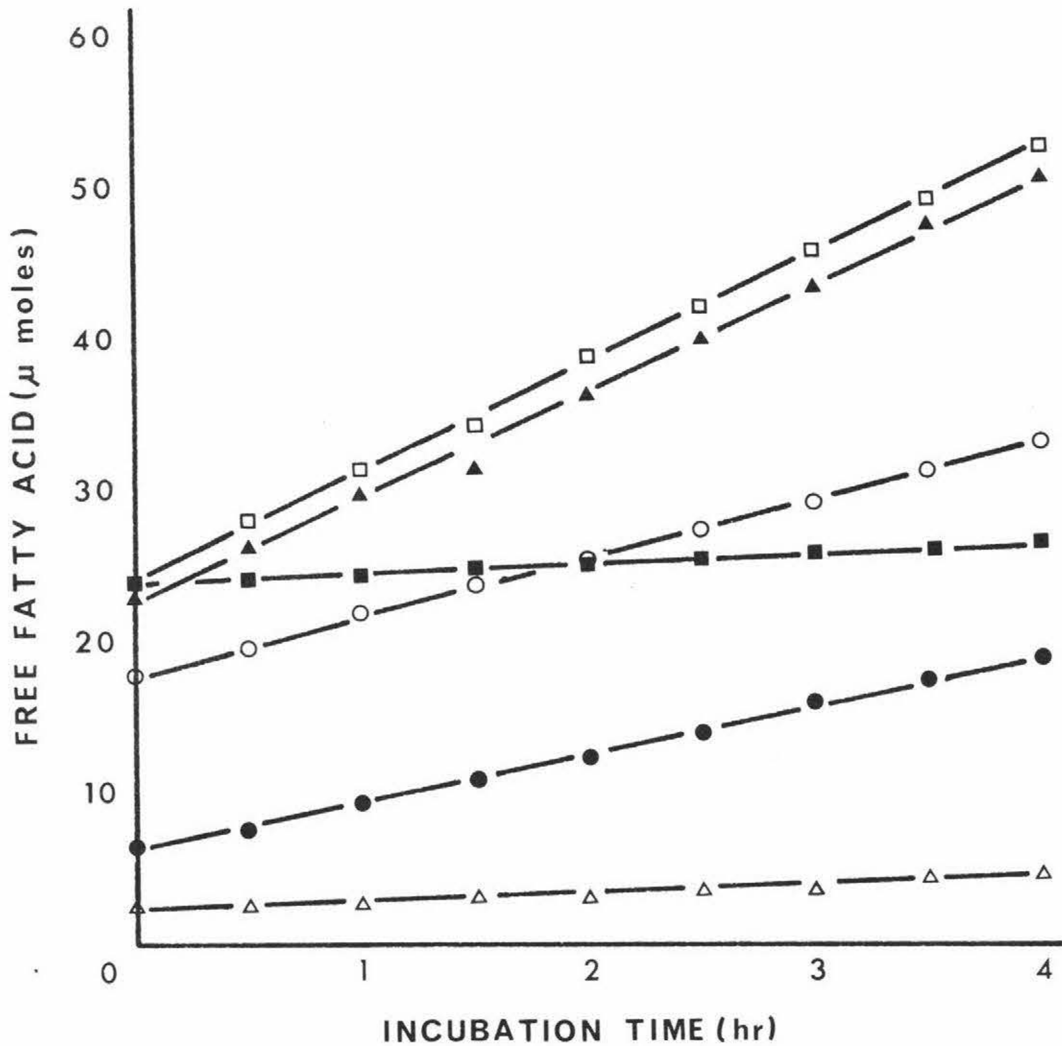



Figure 13. Effect of homogenisation of total rumen contents on the formation of free fatty acid by 500g centrifugate and 500g supernatant fractions. (See text for incubation conditions).

■ Control
□ Strained rumen contents
▲ 500g centrifugate } Unhomogenised
△ 500g supernatant }
○ 500g centrifugate } Homogenised
● 500g supernatant }

would be explained by a greater amount of endogenous fatty acid present in the rumen and a further production of 2.72 $\mu$ moles of fatty acid from endogenous sources was observed in the control (Table 11). However levels of FFA production by fractions prepared from strained rumen liquor are very similar to those in Table 10. The formation of FFA by the 500g centrifugate from strained rumen liquor increased from 22.96 $\mu$ moles to 50.88 $\mu$ moles of fatty acid. Since the 500g supernatant fraction had only formed 2.30 $\mu$ moles of FFA in the 4 hr incubation period, the lipolytic activity of the total strained rumen liquor was almost all recovered in the 500g centrifugate.

On the other hand, the 500g supernatant prepared from homogenised rumen contents formed 12.32 $\mu$ moles of FFA, while the corresponding 500g centrifugate formed 15.52 $\mu$ moles of FFA.

From these results it is apparent that homogenisation of total rumen contents has detached lipolytic microorganisms from the solid ingesta, enabling them to remain suspended in the 500g supernatant.

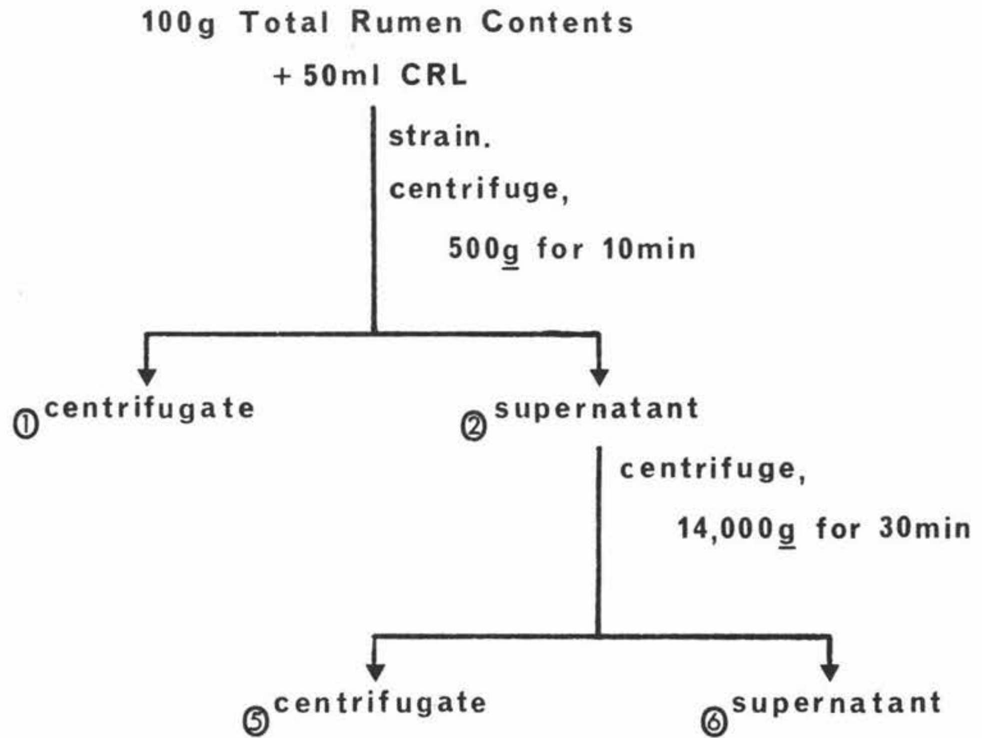
4.3.3. <u>Effect of homogenisation of total rumen contents on the formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein by fractions of rumen contents</u>

A direct comparison of the lipolytic activity of the fractions prepared from rumen contents as outlined in Fig. 14 was made. Activity was assayed in a 60 minute incubation using $^{14}C$-triolein in 0.4 ml emulsified peanut oil added to 20 ml of each fraction (1-8 in Fig. 14). After incubation lipid extracts were chromatographed on thin layers of silica gel and the percentage radioactivity in each lipid component is shown in Table 12.

The results presented confirm the earlier findings that homogenisation increases the lipolytic activity of the 500g supernatant prepared from rumen contents. A 500g centrifugation of strained rumen liquor (unhomogenised) resulted in all the lipolytic activity being confined to the centrifugate, and no activity present in subsequent fractions prepared from the 500g supernatant. However homogenisation of total rumen contents resulted in the formation of 8.8% in the 500g supernatant which was recovered in the 14,000g precipitate of this supernatant. Several washings of this 14,000g precipitate with phosphate buffer, and recentrifugation to obtain a clean bacterial pellet, resulted in no loss of activity.

This method for the preparation of active lipolytic bacteria, was used to obtain starting material for further isolation procedures.

(a) UNHOMOGENISED:




(b) HOMOGENISED:

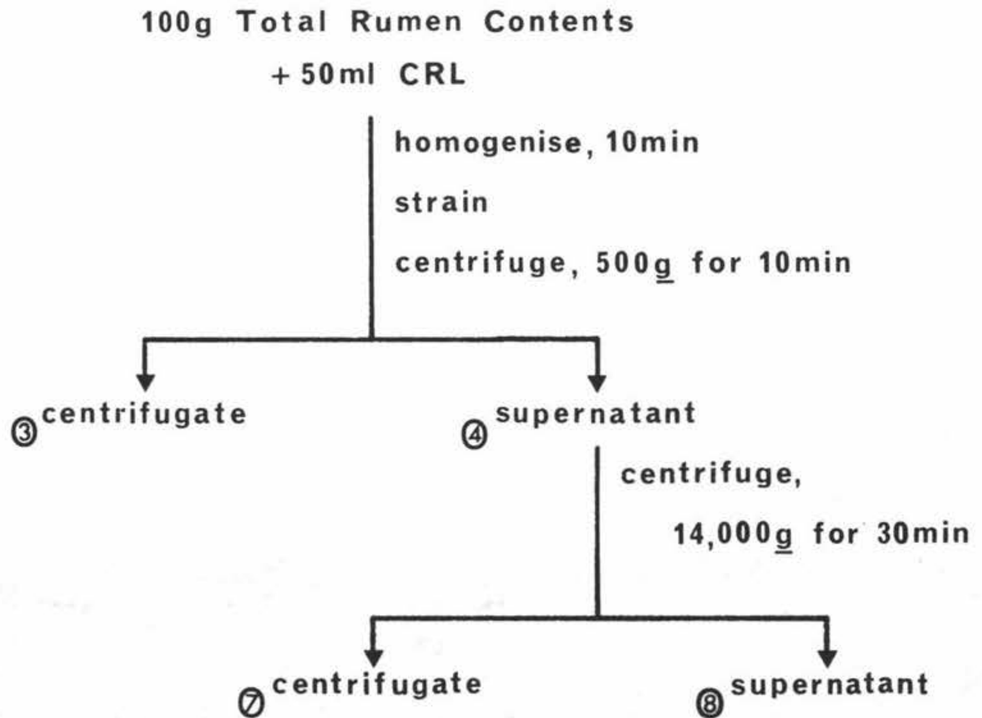



Figure 14. Pathways for the preparation of fractions of rumen contents for incubation. CRL = Clarified rumen liquor. (1) to (8) = fraction numbers for incubation.

TABLE 12

Formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein by fractions of total rumen contents

(See text for incubation conditions)

| Fraction No. | Description | % radioactivity in each lipid component (as % of total radioactivity) | | | |
|---|---|---|---|---|---|
| | | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 1 | Unhomogenised 500g centrifugate | 76.3 | 20.2 | 2.8 | 0.7 |
| 2 | Unhomogenised 500g supernatant | 100.0 | - | - | - |
| 3 | Homogenised 500g centrifugate | 91.2 | 6.3 | 1.6 | 0.9 |
| 4 | Homogenised 500g supernatant | 89.3 | 8.8 | 1.9 | - |
| 5 | Unhomogenised 14,000g centrifugate | 100.0 | - | - | - |
| 6 | Unhomogenised 14,000g supernatant | 100.0 | - | - | - |
| 7 | Homogenised 14,000g centrifugate | 87.8 | 10.2 | - | - |
| 8 | Homogenised 14,000g supernatant | 99.0 | 1.0 | - | - |

unhomogenised = strained rumen liquor

4.3.4. Lipolytic activity of cell-free extracts of rumen bacteria

The lipolytic activity of cell-free extracts of rumen bacteria prepared by osmotic shock and by high frequency sonication was examined. For an appraisal of the separation procedure, the lipolytic activity of each fraction (1-12, in Fig. 15) was measured. During preparation of each fraction, a known volume of phosphate buffer, pH 7.0 was added to resuspended centrifugates to enable direct comparisons to be made between each fraction. A 5 ml aliquot of each fraction was incubated with 5 $\mu$l glyceryl tri(oleate-1-$^{14}$C) ($1.1 \times 10^5$ dpm) in hexane. No unlabelled carrier was added but constant shaking was maintained throughout a 1 hour incubation. Lipids were extracted and reaction products were separated on thin layers of silica gel and scanned on the radiochromatogram. A control incubation consisting of boiled rumen contents with no added substrate showed no lipolytic activity.

The formation of radioactive products of incubation, from a planimetric measurement of the radiochromatogram scans are shown in Table 13.

It is apparent from Table 13 that considerable lipolytic activity was present in each incubation, with the exception of the control, 90,000g supernatant (Fraction 10). The corresponding supernatants after sonication (Fraction 8) and osmotic shock treatment (Fraction 12) showed considerable activity. This lipolytic activity was retained after these fractions had been passed through a Zeitz filter, indicating that they were free of rumen bacteria.

Lipolytic activity is also present in the 14,000g supernatant (Fraction 5). When this fraction was recentrifuged at 30,000g the

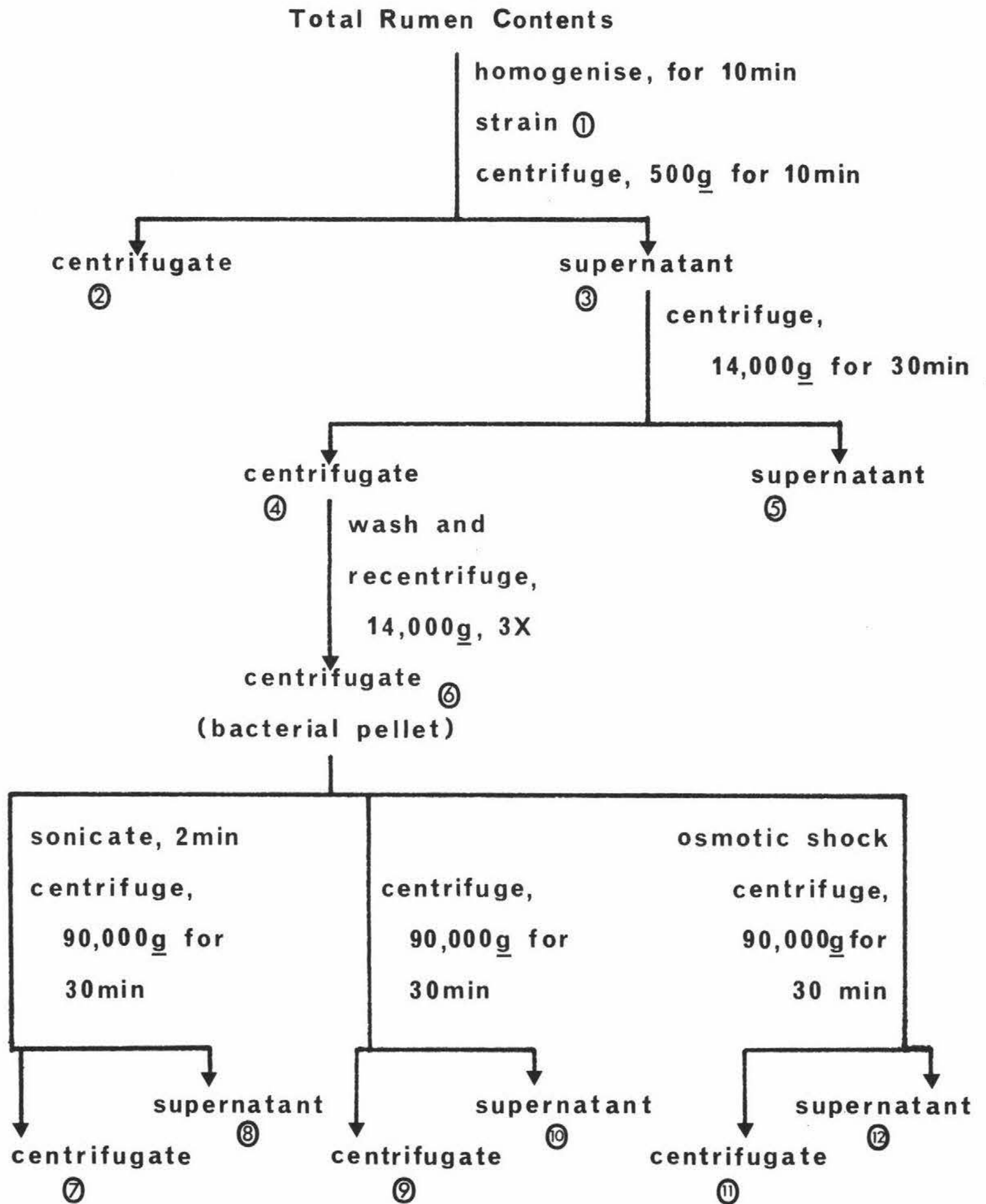



Figure 15. Preparation of bacterial cell-free extracts from total rumen contents. (1) to (12) = fraction numbers for incubation.

TABLE 13

Formation of $^{14}C$-labelled hydrolysis products from $^{14}C$-triolein by fractions of total rumen contents

| Fraction No. | Description | % Radioactivity in each lipid component | | | |
|---|---|---|---|---|---|
| | | Triglyceride | Fatty Acid | Diglyceride | Monoglyceride |
| 1 | Strained rumen contents (after homogenisation) | 11.6 | 75.0 | 9.8 | 3.6 |
| 2 | 500g centrifugate | 31.2 | 59.5 | 4.6 | 4.6 |
| 3 | 500g supernatant | 28.1 | 61.2 | 7.4 | 3.3 |
| 4 | 14,000g centrifugate | 42.6 | 44.3 | 7.8 | 5.2 |
| 5 | 14,000g supernatant | 71.6 | 20.5 | 5.7 | 2.2 |
| 6 | 14,000g centrifugate (after washing) | 49.5 | 47.5 | 3.0 | - |
| 7 | Sonication - 90,000g centrifugate | 66.0 | 21.1 | 8.7 | 4.3 |
| 8 | Sonication - 90,000g supernatant | 60.7 | 22.6 | 14.3 | 2.4 |
| 9 | Control - 90,000g centrifugate | 29.3 | 65.0 | 0.8 | 4.9 |
| 10 | Control - 90,000g supernatant | 100.0 | - | - | - |
| 11 | Osmotic Shock - 90,000g centrifugate | 23.9 | 23.2 | 52.9* | - |
| 12 | Osmotic Shock - 90,000g supernatant | 47.5 | 33.8 | 13.8+ | 5.0 |

* includes monoglyceride diglyceride and an unidentified component which chromatographs between diglyceride and fatty acid on a thin layer chromatogram. Due to the high radioactivity in this region of the chromatogram resolution to single peaks could not be achieved.

\+ includes diglyceride and the same unidentified component as above.

formation of FFA was confined to the centrifugate only. This finding could indicate that the bacteria responsible for lipolysis are relatively small and that the lower centrifugation was not sufficient for total precipitation.

# Chapter 5

## DISCUSSION

### 5.1. Analytical Procedure

In studying the activity of any enzyme it is desirable to have a reliable and reasonably reproducible source of enzyme. This requirement has been satisfied in the present study by giving attention to the maintenance of a dietary regime which could be reproduced prior to each sampling. In experiments conducted during the course of a year, it was observed that when 4 ml of substrate was incubated with 200 ml of rumen liquor 6.30 $\mu$ moles to 6.80 $\mu$ moles of FFA were formed per hour over a 4 hr period of incubation. When the rate of FFA formation was measured radiochemically, it was consistently found that approximately 15% and 60% of the total radioactivity was isolated as FFA after a 1 hr and a 4 hr incubation, respectively.

In addition, the availability of a convenient assay method is an important consideration in enzyme studies. Furthermore, high sensitivity of the assay is very desirable when attempts are being made to purify an enzyme. Studies of lipases have to some extent been hampered by the lack of a rapid and reproducible micro-method for assay. Early workers in this field (Fiore and Nord, 1949; Dole, 1956; Dole and Meinertz, 1960) relied on the titration of the fatty acid products of hydrolysis with alcoholic NaOH, after prolonged incubation of lipid substrate with lipase. Accuracy was limited by the formation of other acidic end-products of metabolism, and by the possible inhibition of enzyme activity by the products of hydrolysis. The introduction of the pH stat enabled a continuous automatic titration of liberated fatty acids (Shahani et al, 1964), and had the advantage that the initial

reaction velocity could be measured in short incubation times. However titration methods have the disadvantage that it is difficult to measure lipase activity close to pH 7.0, because of incomplete titration of fatty acids.

Colorimetric determination of the liberated fatty acids has the advantage of increased sensitivity over titrimetric methods. The use of substrates which give coloured end-products after hydrolysis, or ones which can easily be converted to coloured products were first described by Seligman and Nachlas (1950). Duncombe (1963) developed this method to achieve a sensitivity of 0.05 - 0.5 $\mu$ moles of FFA by colorimetrically determining the formation of a complex of copper soaps of liberated fatty acids in chloroform, with diethyldithiocarbamate reagent. Although relatively time consuming, this method was used satisfactorily in preliminary work for determining the location of the lipolytic fraction in strained rumen liquor.

Radiochemical methods have been developed over the last few years in an endeavour to achieve greater sensitivity and a more rapid analysis procedure. These methods involve incubation of radioactive substrate and the subsequent analysis of the radioactive products of hydrolysis. Kelley (1968) measured the formation of $^{14}$C-labelled fatty acids from $^{14}$C-triglyceride by adsorption on dehydrated hydroxy-charged ion exchange resin, and subsequent determination by liquid scintillation spectrometry. Other lipids were removed by washing with solvent prior to counting.

A major disadvantage of these methods is that only the FFA can be determined readily, and other products of hydrolysis require further analytical procedures. Chino and Gilbert (1965) isolated each of the

radioactive products of hydrolysis by column chromatography and measured the radioactivity in the column effluent. It was found in the present study that thin layer chromatography of the reaction mixture, followed either by a radiochemical scan and planimetric measurement of peak areas, or by removal of the appropriate areas of the chromatogram and measurement of the radioactivity by liquid scintillation spectrometry, is more rapid and sensitive than the column technique, and at the same time retains the advantage of following the formation of diglyceride and monoglyceride intermediates as well as FFA. Both assay techniques were used in the present study, and offer a valuable extension of the published radiochemical methods.

## 5.2. Distribution of Lipolytic Activity in Rumen Contents

The lipolytic activity of strained rumen liquor was first reported by Garton _et al_. (1958), when a marked increase in the level of FFA was observed following the incubation of linseed oil with sheep rumen contents. Under _in vivo_ conditions, the level of FFA present in the rumen of a sheep at slaughter, 7 hr after the last feed was between 80% and 90% of the total lipid (Garton _et al_., 1959). When 1 g of linseed oil was incubated per 100 ml of rumen contents, hydrolysis resulted in the liberation of 60% to 90% of the esterified fatty acid residues from the original oil, in a 24 hr incubation (Garton _et al_., 1961). In this study a 4 hr incubation of $^{14}C$-triolein with unlabelled peanut oil under similar incubation conditions to Garton _et al_. (1961), resulted in the release of 60% of the total FFA's. In contrast to these earlier studies, the rate of FFA formation was reasonably constant between experiments, but there was a considerable variation in the level of endogenous FFA present in the rumen liquor (c.f. Table 6 and

Table 11).

The formation of diglyceride and monoglyceride intermediates in the hydrolysis of triglyceride was not observed in the initial studies by Garton, but Hawke and Robertson (1964) obtained chromatographic evidence for their formation. Silcock (1968) also demonstrated the presence of these intermediates using a similar radiochemical method to the one described in this study. From the data quoted in Chapter 4 it is apparent that both diglyceride and monoglyceride are formed as intermediates in the hydrolysis of triglyceride by rumen liquor, but from the low levels observed, it would appear that they have a transitory existence only.

Pancreatic lipase has been shown to be specific for the hydrolysis of primary alcohol esters (Savary and Desnuelle, 1956), and a similar specificity has been observed for milk lipase (Jensen et al., 1960). On the other hand, Ricinus lipase (Savary et al., 1958) and lipoprotein lipase (Korn, 1961) do not exhibit this specificity. Following the incubation of strained rumen liquor with $^{14}C$-linolenic acid in the 2-position on the triglyceride molecule, Silcock (1968) observed the formation of radioactive diglyceride and monoglyceride. The former had a transitory existence only, but monoglyceride levels tended to increase, followed by the liberation of the remaining esterified fatty acid at a slower rate. It was concluded that hydrolysis by rumen liquor was specific for the primary alcohol esters and that the slower rate of hydrolysis of the resultant monoglyceride, was due to the isomerisation of the fatty acid to a primary position before hydrolysis could take place. However from the results presented in this study there appears to be little evidence for any positional specificity for

hydrolysis, by rumen liquor. Diglyceride levels reached approximately 6% of the total radioactivity after 1 hr of incubation, followed by a decrease to 1% - 2% of the total radioactivity after 4 hr of incubation. The percentage radioactivity in the monoglyceride fraction appeared to reach about half of the diglyceride level, with no further increase.

The rate of FFA formation by strained rumen liquor was linear over the 4 hr period of incubation (Fig. 8b), and studies on the effect of amount of rumen liquor (Fig. 10) and the effect of substrate concentration (Fig. 11) on hydrolysis rate, depict typical enzyme characteristics. From Fig. 11 it would appear that a triglyceride concentration of between 50 and 100 mg/100 ml of strained rumen liquor gives a maximum rate of hydrolysis.

Garton (1964) using differential centrifugation of strained rumen liquor established that rumen bacteria were responsible for the hydrolysis of triglyceride, and consequently it was anticipated that the maximum lipolytic activity would be found in the bacterial concentrate prepared as described in Section 3.6. The results presented in Table 6 and Table 10 are from incubations from the same rumen sample, and they show that the centrifugate from a 500g centrifugation of strained rumen contents has a lipolytic activity almost equal to that of the total strained rumen contents. Rumen bacteria can be precipitated from the 500g supernatant by a further centrifugation at 14,000g. When several preparations of this bacterial concentrate were incubated with triglyceride, it was found that lipolytic activity was very low and often non-existent.

As lipolytic activity was generally confined to the 500g centrifugate of rumen contents it was possible that rumen protozoa were the

principal lipolytic organisms. However, the protozoa isolated from the particulate material by the sedimentation technique of Heald *et al.* (1952), did not exhibit any lipolytic activity. Furthermore, the lipolytic activity of the particulate fraction which remained after the removal of protozoa was undiminished.

It has been established by numerous investigators (King, 1956, 1959; Gouws and Kistner, 1965; Hungate, 1966) that cellulolytic organisms are associated with the particles of ingesta, and from the above fractionation studies it is likely that lipolytic bacteria have a similar association, thus preventing their suspension in the initial 500*g* supernatant.

It was found that subjecting the particulate material to homogenisation was successful in transferring some of the lipolytic activity into the 500*g* supernatant (Table 11 and Table 12). When this supernatant was recentrifuged at 14,000*g* almost all of the lipolytic activity was precipitated in the bacterial concentrate.

The varying difficulty that has been encountered in isolating lipolytic organisms from the bovine rumen could be a reflection of the dietary regime of the animal. Lipolytic bacteria may not adhere as tightly to food particles from a ration of grain and hay as would be the case on a pasture ration, and subsequent isolation of lipolytic bacteria by differential centrifugation may prove satisfactory. It is clear that for the isolation of lipolytic bacteria from the rumen of an animal on a pasture diet, it is an advantage to detach the organisms from the particulate material.

A number of techniques have been used for the preparation of cell-free extracts from rumen bacteria. Kepler and Tove (1967) used a

French pressure cell for the disruption of bacterial cells in the successful isolation of linoleate-12-cis, 11-trans isomerase from Butyrivibrio fibrisolvens. Sonic oscillation is also a technique that has been used for the preparation of cell-free extracts from bacteria. This has been successfully used in the preparation of cellular sub-units of oxidative phosphorylation from Mycobacterium phlei (Brodie and Grey, 1957). Nossal and Heppel (1966) released a number of degradative enzymes from Escherichia coli by osmotic shock treatment. The process of release is completed within about 1 min after contact of the sucrose-treated cells with the shock medium. It is thought that either certain enzymes escape very rapidly through the abnormally permeable cell barrier, or that a group of enzymes in a particular location, such as the sac, are actively ejected by osmotic shock. In the present study it is difficult to interpret whether the released enzyme is intracellular or attached to the cell wall. Osmotic shock is more likely to release intracellular enzymes than cell-bound enzymes, and the activity observed in Fraction 12 (Table 13) would possibly imply that the lipase is intracellular. However, considerable activity was retained by the 90,000g centrifugate which would include cell-wall material. Similar observations were noted following osmotic shock treatment.

However, it is clear that both sonication and osmotic shock have released lipolytic enzymes into the 90,000g supernatant, as a control incubation of rumen bacteria subjected to centrifugation only, did not show any lipolytic activity in the supernatant (Fraction 10, Table 13).

Solubility of the enzymes released by the treatments described above, has been claimed when enzymatic activity remains in the supernatant fraction after a centrifugation of between 120,000g and

140,000g. In the present study the supernatants were prepared from a 90,000g centrifugate only.

### 5.3. Nature of Other Microbial Lipases

Enzymes are generally classified as either, (a) cell-bound, where the enzymes are carried along with the cells by such procedures as centrifugation or, (b) extracellular, where the enzymes are released from the cell by death or lysis, or by some other means yet to be described.

Lipase activity has often been observed in the culture fluids of many microorganisms, and the enzyme is generally regarded as being extracellular (Hugo and Beveridge, 1962; Khan, Chandan, Dill, and Shahani, 1964; Motai, Ichishima and Yoshida, 1966). Some extracellular lipase-forming systems also appear to have a small amount of cell-bound enzyme. Very small amounts of cell-bound enzyme were detected in *Geotrichum candidum* and *Achromobacter lipolyticum*, but almost equal quantities of cell-bound and extracellular lipase were found during the growth of *Aspergillus niger* (Chandan, Carrancedo and Shahani, 1962). There are few reports in the literature where intracellular lipase activity only, or extracellular lipase activity only, has been detected. Hobson and Summers (1966) isolated an unidentified organism which appeared to produce two different enzymes, one cell-bound and the other secreted into the culture medium, and Khan *et al* (1964) reported that the extracellular lipase from *A. lipolyticum* had a different pH optimum to that of the cell-bound enzyme from the same organism (Shahani, Sarda, Desnuelle and Azoulay, 1964), and is presumably not the same enzyme.

From the results of the present study, it appears that the lipolytic enzymes are cell-bound, and are not released into the super-

natant. Clarified rumen liquor did not exhibit any lipolytic activity in this study, although the supernatant from a 14,000g centrifugation of homogenised rumen contents (Fraction 5, Table 13) did show some hydrolysis of triglyceride. However a further centrifugation at 30,000g transferred all the activity to the centrifugate, suggesting that possibly some of the lipolytic organisms of the rumen are very small bacteria.

## 5.4. The Relationship Between Hydrolysis and Hydrogenation of Lipid in the Rumen

Hydrolysis and hydrogenation are enzymatically controlled reactions (Reiser, 1951; Garton **et al**, 1961) and the latter authors suggested that triglyceride hydrolysis was carried to completion before the substrate was released from the enzyme thus precluding the isolation of free diglyceride or monoglyceride intermediates in the hydrolysis process. However these intermediates have been isolated more recently by Hawke and Robertson (1964), Silcock (1968), and in the present study.

Silcock (1968) concluded that fatty acids were found to be more saturated when free in the rumen liquor than when in esterified form, but he did not discount the possibility of hydrogenation of esterified fatty acids. However in more precise studies (Hawke and Silcock, 1969) it was found that hydrogenation can only take place with free fatty acid substrate. This finding could imply that hydrolysis is the limiting factor in the hydrogenation of dietary unsaturated fatty acids.

The importance of hydrolysis to dairy products, thus becomes obvious. A slow rate of hydrolysis could mean a lower rate of hydrogenation in the rumen, and the subsequent appearance of increased

unsaturation of depot fats and milk lipids, which is undesirable to both the meat and butter industry.

The seasonal variations in the levels of unsaturated fatty acids in butterfat has been observed by a number of workers (McDowall, Reid and Patchell, 1957; Hawke, 1963), and has been explained to a certain extent by fluctuations in the levels of unsaturated fatty acids of the diet. Hawke (1963) fed monozygotic twin cows on a diet of short rotation ryegrass, grown to two stages of maturity. The young ryegrass diet contained higher levels of unsaturated fatty acids, which were subsequently reflected in higher levels of unsaturated fatty acids in the milk fat. A similar effect was observed by feeding linseed oil in addition to the normal grass diet (McDowall et al, 1957). This increase in unsaturated lipids of the milk fat has been explained by decreased over-all hydrogenation in the rumen. In the light of this discussion however this effect may also be explained by a limited hydrolysis rate.

# Chapter 6

## SUMMARY

1. A study has been made of the hydrolysis of triglycerides by rumen contents under *in vitro* conditions using peanut oil and synthetic glyceryl tri(oleate-1-$^{14}C$) as substrate.

2. A radiochemical technique has been developed for assaying lipase activity. The products of hydrolysis of glyceryl tri(oleate-1-$^{14}C$) were separated by thin layer chromatography and the radioactivity measured by two alternative techniques. The appropriate areas of the chromatogram were scraped into counting vials for liquid scintillation counting, or the chromatogram was scanned by a radiochromatogram scanner, followed by planimetric measurement of peak areas.

3. The hydrolysis of triglyceride via diglyceride and monoglyceride intermediates to free fatty acid, by strained rumen liquor has been clearly demonstrated.

4. The organisms responsible for lipolysis appeared to be closely associated with the particulate material in the rumen. After homogenisation of total rumen contents, lipolytic activity was transferred to the bacterial pellet (14,000g centrifugate).

5. Lipolytic activity was found in cell-free extracts of rumen bacteria, prepared from a 90,000g centrifugation, after the organisms had been subjected to osmotic shock treatment or sonication.

6. That the lipolytic enzyme(s) are intracellular and appear to exhibit no specificity of hydrolysis for fatty acids in the $\alpha$ or $\beta$ position of triglyceride has been discussed.

7. The importance of hydrolysis as a prerequisite for the hydrogenation of dietary unsaturated fatty acids has been discussed.

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