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LIPOLYTIC ENZYMES FROM THE BOVINE RUMEN

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CHAPTER 1

INTRODUCTION

1.1. Lipid metabolism in the ruminants and significance of hydrolysis and hydrogenation in the rumen.

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

It is apparent from earlier studies that the lipids of ruminants differ in several respects from those of non-ruminant herbivorous animals in particular. Occurrence of unusually high proportions of stearic acid and the presence of trans acids and isomeric forms of oleic, linoleic and linolenic acid in depot fats and tissue lipids are peculiar to ruminants.

Furthermore milk fat of ruminants contains a number of branched-chain fatty acids and a mixture of volatile fatty acids. These differences in the lipid compositions of ruminant animals from those of non-ruminants may be explained by the fact that the microorganisms in the rumen can effect extensive changes to the dietary lipids. These changes include the hydrolytic release of esterified fatty acids (Garton et al., 1958, 1959 and 1961; Wright, 1961) and the hydrogenation of unsaturated fatty acids (Reiser, 1951). Because of the high content of C_{18} - unsaturated fatty acids in the most common feeds of ruminants, the microbial activity results in the accumulation of free stearic acid as the end product of complete hydrogenation and geometrical and positional isomers of oleic, linoleic and linolenic acids as the end product of incomplete hydrogenation (Shorland et al., 1955 and 1957). At the same time, hydrolysis of triglyceride and galactosyldiglyceride make glycerol and galactose available for fermentation by the rumen microorganisms. The products of glycerol fermentation by the microorganisms are CO_2, acetic acid, propionic acid and butyric acid. The rate of utilisation of glycerol, calculated to be at least 0.065 μmoles/ml/min., is due to bacterial metabolism (Wright, 1969). A mixture of acetic, propionic and butyric acids results from the fermentation of galactose by several rumen bacterial species (Hobson and Mann, 1961) These VFA further undergo metabolic changes to provide extra energy to the animal. Very little, if any degradation of liberated long-chain fatty acids from the hydrolysis of lipids apparently takes place in the rumen. There is no evidence that acids of chain length C_{16} and greater are absorbed to any appreciable extent from this part of the alimentary tract (Garton et al., 1961; Hobson and Mann, 1961; Wood et al., 1963).

Little or no resemblance between the fatty acids of dietary fat and the fat in the rumen indicates that extensive modification of dietary fat by the rumen microorganisms occurs in the rumen. Lipid content of digesta which passes from the rumen to the small intestine via the true stomach is mainly long-chain fatty acids, in particular stearic and oleic acid. These fatty acids, readily absorb from the small intestine, form a considerable proportion of the fatty acids in the lipids of thoracic - duct lymph which drains into systemic circulation (Garton, 1967).

It was noticed that depot fat of steers and goats contained more stearic acids and less oleic acids on diets containing triglyceride rich in C_{18} unsaturated fatty acids. Hydrogenation of unsaturated fatty acids in the rumen was first observed by Reiser (1951). This process attributed to the deposition of stearic acid in the depot fats which arose from bacterial hydrogenation of C_{18} unsaturated fatty acids. Linolenic acid is almost absent from the depot fats of ox, sheep in contrast to the non-ruminants which contains a very high proportion of linolenic acid in their depot fats (Shorland, 1952). Linolenic acid, the predominant fatty acid constituent of pasture, appeared only in traces in the depot fats of ruminants. A particularly effective hydrogenation in the rumen has been observed by Shorland _et al._,(1955). They found that more than 50% of linolenic was converted into stearic acid. The presence of _trans_ acids in the ruminant depot fats (Hartman _et al._, 1954) was explained on the basis of bacterial action in the rumen. Incubation of linolenic, linoleic and oleic acids under CO_2 with sheep rumen contents resulted in the production of stearic acid, _trans_ and positional isomers of unsaturated acids as well (Shorland _et al._, 1957).

Depot fats of foetal lambs do not contain such a high proportion of stearic acid as the maternal ewe (Body and Shorland, 1964). The young animal was found to absorb dietary unsaturated fatty acids into its depot fats, which demonstrates that the development of an active rumen is essential for hydrogenation of unsaturated fatty acids (Siren, 1962). Ogilvie et al. (1961) studied the effect of duodenal administration of linseed oil on the composition of ruminal depot fats and observed that the main fatty constituents of the oil - linoleic and linolenic acids are capable of being deposited in the depot fats. Normally those fatty acids are in the range of 1 to 2%. A distinct increase in proportion of stearic acid of both milk fat and adipose tissue was observed when soybean oil was fed orally to cows but on intravenous injection of the oil, the proportion of polyunsaturated acids in milk fat was increased (Tove and Mochrie, 1963).

Of the known naturally occurring fats, the ruminant milk triglycerides are among the most complex in fatty acid composition. The low concentration of polyunsaturated fatty acids in ruminant milk fat and adipose tissue fats is primarily due to biohydrogenation of dietary C_{18} di- and tri- unsaturated fatty acids to more saturated forms by the microorganisms in the rumen. However, higher levels of unsaturated fatty acids in milk fat were observed when monozygotic twin milking cows were grazed on young ryegrass (Hawke, 1963) which contained more lipid and more unsaturated fatty acids than mature ryegrass (McDowell et al., 1961). This increase in unsaturated lipids in the milk fat was related to the higher levels of unsaturated acids in the diet and the consequence of a decrease in the overall hydrogenation in the rumen. On incubation of linoleic acid with rumen contents, the product of hydrogenation by the

microorganisms were mainly oleic acid with a small concentration of stearic acid. This was explained on the basis that high concentration of linoleic acid completely inhibited the conversion of oleic acid to stearic acid (Moore et al., 1969). Recently it has been shown that lipolysis and hydrogenation in the rumen can be controlled by protecting the lipids from the action of lipases and hydrogenases (Scott et al., 1970). When formaldehyde treated polyunsaturated lipid-protein complex were added to the diets of ruminants the proportion of polyunsaturated acids in the plasma increased from approximately 4% to 25-30% within 24 hours post feeding and led to an increased incorporation of these acids into glycerides of milk and body fats.

An increased yield of stearic and oleic acid was observed in milk fat when cows were fed oil containing stearic, oleic, linoleic and linolenic acids or these acids in free form (Storry, 1970). Storry et al. (1967) supplemented the diet of cows with coconut oil and observed an increase in the concentration of lauric and myristic acids in plasma triglyceride and in the amount of these acids in milk fat. A variable relationship exists between the dietary intake of palmitic acid and its yield in milk fat (Storry, 1970). The addition of lipids to the rumen influences the pattern of fermentation of other dietary constituents (Robertson and Hawke, 1964a; 1964b) which in turn may have important effects on the metabolism of ruminants.

The food of grazing ruminants is mainly of pasture species such as ryegrass, clover etc. and to a lesser extent the leaves of many other plant species. Although the lipid content of leaf tissue is only about 5 to 10% of the total dry matter, the quantity of lipid consumed by adult

ruminants is quite significant - for example, a cow eating 100 lb. of pasture daily will ingest approximately 500g of lipids and during the period of pregnancy, lactation and stall-feeding it may receive a diet which provides 1 kg of lipids daily (Garton, 1967).

1.2. Relationship between hydrolysis and hydrogenation in the rumen.

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

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

(ii) hydrogenation of unsaturated fatty acids which are the main fatty acid components of plant lipids (Weenink, 1961).

Comparisons of the degree of saturation of the free fatty acids and the esterified lipids of rumen contents suggest that biohydrogenation of the free fatty acids does not occur until they are hydrolysed from dietary glycerolipid (Garton et al., 1961; Hawke and Robertson, 1964; Patton and Kester, 1967). The requirement of free fatty acid substrate in the rumen was further confirmed by Hawke and Silcock (1969). In more precise studies, investigation of the rate of lipolysis and hydrogenation was carried out by the use of a synthetic triglyceride 2 ($1-^{14}C$) linolenoyl - 3 - oleoyl - 1 - palmitoylglycerol in incubation with rumen content. (Hawke and Silcock, 1970). The authors found no detectable hydrogenation products in the triglyceride fraction which remained unhydrolysed and in the partial products of hydrolysis whereas hydrogenation of ($1-^{14}C$)-linolenic acid occurred in the free fatty acid fraction producing a mixture of ^{14}C- stearic, monoenoic and dienoic acids. From these evidences it was concluded that biohydrogenation of unsaturated fatty acids of dietary lipids in the rumen proceeds only after the fatty acids have been removed from ester combination by lipolysis. Consequently, the extent of

hydrogenation of unsaturated fatty acids is dependent on the activity of lipolytic microorganism in the rumen.

1.3. Lipases

1.3.1. Terminology

A group of esterases, called lipases, are of primary importance in catalysing the hydrolysis of glycerol esters of fatty acids to fatty acids and glycerol. Usually the term lipase refers to any enzyme which hydrolyses various esters. The report of the commission on enzymes of the International union of Biochemistry (1961) defines lipase (E.C.3.1.1.3) as a "glycerol ester hydrolase" and the use of emulsified substrates are recommended because they are active in heterogeneous systems (Sarda and Desnuelle, 1958) whereas estrases seem to hydrolyse substrates in solution (Aldridge, 1954). This distinction between lipases and esterases does not imply to a different catalytical mechanism between the two groups of enzymes (Oosterbaan and Jansz, 1965). The above commission further defines a unit of lipase as being that amount of enzyme which, acting on an ester emulsion under the condition of the test, libsates 1 microequivalent of acid per min. The emulsion should be of such nature that gives the maximum reaction rate.

Lipases are widespread in plants, animals and microorganisms. Pancreatic lipase has been the most extensively studied and our knowledge of the mechanism of lipase action is almost entirely derived from studies on pancreatic lipase.

1.3.2. Detection and isolation of lipolytic organisms

A) From the rumen

It has been shown that triglyceride of long-chain fatty acids can be rapidly hydrolysed by mixed rumen microorganisms (Garton et al., 1958; 1961; Wright, 1961). Hobson and Mann (1961) detected lipolytic organisms from the sheep rumen by diluting fresh rumen contents into various media containing linseed oil. For the detection of total acidity, extraction of fatty acids was followed by their titration with ethanolic NaOH solution. Alternatively, clear zones around the colonies in linseed oil-agar or tributyrin-agar roll tube were taken to indicate lipolysis or esterase activity. The isolated lipolytic bacteria were strictly anaerobic curved Gram-negative rods and were active in the hydrolysis of tributyrin and linseed oil as well as fermentation of glycerol. The lipolytic bacteria did not utilise the liberated long-chain fatty acids, a finding there is in agreement with the observation of Garton et al. (1961). These bacteria appeared to be normal inhabitants of the sheep-rumen when the animals were fed a number of different rations. They differed from all known species in their limited fermentation reactions but morphologically they were similar to many types of rumen bacteria. The authors suggested that these bacteria were amongst the more important lipolytic bacteria in the rumen. Hobson and Summers (1966) studied the relationship between growth rate and the activity of a lipolytic bacterium isolated from the rumen and in batch culture observed that lipolytic activity was associated with growing cells.

B) From various sources

A detailed review on earlier work for the detection and isolation of microbial lipases have been published by Lawrence (1967a). However,

Fryer et al. (1967b) described two double layer techniques for the detection of lipolytic organism in which the organisms are grown on nutrient agar overlaid on;

i) tributyrin agar, or

ii) a thin layer of milk fat saturated with victoria blue.

Using these two techniques the authors tested 22 strains of Gram-positive cocci, 20 strains of Gram-negative rods, and 3 strains of micrococci for the lipolytic activity. These techniques offerred the advantages that there was no danger of inhibition of microbial growth by dyes or substrates, the rate of lipolysis could be followed from the beginning of incubation and the colonies recovered after detection. If necessary the organisms could be grown on a carbohydrate media. The tributyrin-agar method has the further advantage that the sensitivity of the assay could be increased by decreasing the concentration of tributyrin.

1.3.3. Nature of microbial lipases

The ability to produce some extra-cellular lipase appears to be a general property of most, if not all, growing bacteria under suitable conditions. Although the extent to which lipase is formed varies very considerably even between strains of the same species of organisms (Lawrence, 1967a) and is markedly influenced by different nutritional and physical conditions (Lawrence et al., 1967b). Extracellular lipases are found in the supernatant fluids of cultures of Pseudomonas fragi (Alford and Pierce, 1963; Mencher et al., 1965; Mencher and Alford, 1967; Lawrence et al., 1967b; Lu and Liska 1969a), Micrococcus freudenreichii (Lawrence et al., 1967b), lactic acid bacteria (Fryer et al., 1967a), Staphylococcal lipase (Tirunarayanan and Lundbeck, 1968; Vadehra and Harmon, 1969). Most of the organisms considered to produce extracellular

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enzymes are Gram-positive bacteria (Pollock, 1962). Some organisms capable of producing extracellular lipase appear to contain a small amount of cell-bound enzyme (Lawrence et al., 1967b). Presence of the latter was explained on the basis of the inefficiency of the lipase forming system to release most of the new enzyme into the culture fluid with no specific intracellular function (Lampen, 1965). During the growth of P.roqueforti and Asperigillus niger the presence of almost equal quantities of cell-bound and extracellular lipase was observed (Chandan et al., 1962). There are reports where extracellular lipase have different optimum pH (Shahani et al., 1964) to that of cell-bound lipase from the same organism (Khan et al., 1964). The rumen bacterium reported by Hobson and Summers (1966) was found to produce two enzymes - an esterase associated mainly with the cells and a lipase which mainly secreted into the culture medium.

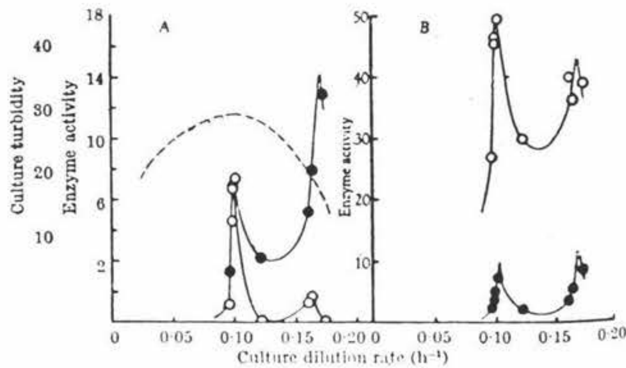

Figure 1. The lipase (A) and esterase (B) activities of cells (O) and supernatants (●) from a continuous culture growing at different rates. Substrates, naphthyl stearate (A), naphthylacetate(B). Superimposed on A is a graph of the steady-state cell concentrations at different growth rates (Hobson & Summers, 1966).

1.3.4. Induced and noninduced lipases

Enzymes in some microorganisms are not formed by a genetic system if the substrate of the enzyme is absent e.g. lipases are synthesised in _Candida paralipolytica_ under the influence of inducer lipids and related substances (Ota _et al._, 1968) whereas the enzyme was undetectable in non-induced microorganisms. Lipase production by _Torulopsis ernobii_ was increased markedly on the addition of fats, oils and higher fatty acids to the base medium at concentrations of 0.2 - 0.6% (Yoshida _et al._, 1968).

The formation of lipase by some microorganisms are not inducible i.e. lipases are formed by organisms in the absence of lipid in the medium. _Micrococcus freudenreichii_ and _Pseudomonas fragi_ produced lipase in media free of triglyceride substrate (Lawrence _et al._, 1967b). However, low concentrations of tributyrin or trioctanoin did not show any significant effect on lipase production although higher concentrations were inhibitory.

1.3.5. Purification of lipases

A) Pancreatic lipase

Pancreatic lipase is capable of acting on emulsified (Benzonana and Desnuelle, 1968) and micellar (Entressangles and Desnuelle, 1968) substrates. Attempts have been made by many investigators to purify the enzyme. Since 1957, Desnuelle and his colleagues in Marseilles have contributed remarkedly in the purification of hog pancreatic lipase. Their earlier methods involve extraction of lipase from pig pancreatic, its selective precipitation with $(NH_4)_2SO_4$ and acetone followed by electrophoresis on starch. Enzymic activity was followed by potentiometric titration of fatty acids liberated from emulsified triglyceride.

TABLE 1

Effect of lipid and related substances on the growth and lipolytic activity of Candida paralipolytica (Ota et al., 1968)

Addition ($1\% \frac{W}{V}$)	Lipase Act. (0.05 M-NaOH ml)	Growth (Packed vol. ml)
None	0.00	0.103
Castor oil	2.17	0.035
Olive oil	0.85	0.152
Soybean oil	0.48	0.176
Linseed oil	0.52	0.199
Rapeseed oil	0.35	0.163
Coconut oil	0.03	0.146
Tung oil	0.05	0.001
Lard	0.42	0.110
Triacetin	0.00	0.074
Tri-n-butyrin	0.00	0.055
Tripalmitin	0.00	0.096
Tristearin	0.02	0.102
Triolein	2.33	0.080
Monolein	0.12	0.038
Stearic acid	0.01	0.182
Linoleic acid	0.05	0.078
Tween 20 +	0.58	0.119
Span 20 +	1.25	0.070
n-octane *	0.00	0.036
n-Dodecane *	0.00	0.020
Pentene 2 *	0.00	0.001
Octadecene -1 *	0.02	0.100
Cyclohexane *	0.00	0.001
Saponin +	0.13	0.009
Cholesterol +	0.66	0.104

* ($1\% \frac{V}{V}$) + ($0.5\% \frac{W}{V}$)

They obtained a 35 - fold purification with a yield of 30% with respect to the initial extract or a 63 times purification with a yield of only 3% (Sarda et al., 1957). A 135 - fold purification was achieved with an overall yield of 20% when differential absorptions of lipase on tricalcium phosphate and an aluminium hydroxide and zone electrophoresis at pH 5.25 in starch columns were used. The purest fraction appeared to be homogeneous by both chromatographically and electropharetically (Marchis-Mouren et al., 1959). The procedure seemed to be time consuming and in each preparation not more than 1 mg of purified lipase could be obtained. Use of lyophilised supernatants of fresh pancreas homogenates as the starting material improved the purification technique. The lipase peak obtained by DEAE-cellulose chromatography was dialysed and then lyophilised and chromatographed on Sephadex G-200. This last step freed the enzyme from all remaining nucleotides and some protein impurities but the specific activity of the final product (60 to 65% lipase) did not increase due to inactivation of lipase during dialysis or lyophilisation (Benzonana et al., 1964). The techniques described so far for the purification of pig pancreatic lipase supply insufficient enzyme to allow studies of structure. However, Sarda et al. (1964) described more satisfying techniques comprising centrifugation of pancreas homogenates at 100,000g for 60 min., lyophilisation of the clear extract to give a stable powder, $(NH_4)_2SO_4$ fractionation of this powder followed by filtration through Sephadex G-200, during which the lipase showed an abnormally high rate of migration and was highly purified during passage through the column. Verger et al. (1969) purified two lipases existing in porcine pancreas and pancreatic juice by a method involving the following steps: delipidation of pancreas homogenates by solvent extraction, fractional $(NH_4)_2SO_4$ precipitation, removal of an acidic phosphatide by extraction and partition

between butanol and $(NH_4)_2SO_4$, chromatography on DEAE-cellulose at pH 9.0, gel filtration with Sephadex G-100 followed by the separation of the two lipases by chromatography on CM-cellulose. This procedure was used on a relatively large scale.

TABLE 2

Main steps of lipase purification

(Benzonana et al., 1964)

Steps	Lipase				Enzymes in the fractions (gm per 100 gm protein)			
	Total Number Units	Estimated Weight mg	Recovery	Sp. Activity	Lipase	Amylase	Chymotripsinogen	Trypsinogen
Pancreas homogenates 300g	25 $\times 10^5$	278	-	90	-	-	-	-
Supernatant	22.2×10^5	248	100	125	1.4	6.3	10.8	15.0
$(NH_4)_2SO_4$ precipitate	21.8×10^5	243	98	180	2.0	6.8	4.7	8.2
Acetone precipitate	14.5×10^5	161	65	880	9.8	3.7	0.8	4.5
DEAE-Cellulose chromatography	6.2×10^5	70	28	5,500	6.1	0.7	0.0	0.0

B) Microbial lipases

Earlier attempts to purify microbial lipases were carried out by precipitation with ehtanol followed by electrophoresis (Fiore and Nord, 1950); precipitation with $(NH_4)_2SO_4$ absorption on calcium phosphate gel and chromatography on calcium phosphate-celite 535 (Tatsuoka et al., 1959).

In the last few years sephadex, polyacrylamide gels, DEAE-cellulose have been successfully used for the purification of and characterisation of lipases.

Rhizopus arrhizus lipase was purified by fractionation through Sephadex G - 100 followed by ultracentrifugation and then again chromatography on sephadex G - 100. The lipolytic fraction was concentrated under reduced pressure, recycled on a smaller column and lyophilised (Laboureur and Labrousse, 1968). Partial purification of Micrococcus and Pseudomonal lipase was achieved by $(NH_4)_2SO_4$ precipitation of culture supernatant and then filtration by Sephadex G - 100 and G - 200 (Lawrence et al., 1967b). Sephadex G - 100 was used for the purification of concentrated Staphylococcal lipase (Tirunarayanan and Lundbeck, 1968). Lipase from Pseudomonas fragi was purified by fractionation of the culture supernatant with $(NH_4)_2SO_4$ and acetone precipitation. Filtration through sephadex G - 200 followed by DEAE-cellulose chromatography gave further purification. The purified lipolytic fraction was electrophoretically homogeneous. The yield was 1.8% of the original activity with a specific activity 100 times that of the starting culture filtrates (Lu and Liska, 1969a).

1.3.6. Purification of lysolecithinase from the rumen

Attempts to obtain cell-free lipolytic enzyme preparations from the rumen bacteria have so far not been successful although a soluble enzyme preparation of washed rumen microorganisms was obtained by Dawson (1959). The enzyme was purified by $(NH_4)_2SO_4$ precipitation and adsorption on calcium phosphate gel. The purified enzyme thus prepared was readily active in the hydrolysis of lysolecithin.

1.3.7. Methods of determining lipase activity

The rate of lipolysis can be followed by measuring the rates of disappearance of triglyceride or the rate of fatty acid production. Measurement of the formation of glycerol, mono- and diglycerides is more difficult for following the rate of lipolysis and is not commonly used.

A. Colorimetric methods

The methods described by a number of investigators for the colorimetric micro-determination of fatty acids depend on the formation of a copper soap of the fatty acid, its extraction into an organic solvent followed by the estimation of copper (Iwayama, 1959; Baker, 1961; Duncombe, 1963). Copper may be replaced by cobalt which is then determined with α- nitroso - β - naphthol (Novak, 1965). The coloured complex with free fatty acids was formed by the use of rhodamine B and uranyl ion and the complex was extracted into toluene and measured colorimetrically (Mackenzie et al., 1967). Mahadevan et al. (1968) showed that the sensitivity of Duncombe's procedure could be increased by the use of 1,5 diphenylcarbohydrazide as colour complex agent in place of diethyldithiocarbamate. Meyer-Bertenrath and Kaffarnik (1968) described a method for measuirng the lipase activity in the serum and other fluids by using dilauric acid ester of fluoresceine as substrate. As this ester is colourless and non-fluorescent, hydrolysis of the ester liberates fluoresceine which can be determined precisely by colorimetric or fluorometric techniques.

B. Titrimetric measurements of liberated fatty acids

Several methods which have been described for measuring lipase activity in the hydrolysis of triglyceride are based on the titration of

liberated free fatty acids with alcoholic sodium hydroxide solution (Dole 1956). Hobson and Mann (1961) used this method to measure the lipolytic activity of rumen microorganisms grown in media containing linseed oil. At the end of incubation period the whole culture was acidified, extracted with ether and the total acidity determined by titration with sodium hydroxide solution. A similar procedure was carried out for studying the lipolytic activity of Staphylococcus aureus (Vadehra and Harmon, 1965). Instead of using the organisms themselves, disintegrated cells or cell-free supernatants of the growth medium have been incubated with triglyceride. Ether extraction followed by the titration of liberated free fatty acids with ethanolic sodium hydroxide determined the total acidity (Alford and Pierce, 1963).

The continuous automatic titration of the fatty acid produced by the action of lipase on an appropriate substrate in a pH - stat has been used by many investigators (Shah and Wilson, 1965; Lawrence et al., 1967b; Tirunarayanan and Lundbeck, 1968; Downey and Andrews, 1969). The major advantage of this procedure is that the initial velocity of lipolysis can be measured within a short period of incubation and no extraction of free fatty acids is involved. However, this method suffers from a drawback, that it is difficult to measure the lipase activity at $pH < 7.0$ perhaps due to incomplete titration of long-chain fatty acids, and the method cannot be used to follow the lipolysis in a buffered culture medium (Lawrence, 1967b).

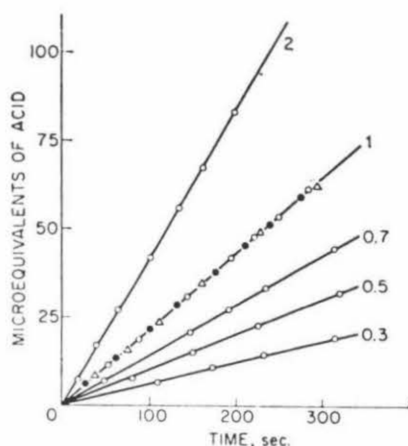

Figure 2. Potentiometric test for the determination of lipase activity. The figures indicate the relative amounts of lipase used in each assay (From Desnuelle, 1961).

C. Manometric methods

Manometric methods have been described (Aldridge, 1954; Wills, 1961) to follow the rate of fatty acid production by determining the rate of liberation of CO_2 from a bicarbonate buffer. The technique can be used as an alternative to the pH - stat but insoluble fatty acids do not readily liberate CO_2 from the bicarbonate buffer (Lawrence, 1967b).

D. Clarification or turbidimetric method

A rapid turbidimetric method has been devised by Rottem and Razin (1964) in which the lipolytic activity is followed by measuring the rate of clarification of the tributyrin suspension. As the hydrolysis of tributyrin proceeds, the products of hydrolysis e.g. monobutyrin, glycerol or butyric acid become water-soluble. So the rate of clarification of tributyrin suspension is a direct measurement of the rate of disappearance

of triglyceride. The method is also applicable for higher triglyceride (Grossberg et al., 1953). A thin-layer agar diffusion technique developed by Lawrence et al. (1967a), permits the rapid quantitative comparison of the relative activities of lipase preparation against low concentrations (0.1% $\frac{V}{V}$) of triglyceride, usually tributyrin. Because of the solubility of monobutyrin, glycerol and butyric acid, lipolytic activity produced a zone of clearing around a hole containing the enzyme solution which was made in solidified tributyrin-agar emulsion.

E. Radio-chemical techniques

The radio-chemical techniques recently developed for following the lipolytic activity involve separation of the products of hydrolysis of radioactive triglyceride and unreacted triglyceride followed by the measurement of radioactivity of individual components. ^{14}C - triolein is used as test substrate it is readily emulsified and is available commercially. Chino and Gilbert (1965) used a florisil column to separate monoglyceride, diglyceride, triglyceride and free fatty acids. Clarke and Hawke (1970) separated the products by thin-layer chromatography on silica gel. The radioactivity of each component was measured by two alternative techniques ;

(a) The chromatogram was scanned by a radio chromatogram scanner and the peak areas were measured planimetrically

(b) The appropriate areas of each component were scraped into counting vials and counted in a liquid scintillation counter.

Finally, a radioactive assay procedure for triglyceride lipase described by Kaplan (1970) was based on the differential extraction of triglyceride and fatty acids by alkaline solvents followed by the measurement of radioactivity in a Packard TriCarb Scintillation counter.

1.3.8. Specificity of lipases

The hydrolytic activity of a lipase may be influenced by the nature of the alcohol moiety and the structure of the fatty acids. In addition, the effect of the stereochemistry of substrates such as triglycerides must be considered.

A. Effect of alcohol moiety

Some studies have been made on the rate of hydrolysis of esters of different alcohols by microbial lipases. Lipases from *Pseudomonas fragi* hydrolysed methyl butyrate - the rate of hydrolysis was about one-fifth the rate of triolein and one-thirteenth that of coconut oil (Lu and Liska, 1969b). A weak esterase activity found in the lipase preparation from *Rhizopus delemar* would be considered to be due to the activity of lipase (Fukumoto *et al.*, 1964). A partially purified lipase of *Micrococcus freudenreichii* was active against *O* - nitrophenylbutyrate and emulsified triglyceride (Lawrence *et al.*, 1967b) and Stephylococcal lipase was preferentially active in the removal of fatty acids having between four and six carbon atoms, either in the form of glycerol esters (triglyceride) or simple esters of butyric acids (Tirunarayanan and Lundbeck, 1968).

Pancreatic lipase was found to hydrolyse methyl oleate but the rate of hydrolysis was $^{1}/30$th of that of triolein. Even the hydrolysis of tributyrin was much faster than that of methyl butyrate despite the latter being in the form of an emulsion (Sarda and Desnuelle, 1958).

B. Effect of structure of fatty acids

1) Unsaturation

The lipase from Geotrichum candidum has been found to possess a high degree of specificity towards esterbonds involving oleic acid regardless of position in the triglyceride (Alford et al., 1964). Furthermore, the lipase removed very little elaidic acid from glyceryl - 1 - elaidate - 2, 3 - dioleate (Jensen et al., 1965). The degree of unsaturation of the chains of fatty acids from zero to two double bonds did not have any appreciable influence on the rate of hydrolysis by pancreatic lipase (Savary and Desnuelle, 1956).

TABLE 3.

Lipolysis of synthetic triglyceride by lipase from Geotrichum candidum Alford et al., (1964).

	Weight percent of fatty acids as		
Triglyceride	Palmitic	Stearic	Oleic
2 - stearyldiolein	-	1	99
2 - oleyldistearin	-	2	98
2 - palmitoyldiolein	1	-	99
2 - oleyldipalmitin	20	-	80
2 - oleylpalmitostearin	9	1	90
2 - palmitoyldistearin	50	50	-
2 - stearoyldipalmitin	99	1	-
1 - oleoyldistearin	-	2	98
1 - stearoyldiolein	-	1	99
1 - oleoyldipalmitin	35	-	65
1 - palmitoyldiolein	5	-	95

II) Chain length of fatty acids

It has been consistently observed that microbial lipases show a greater activity towards glycerides containing short-chain fatty acids than those containing long-chain fatty acids. Maximum rate of hydrolysis were observed when tributyrin or tripropionin was used as substrate (Rottem and Razin, 1964; Shah and Wilson, 1965; Tirunarayanan and Lundbeck, 1968). However, purified lipase from Pseudomonas fragi hydrolysed trilaurin most rapidly and followed in order by tricaprin, tripalmitin, tributyrin, tricaproin and tristearin (Lu and Liska, 1969b). Pancreatic lipase removes short-chain fatty acids more rapidly than long-chain fatty acids (Entressangles et al., 1961; Wills, 1961). Hydrolysis of tributyrin was more rapid than any other triglyceride by human milk lipase (Schønheyder and Volqvartz, 1943).

It follows from the foregoing consideration that variations in activity towards various natural lipids by a microbial lipase are possible because of the particular specificity of the enzyme. Lipase from Mucor pusillus showed activity in the hydrolysis of butter fat, vegetable lipids and selected synthetic triglyceride (Somkuti and Babel, 1968). Among the natural lipids it showed highest activity in the hydrolysis of coconut oil and the activity decreased in the following order:
Coconut oil , Butter fat , Safflower oil , Cottonseed oil , Olive oil and Corn oil. Analysis of rumen liquor incubated with linseed oil and olive oil showed that hydrolysis of the latter is slightly more rapid than that of the former (Wright, 1961). In some cases it is difficult to decide whether these are differences due to structure or whether dispersion and emulsification are affecting the rate of lipolysis. In this connection it has been observed that vegetable fats are hydrolysed more readily by

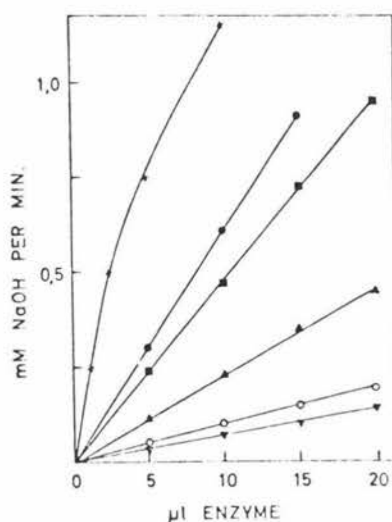

Figure 3. Hydrolysis of triglyceride by Staphylococcal lipase (Tirunarayanan & Lundbeck, 1968).

×——× tributyrin; ▲——▲ triacetin; ●——● tricaproin; ○——○ tricaprin; ■——■ tripropionin; ▼——▼ triolein.

pancreatic lipase than animal fats such as beef fat or whale oil (Wills, 1965).

C. Effect of position of fatty acid in the triglyceride

Microbial lipases have been found to differ in the site of their attack on triglyceride. Some microbial lipases e.g. Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas geniculata, Candida paralipolytica hydrolyse primarily the ester bonds in 1 and 3 positions of triglyceride (Alford et al., 1964) in a manner similar to that of pancreatic lipase. Lipases of some microbial sources, however, show positional specifities towards the 2 - position as well as the 1 and 3 positions of the triglyceride. For example Fukumoto et al. (1963) observed that the crystalline lipase from Asperigillus niger almost completely hydrolysed olive oil, indicating that the enzyme can attack not only the primary ester bond in the triglyceride but also those at the secondary positions smoothly. The lipase from Staphylococcus aureus and Asperigillus flavus appears to be similarly non-specific (Alford et al., 1964).

1.3.9. Factors affecting the activity of lipases

A. Effect of pH

The effect of pH on the rate of hydrolysis is the result of its combined effects on the enzyme itself, on the stability of enzyme, the velocity of enzyme-substrate combination and breakdown and the properties of the substrate/aqueous interface in case of diphasic systems (Lawrence 1967b). In some cases the optimal pH depends on the nature of the substrate being hydrolysed. Lipase from Mycoplasma gallisepticum showed maximum activity for mono-, di- and tributyrin at pH 7.5 but for trilaurin and triolein the optimal pH was 8.0 (Rottem and Razin, 1964).

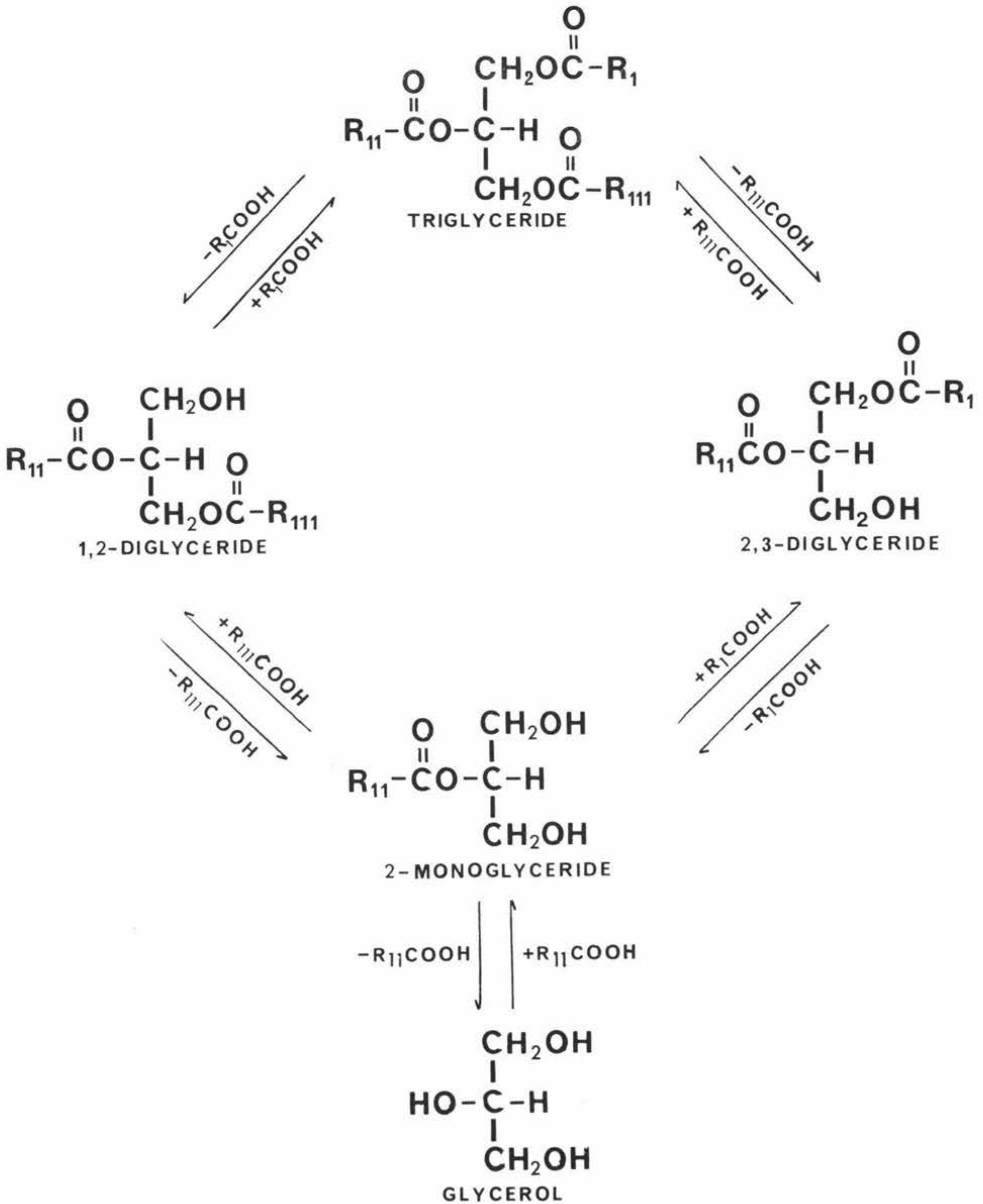

Figure 4. Major pathway for the hydrolysis of triglyceride by pancreatic lipase. Complete hydrolysis of triglyceride proceeds with the rapid splitting of the 1 & 3 linkages followed by slow hydrolysis of the 2- monoglyceride. R_1, R_{11}, R_{111} represent fatty acids of the same or different structure (From Mahler & Cordes, 1967).

pH optima of rat serum lipase were 7.2, 7.6 and 8.05 for ethyl butyrate, tripropionin and tributyrin respectively (Tuba and Hoare, 1950).

Other pH optimals quoted are:
9.0 for Mucor lipoluticus Aac - 0102 lipase (Nagaoka et al., 1969); 8.0 - 8.5 for Micrococcus freudenreichii and Pseudomonas fragi lipase (Lawrence et al., 1967b); 9.0 for Penicillium erustosum lipase (Oi et al. 1967); 7.5 - 8.8 for Staphylococcal lipase with tributyrin (Tirunarayanan and Lundbeck, 1968); 10.0 for E.Coli phospholipase but phospholipase A from the same organism showed two pH optimas-5.0 and 8.4 (Proulx and Fung, 1969). Pancreatic lipase has presented an optimum pH of 8.2 (Alichanidis, 1969).

B. Effect of temperature

With a few exceptions microbial lipases are most active within temperature range 30° - 40°C e.g. the lipase of Penicillium oxalicum is almost active at 37° - 40°C (Kirsh, 1935); of Mycoplasma gallicepticum at 37°C (Rottem and Razin, 1964). However, lipases from different micro-organisms are active at temperatures below 0°C e.g. lipases from Pseudomonas fragi, Staphylococcus aureus, Geotrichum candidum, Candida paralipolytica and Penicillium Sp. showed considerable activity in the hydrolysis of emulsified corn oil, coconut oil and lard in 2 - 4 days at -7°C, in 7 days at -18°C and over a 3 week incubation period activity was exhibited by some of these cultures at -29°C particularly towards corn oil (Alford and Pierce, 1961). Pancreatic lipase showed an optimum temperature at 37°C in an incubation period of 15 min. (Alichanidis, 1969).

Studies have been made on the stability of lipases at different

temperatures e.g. lipase from Mucor pusillus lost 50% of its activity when heated at 58°C for 45 min. (Somkuti and Babel, 1968) at pH 5.5. A preparation of Pseudomonal lipase obtained by precipitation with $(NH_4)_2SO_4$ lost all activity at 100°C for 3 min. and the thermostability of lipase from Micrococcus freudenreichii was dependent on its degree of purification (Lawrence et al., 1967b). Lipases from Penicillium crustosum were found to be stable below 45°C (Oi et al., 1967). Activity of pancreatic lipase completely ceased on incubation for 15 min. at 80°C (Alichanidis, 1969).

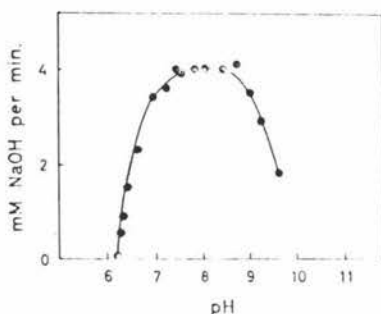

Figure 5. pH-activity relationship for the hydrolysis of tributyrin by Staphylococcal lipase (Tirunarayanan & Lundbeck, 1968).

C. Effect of emulsification of substrate

It has been shown that pancreatic lipase acts preferentially on emulsified esters (Sarda and Desnuelle, 1958). The authors compared the rate of hydrolysis of a true solution of tracetin and emulsified triacetin in gum arabic. In a true solution, at low concentrations of triacetin, the rate of hydrolysis is very slow but the rate increased very sharply as the concentration of triacetin is increased to form a heterogeneous system. Under these conditions, lipolysis must occur only at the interface between the lipid droplet and the aqueous phase. The rate of hydrolysis is, in part determined by the area of this interface - the higher the degree of emulsification the smaller the individual lipid droplets and the larger the surface area. When the emulsion is finely dispersed the interfacial area is larger and for the same weight of substrate larger interfaces will give higher rates of hydrolysis by absorbing more lipase. Consequently, if the same weight of substrate (triolein) is emulsified to give different dispersions, the rate of hydrolysis is fastest when the interfacial area is greatest (Sarda _et al._, unpublished experiments cited by Desnuelle, 1961).

Although an increase in surface area due to emulsification significantly increases the rate of lipolysis, the effect is complex and depends on the exact chemical nature of the emulsifying agent (Wills, 1965). Bile salts, egg albumin, gum arabic, soaps and synthetic detergents have been used as emulsifying agents to increase the rate of lipolysis. Some of these agents activate, while others inhibit lipase. Rate of shaking of the reaction mixture is an additional factor of importance in investigating lipase. The degree of emulsification is less important if the triglyceride suspension in the aqueous phase is rapidly shaken so that a fresh interface is constantly being made available to the lipase (Wills,

1965).

Since microbial lipases like pancreatic lipase usually act on water insoluble substrates, the degree of emulsification also accelerates the rate of hydrolysis by microbial lipases (Hugo and Beveridge, 1962). However, <u>Asperigillus niger</u> lipase was found to be inactive in an emulsified system but activity was obtained simply by shaking the reaction mixture (Iwai <u>et al</u>., 1964).

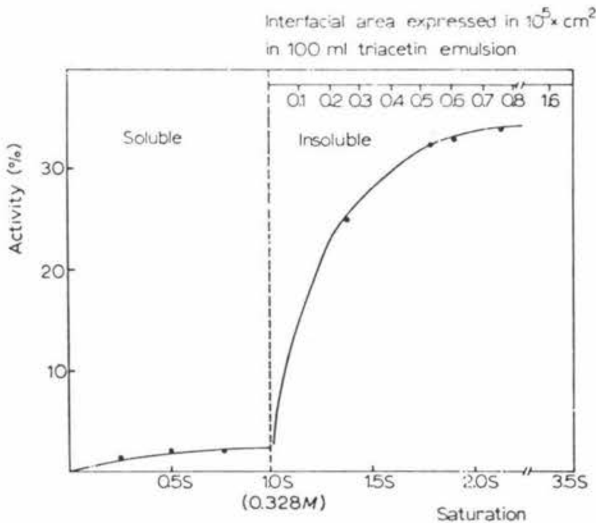

Figure 6. The influence of saturation on the hydrolysis of triacetin by lipase from swine pancreas. The numbers at the upper borderline give the interfacial area expressed in 10^5 x cm^2 in 100 ml triacetin emulsion (From Oosterbaan & Jansz, 1965).

D. Effect of Sonication on pancreatic lipase

Sonication for a short period of time was found to increase the activity of pancreatic lipase (Goodman and Dugan, Jr., 1970). Lipase was rapidly inactivated by sonication at 50°C, although it was stable at 30°C and at lower temperatures. At 40°C a slight inactivation of the enzyme was observed during the first 5 min. of sonication, however as the sonication time increased, the enzyme lost activity. When an emulsion of olive oil - gum arabic in lipase was sonicated for 4.5 min. at 38°C, 2.7 times as much fatty acid was liberated than in the control. This significant increase in rate of hydrolysis was ascribed to the formation of a better emulsion of olive oil and gum arabic due to sonication leading to a greater turnover rate of substrate at the oil - water interface. The enzyme was active in the hydrolysis of tripalmitin when the latter was dissolved in methylmyristate and sonicated with the enzyme at 45°C. On the otherhand stirring of lipase and tripalmitin did not lead to the liberation of free fatty acids.

E. Effect of metal ions

Certain metal ions e.g. calcium and magnesium were found to accelerate the hydrolysis of triglyceride by lipase probably by removing the liberated fatty acids as insoluble soaps. Ca^{++} have a function in maintaining the stability of pancreatic lipase (Wills, 1961). Ca and Mg ions increased the activity of Penicillium crustosum lipase. Oi et al. (1967) suggested that calcium activated the enzyme by stabilising its active configuration. Calcium was found to have a catalytic effect 3 to 4 times greater than Mg for staphylococcal lipase when tributyrin was used as substrate (Tirunarayanan and Lundbeck, 1968). These two metal ions also increased the activity of phospholipase A from E.Coli (Proulx and

Fung, 1969). Hydrolysis of triglyceride by pancreatic lipase goes to completion more readily in the presence of calcium (Desnuelle et al., 1950). However, lipases of some microorganisms are not stimulated by divalent ions e.g. lipases from Micoplasma (Rottem and Razin, 1964) and Micrococcus freudenreichii (Lawrence et al., 1967b).

1.3.10. Effect of metals and non-metallic inhibitors on lipases

In common with many other hydrolytic enzymes, microbial lipases are inhibited by several metal ions. Lipases from Micrococcus freudenreichii and Pseudomonas fragi are strongly inhibited by zinc and mercuric ions and partially, by copper, nickel, cadmium and beryllium in that order of decreasing effectiveness (Lawrence et al., 1967b). Cu^{++} and Hg^{++} strongly inhibited pancreatic lipase (Wills, 1960). The inhibition of zinc and mercuric ions towards both the above mentioned microbial lipases are counteracted by histidine and less effectively by EDTA (Lawrence et al., 1967b). Again EDTA was active in removing the toxicity of Fe^{+++} towards lipase from Pseudomonas fragi (Lu and Liska, 1969b) but it inhibited staphylococcal lipase in hydrolysis of various substrates, although the inhibition was counteracted by Ca and Mg (Tirunarayanan and Lundbeck, 1968). Sodium laurylsulphate was an inhibitor for Mucor lipolyticus lipase F_3 towards olive oil (Nogaoka and Yamada, 1969), E.Coli phospholipase A and lysophospholipase (Proulx and Fung, 1969) and lipase I and II of Penicillium crustosum (Oi et al., 1967). Diethyl-p-nitrophenylphosphate was most effective inhibitor of lipase of Micrococcus freudenreichii (Lawrence et al., 1967b). Iodoacetate and N-ethylmaleimide were moderate inhibitors of Pseudomonas lipase but relatively high concentrations of p-chloromercuribenzoate did not inhibit the enzyme completely (Lu and Liska, 1969b). The following illustrations show the interesting behaviour of some

compounds as inhibitors or accelerators of the activity of 2 lipase fractions prepared from Penicillium crustosum. Lipase I is slightly inhibited by sodium deoxycholate (inhibition is reversed by calcium ions) while lipase II is stimulated to some degree by the same agent. On the otherhand the activity of lipase I towards tributyrin is decreased by tween 20 and polyvinylalcohol but the same agents inhibited the activity of lipase I towards olive oil and that of lipase II towards tributyrin. Although lipase I was unaffected by span 80, it inhibited lipase II in the hydrolysis of tributyrin (Oi et al., 1967).

During lypolysis the liberated free fatty acids inhibit lipases of different microorganisms to differing extents. Oleic acid was found to inhibit the lipase activity of Pseudomonas aeruginosa (Sierra, 1957), Pseudomonas fragi (Smith and Alford, 1966) and Micrococcus freudenreichii (Lawrence et al., 1967b). The growth of Streptococcus cremoris was markedly inhibited by oleic acid (Anders and Jago, 1964).

A marked reduction in lipolytic activity towards triglyceride was was observed when penicillin or terramycin were added to rumen contents (Wright, 1961).

CHAPTER 2

AIM OF THE PRESENT STUDY

Since it has been firmly established that biohydrogenation in the rumen only takes place after the constituent fatty acids of dietary lipids are released in free form, it is of importance, therefore, to obtain an understanding of the nature and mechanism of activity of microbial lipases which are responsible for the release of fatty acids from the dietary lipids. To this end, attempts have been made to isolate lipases from mixed rumen bacteria and to isolate the lipolytic organisms present.

CHAPTER 3

MATERIALS AND METHODS

3.1. Solvents and reagents

All solvents used were purified by distillation and reagents were of analar or reagent grade unless otherwise mentioned. Glyceryl tri (oleate - 1 - ^{14}C) and linolenic - 1 - ^{14}C were obtained from the Radiochemical Centre, Amersham, England.

3.2. Preparation of medium constituents

The anaerobic techniques followed are basically those of Hungate (1969). Commercial gases were freed from oxygen by passage through a column of hot reduced copper filings. Sterile oxygen-free CO_2 or N_2 were used to displace air from the solutions, media and cultures.

Clarified rumen liquor was prepared from rumen contents taken from a fistulated cow. The contents were first strained through one layer of cheese-cloth to remove coarse food particles. The strained fluid was autoclaved under anaerobic conditions for 15 min. at 15 lb/in^2 and centrifuged at 20,000xg for 20 min. under CO_2. The clear supernatant was used as a component of the medium for the culture of total rumen bacteria.

Mineral salt solutions described by Bryant and Burkey (1953) were used. These solutions had the following compositions (g/100ml):

Salt solution No. 1: K_2HPO_4, 0.3;

Salt solution No. 2: KH_2PO_4, 0.3; $(NH_4)_2SO_4$, 0.6; NaCl 0.6; $MgSO_4$, 0.06; $CaCl_2$ 0.06.

The required quantity of dry material was weighed into bottles previously

flushed with oxygen-free CO_2. CO_2-saturated boiled and cooled water was added to the bottles and then they were stoppered with precautions to prevent the entry of atmospheric oxygen.

Sterile 10% sodium bicarbonate solution was prepared anaerobically by using a sterile membrane (cat. No. XX4004700 Milli-pore corp.) filter. Distilled water was boiled and cooled under a stream of oxygen-free CO_2 in a flask. Sodium bicarbonate was placed into a bottle which was flushed with CO_2 for at least 5 min. before the required quantity of water was added to the bottle with a 100 ml bulb pipette. When the Sodium bicarbonate was dissolved, the sterile filter assembly was flushed free of oxygen with a stream of oxygen-free CO_2 gas. The Sodium bicarbonate solution was transferred to the filter and the gas used to supply pressure for filtration. The sterile filtrate was collected in previously flushed sterile tubes and then stoppered.

Sterile Sodium sulphide - cysteine hydrochloride reducing agent was prepared according to the method of Bryant and Robinson (1961a) and sterilised by filtration under oxygen-free nitrogen using the methods described for the preparation of sterile sodium bicarbonate solution.

3.3. Preparation of media

The ingredients of the media other than those provided in salt solutions 1 and 2 were weighed and placed into a round bottomed flask having a long neck. The flask was gassed with oxygen-free CO_2 for at least 5 min. before the addition of CO_2-saturated boiled and cooled water with a 100 ml bulb pipette. Appropriate quantities of salt solutions 1 and 2 were added to the flask next and finally a 50 ml syringe was used

to add clarified rumen fluid. The components were mixed thoroughly and brought to the boil under a stream of oxygen-free CO_2. This medium was distributed to culture tubes in 4.15 ml (medium A and B) and 8 ml quantities (medium C) using the procedure described by Hungate (1969). The stoppered tubes of medium under CO_2 were autoclaved for 15 min. at 15 lb/in^2 in a vertical rack with a lid (Labtool Specialties, Ypsilanti, Michigan, U.S.A.). Any tubes showing a trace of pink oxidised resazurin were rejected as the strictly anaerobic bacteria will not grow under these conditions.

3.4. Composition of media

Medium A: It was the rumen fluid agar medium described by Coleman (1962). It was used for the culture of the total rumen bacteria and the isolation of tributyrin-splitting bacteria from the rumen. The medium had the following composition (g/100 ml or ml/100 ml); Agar, 1.5; glucose, 0.025; cellobiose, 0.025; starch, 0.025; salt solution no. 1, 7.5; salt solution no. 2, 7.5; clarified rumen liquor, 40; water, 60; resazurin (0.1% solution), 0.1.

Medium B: This was a semi-synthetic medium described by Bryant and Small (1956). It was used here in an attempt to avoid the high concentrations of free fatty acids in rumen fluid medium. The composition of the medium was as follows (g/100 ml or ml/100 ml); glucose, 0.5; tripticase, 1.5; yeast extract, 0.5; salt solution no. 1, 7.5; salt solution no. 2, 7.5; Agar, 1.5; resazurin (0.1% solution), 0.1.

Medium C: This was a synthetic medium described by Bryant and Robinson (1961b). It was used for the determination of the lipolytic activity of tributyrin-splitting rumen bacteria with respect to monogalactosyldiglyceride.

TABLE 4

Bryant and Robinson's (1961b) synthetic medium

Component	Concentration in mg/100 ml or in ml/100 ml
Cellobiose	300.0
Casein hydrolysate	200.0
KH_2PO_4	90.0
NaCl	90.0
$(NH_4)_2SO_4$	90.0
$CaCl_2$	2.0
$MgCl_2$, $6H_2O$	2.0
$MnCl_2$, $4H_2O$	1.0
$CoCl_2$, $6H_2O$	0.1
Pyridoxamine 2HCl	0.2
Riboflavin	0.2
Thiamine HCl	0.2
Nicotinamide	0.2
Ca-D-pantothenate	0.2
p-Aminobenzoicacid	0.01
Folic acid	0.005
Biotin	0.005
Resazurin (0.1% solution)	0.1

3.5. Inoculation of medium

A series of 9 tubes containing medium A which showed no trace of oxidation were selected for the dilution of fresh rumen fluid and culture of the bacteria. The contents of the tubes were melted and kept in a water bath at 50°C. Sterile sodium bicarbonate (0.25ml) and sodium sulphide-cysteine hydro-

chloride reducing agent (0.1 ml) were added to each of these tubes using sterile disposable tuberculin syringes fitted with 21 gauge needles. Rumen fluid (0.5 ml) was injected to give a 1:10 dilution into the first tube and mixed. Serial 10 fold dilutions were prepared by transferring 0.5 ml of this to subsequent dilutions to 4.5 ml of molten medium 'A' using a fresh syringe for each dilution. The inoculated agar was evenly spread over the inside of the tubes as they were rolled under a stream of cold tap water. The first tubes to be cooled were those containing the highest dilution. All incubations were carried out at 39°C.

3.6 Isolation of pure cultures

The technique used for picking colonies was described by Hungate (1950).

3.7. Subculture and storage of cultures

Pure cultures of B_1, B_2 and B_3 (strain 1, 2 and 3 respectively) were stored at room temperature and subcultured in medium A with agar at an interval of 15 to 20 days.

3.8. Rumen sampling

For the isolation of lipolytic fractions and lipolytic microorganisms from rumen content, rumen samples were collected from a lactating Jersey cow fitted with a rumen fistula. The cow was fed on clover-ryegrass pasture. Before sampling the cow was withheld from feeding for about 3 hours. The samples were taken from a central position in the rumen and placed directly into a previously warmed thermos flask. To minimise the sampling errors, a large quantity of the sample (approximately 2 litres after homogenisation and straining) was taken on each occasion and mixed

thoroughly before homogenisation.

3.9. Procedure for the preparation of lipolytic fractions from total rumen contents for the purification of lipases.

3.9.1. Homogenisation and straining of rumen content

Total rumen contents were homogenised in a Waring blender for 2.5 min. The operation was carried out in two stages in order to avoid the possible inactivation of the enzyme by warming during homogenisation. Sufficient solid materials from the total rumen contents were discarded in order to obtain a suitable consistency during homogenisation. The mixture was strained through one layer of cheese-cloth immediately after homogenisation.

3.9.2. Separation of protozoal and debris fraction

Strained rumen contents were centrifuged in a Sorvall Model SS-3 centrifuge (GSA rotor) at 500xg for 10 min. to precipitate protozoa and debris.

3.9.3. Preparation of bacterial fraction

The supernatant obtained from centrifugation of strained rumen content at 500xg was further centrifuged at 20,000xg for 30 min. in the Sorvall centrifuge (SS-34 rotor). The precipitate (bacterial pellet) was washed twice with 0.06 M phosphate buffer (pH 6.9 - 7.0), recentrifuged at 20,000xg for 20 min. and suspended in sufficient 0.06 M phosphate buffer to make up to the original volume of rumen contents.

3.9.4. High frequency sonication

A suitable quantity of washed bacterial cell was taken in a small beaker and suspended in 0.06 M phosphate buffer to form a thick slurry. The beaker was placed in an ice bucket and subjected to sonication in a MSE 100 watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London) for one min. at a power output of 8 microns peak-to-peak and the process was repeated after cooling to give the required length of sonication time. This intermittent procedure was adopted in order to avoid the possible denaturation of the enzyme by heat produced by sonication.

3.9.5. Ultrafiltration

A suspension of sonicated bacterial pellet in 0.06 M phosphate buffer made up to the original volume of rumen contents was centrifuged in Beckman Model L preparative ultra centrifuge at 90,000xg for 30 min. The supernatant fraction was filtered through a Zeitz filter to give a bacterial free extract.

3.9.6. Dialysis

The filtered supernatant was dialysed against 5 litres of distilled water with changes after 1, 3 and 6 hours followed by an overnight dialysis against 5 litres of distilled water. Dialysis was carried out in cellulose tubing (Visking Co., Chicago) with stirring at $4^{\circ}C$.

3.9.7. Freeze-drying

The dialysed material was transferred to a round bottomed flask and rotated in an ethanol tank ($-38^{\circ}C$) in order to get an evenly distributed deposit of frozen material about the inside wall of the flask. The flask was then attached to the vacuum system of the freeze-drier (Cuddon freeze-

dry, Blenheim, New Zealand).

3.9.8. Gel-filtration

A column (71 cm x 1.8 cm internal diameter) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) was packed up to a height of 68 cm. with swollen gel in the cold. A plug of glass wool was placed at the bottom of the column in order to support the gel. The column was equilibriated with 0.15 M-NaCl solution and the sample (lyophilised material) dissolved in 1.5 ml of 0.15 MNaCl, was carefully added to the top of the column with a Pasteur pipette without disturbing the surface. The sample was allowed to enter the bed followed by a few ml of the eluant (0.15 M-NaCl) to rinse the edges of the column. Finally the space above the bed was filled with the eluting solvent and fractions of approximately 4 ml were collected with a flow rate of 12 to 18 ml/hour.

3.9.9. $(NH_4)_2SO_4$ precipitation

Sufficient lyophilised material was taken in half-saturated $(NH_4)_2SO_4$ to give a 1.12% solution and held at 4°C for several hours. This mixture was centrifuged at 90,000xg for 15 min. The supernatant was dialysed overnight and freeze-dried. The precipitate was washed with half-saturated $(NH_4)_2SO_4$ and centrifuged at 60,000xg for 30 min. The centrifugate was dissolved in water in the cold and centrifuged at 25,000xg for 30 min. to give a clear supernatant which was freeze-dried.

3.10 Determination of protein

The protein content of lyophilised material prepared from sonicated bacterial pellet was estimated by Biuret method. Lowry method was

adopted to determine the protein content of lyophilised $(NH_4)_2SO_4$ precipitate or supernatant. In both cases bovine serum albumin was used as the standard protein solution.

3.11. Thin-layer chromatography

3.11.1. Preparation of chromatographic plates

A weighed amount of the silica gel (E. Merck AG. Darmstadt) with twice the volume of distilled water shaken vigorously and the resultant slurry added to the Desaga applicator which was adjusted to give layers of required thickness. For qualitative analyses of fatty acids, mono-, di- and triglycerides layers of 0.25 mm thickness used while 0.5 mm layers were used for the purification of triglycerides, 1, 2-diglycerides and methyl esters of fatty acids. The plates were activated at a temperature between 110 to 115°C in an oven for about 1 hour. No special conditions were used for the storage of the prepared plates.

3.11.2. Elution solvents

Hexane (B.P. 66 - 70°C), diethyl ether and acetic acid were used as eluting solvents. Hexane and ether were dried over anhydrous sodium sulphate. The solvent mixture used to develop the chromatogram were:

Experiment	Component (% by volume)		
	Hexane	Ether	Acetic acid
Separation and identification of fatty acids, mono-, di-, and triglycerides. Purification of glyceryl tri (oleate-1-^{14}C).	70	30	1
Purification of 1, 2 - diglyceride	60	40	1
Purification of methyl ester of oleic acid.	80	20	-

The samples dissolved in hexane were applied to the chromatographic layer about 1.5 cm from one edge of the plate. The layers were developed in a chromatography tank lined with filter paper to assist equilibriation. After chromatography the plate was dried and sprayed with 0.1% $(\frac{W}{V})$ solution of 2,7-dichlorofluorescein in ethanol and viewed under UV-light. Components were identified by comparison with known compounds chromatographed simultaneously. In the preparative procedure, the zones containing the required component were scraped into centrifuge tubes, extracted with ether by shaking, centrifuged, the supernatants removed by decantation and the solvents evaporated to dryness.

3.12. Incubation procedure for radio-chemical assay

A suitable aliquot (usually 5 ml, except otherwise mentioned) of each fraction to be tested for lipase activity was incubated with ^{14}C - triolein dissolved in small volume of hexane in an Ertenmeyer flask or in a test tube. Anaerobic conditions were maintained by bubbling N_2 through the incubation mixture. Anaerobic conditions were not maintained during the assay of lipolytic activity of fractions obtained from Sephadex G - 200. Mild shaking was continued throughout the incubation period. In all experiments the incubation temperature was 39°C.

3.13. Radiochemical methods

3.13.1. Radioisotope counting

Aliquots of radioactive sample in hexane were evaporated to dryness in counting vials or radioactive components from the thin-layer plate were scraped into the counting vials and 10 ml of toluene scintillation fluid (toluene containing 2,5 diphenyloxazole (0.6%) and 1,4-bis-2 (5-phenyloxazolyl)-benzene (0.05%)) was added to the vials and counted in a

Packard Model 3375 liquid scintillation spectrometer. The efficiency of the system was determined by using a standard sample of U - ^{14}C - hexadecane.

3.13.2. Determination of radioactive lipids from the thin-layer plates

i) Scanning

To determine the distibution of radioactivity among components of a mixture of free fatty acids, mono-, di- and tri-glycerides 20 cm x 5 cm plate were used. The individual areas of radioactivity were determined by a Packard Model 7200 Radiochromatogram modified to take thin-layer plates using the following settings: Voltage, 300; time constant, 30 sec; chart speed, 6 cm/hour; gas flow, 110 ml/min. Radioactive compounds were identified by comparison with standards chromatographed and sprayed with 0.1% ($\frac{W}{V}$) solution of 2,7 - dichlorofluorescein in ethanol. The relative estimates of the proportions of radioactivity present in each lipid component was obtained with a planimeter (G. Coradi A.G. Zurich).

ii) Removal of zones

Alternatively, the determination of the relative radioactivity of the components was achieved by scraping the zones corresponding to the standard compounds run simultaneously into counting vials. Scintillation fluid was added to each of the vials and counted in a Packard Model 3375 liquid scintillation spectrometer.

3.14. Preparation of emulsions

It was found necessary to emulsify the triglyceride under standard conditions to get a reproducible rate of hydrolysis. Lissapol NDB

(Imperial Chemical Industry Ltd.) and gum acacia (B.D.H.) were used as emulsifying agents.

Equal volumes of peanut oil and water with 1% lissapol NDB were mixed and the mixture was homogenised for 5 min. in a MSE homogeniser (Measuring and Scientific Equipment Ltd., London) and immediately pipetted into the incubation flasks. Alternatively, 10 g of gum acacia was added to 80 ml of ice-cold water and mixed thoroughly. 1 ml of lipid (triolein or tributyrin) was added and the resultant mixture was homogenised in the MSE homogeniser for 5 min. at full speed. Water was added to give a final volume of 100 ml (Downey and Andrews, 1969). The emulsions were used on the day they were prepared.

Emulsification of monogalactosyldiglyceride was carried out according to the following procedure: An aliquot of ether with 25 mg or 8.5 mg of galactolipid was added to a homogeninsing vessel containing 0.5 g of powdered gum acacia and 8 ml of hot CO_2-saturated water. The contents of the vessel were homogenised for 15 min. at full speed using the MSE homogeniser under a stream of oxygen-free CO_2. Sterile reducing agent and 10% sodium bicarbonate solution was added to the emulsion in the proportion of 1% $(\frac{V}{V})$ and 5% $(\frac{V}{V})$ respectively.

Tributyrin agar or peanut oil-agar emulsion was prepared according to the procedure of Fryer et al. (1967b) with some modification. Tributyrin or peanut oil (0.2% $\frac{V}{V}$) was emulsified in a hot aqueous solution containing 1.5% agar and 0.0001% resazurin using a MSE homogeniser at full speed for 5 min. under a stream of oxygen-free CO_2. Aliquots of 2 ml were distributed into culture tubes under oxygen-free CO_2 (Hungate, 1969) and sterilised for 15 min. at 15 lb./in^2.

3.15. Double layer technique for the detection of tributyrin-splitting bacteria from the rumen.

Tributyrin-agar or peanut oil-agar as prepared in tubes (section 3.14) was melted and kept at 50°C. Sterile sodium bicarbonate solution (0.1 ml) and reducing agent (0.02 ml) was added to each tube by means of a disposable syringe and the contents of the tubes were thoroughly mixed. Each tube was held horizontally in running cold tap water and the emulsion was kept flowing over the inner surface of the tube until the tributyrin-agar or peanut oil-agar emulsion was set. This operation gave an opaque thin coating of the emulsion over the inner surface of the tube. The same operation was continued for all the tubes and the agar was allowed to set at the room temperature for 20 - 25 min. and then transferred into the water bath (50°C). Tenfold dilutions of rumen fluid or a bacterial preparation from rumen solids (Sub-section 3.9.3.) in a known volume of 0.06 M phosphate buffer, were prepared in medium A using the methods described in section 3.5. Starting from the highest dilution, an aliquot of 2 ml from each tube was transferred to each of the previously warmed tubes containing a thin-layer of tributyrin-agar emulsion. The emulsion was overlaid with the basal medium by rotating the tube under a stream of tap water. Finally, the tubes were incubated at 39°C.

3.16. Procedure for following the hydrolysis of grass galactolipid by tributyrin-splitting bacteria

3.16.1. Comparison of the rate of hydrolysis of galactolipid by strain 1, 2 and 3

For each strain, 1 ml of 96 hours broth culture in medium C was inoculated into another 8 ml of medium C and incubated for 20 hours. A syringe was used to add a suitable aliquot of galactolipid emulsion pre-

pared as described in section 3.14 to each tube of bacterial culture and at the same time to two uninoculated control tubes. The tubes were incubated at 39°C. The uninoculated control tubes were used to check the non-enzymatic hydrolysis of the substrate.

3.16.2. Time course for the hydrolysis of galactolipid by strain 1

1 ml of 40 hours broth culture in media C was inoculated into 4 tubes containing 8 ml of media C and incubated for 6 hours with an uninoculated control (9ml). A suitable aliquot of galactolipid emulsion as prepared in section 3.14 was added to each tube and the tubes were re-incubated. In both the experiments (section 3.16.1 and 3.16.2) reactions in the tubes were stopped after appropriate intervals using the procedure described in section 3.17; except hexane was used in place of chloroform. A quantitative determination of the proportion of liberated free fatty acids in each incubation was carried out colorimetrically using the method described in section 3.18.

3.17. Termination of lipolysis and extraction of lipid

The reaction was stopped by adding an equal volume of ethanol to the incubation mixture and heating for 5 mins. in the water bath at a temperature between 50°C to 60°C. The pH was brought to 4.0 by addition of 0.1 M-HCl. The resultant mixture was transferred to a separating funnel and lipids were extracted with chloroform (3 separate extractions). The combined extracts were washed with distilled water, dried with anhydrous sodium sulphate and evaporated to dryness. The dried lipids were dissolved in a known volume of hexane and stored in the refrigerator at 4°C until required for analysis.

3.18. Colorimetric assay of lipolytic activity

A standard graph was prepared using solutions of palmitic acid in the range of 80 to 400 mμ moles/ 5ml chloroform. The method of Duncombe (1963) modified by Mahadevan et al. (1969) was used with the following changes:

i) Shaking of the lipid solutions with copper reagent for 2 min. in a vortex shaker set at top speed was followed by hand shaking for 1 min. in place of shaking only for 30 sec. in the vortex shaker.

ii) Centrifugation at 1500 rpm for 5 min. was followed by the removal of the water layer with the aid of a suction pump.

Colorimetric estimation of free fatty acids was carried out using the graph shown in fig. 7. Appropriate standard solutions of palmitic acid were included with each series of determinations.

3.19. Assay of lipolytic activity using the pH-stat.

The activity of lyophilised material prepared from sonicated bacterial pellet towards emulsified triolein and tributyrin was followed by the titration of the acids liberated from the substrates with 1.9 mM-NaOH solution by using a pH-stat (type TTT11A, Radiometer, Copenhagen, Denmark).

A suitable quantity of enzyme solution was placed into the reaction vessel. 2 ml of substrate emulsion was added followed by distilled water to bring up the final volume to 4 ml. The pH of the assay mixture was adjusted to the desired value by adding 0.1 M-NaOH or 0.1 M-HCl as necessary. N_2 was continuously bubbled through the incubation mixture at

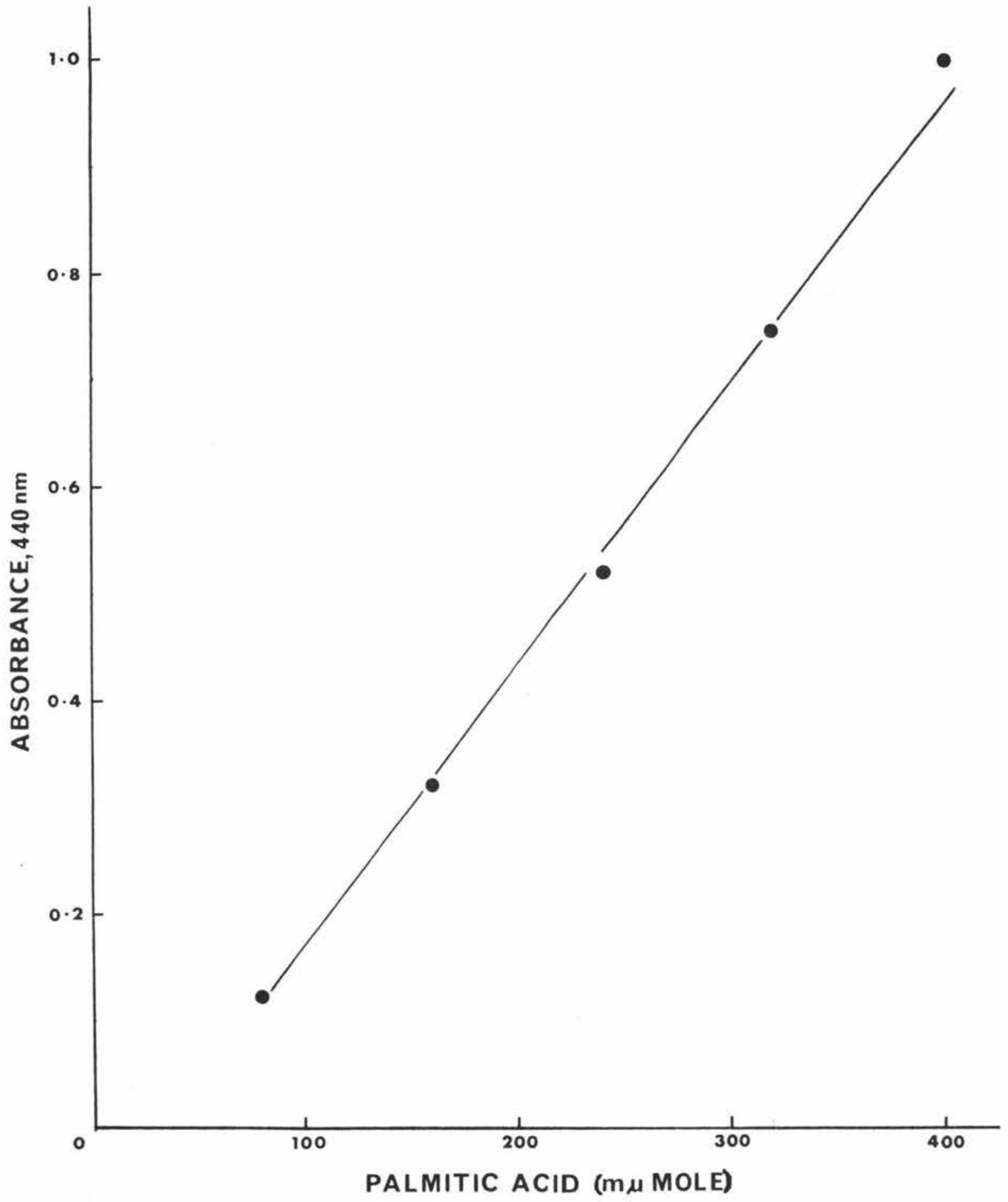

Figure 7. The relationship between extinction at 440 nm and free fatty acid.

$39^{\circ}C$ to maintain anaerobic conditions. The burette containing NaOH solution was fitted with a CO_2 absorbing tube (carbsorb, B.D.H.). Recording of the additions of NaOH solution were commenced 5 min. after mixing the substrate and enzyme solution. To check for non-enzymatic hydrolysis control experiments (without enzyme) were also run. The assays for one particular set of experiments were performed in rapid sequence in order to minimise the possible effects of enzyme instability.

3.20. Preparation of fatty acids from lipids

Saponification of purified monogalactosyldiglyceride (kindly supplied by Dr. J.C. Hawke) and methyl oleate were carried out by refluxing with an appropriate volume of 6% $(\frac{W}{V})$ KOH in ethanol on a sand bath for 1 hour 30 min. After removal of most of the ethanol in *vacuo*, the potassium salts were transferred to a separating funnel and acidified with 6 M - HCl. The fatty acids were extracted with diethylether. The ether extract was washed 3 times with water, evaporated to dryness in *vacuo* and stored at $-20^{\circ}C$ until required.

3.21. Methylation of fatty acids by diazomethane

Diazomethane was produced as an ether solution from N-methyl-N-nitroso-toluene-para-sulphonamide. Methylation was carried out at $0^{\circ}C$ for 20 - 30 min. The excess diazomethane was removed by heating the reaction at about $55^{\circ}C$. Purification of the methyl esters of fatty acids on thin-layer plate (sub-section 3.11.2) was followed by their storage at $-20^{\circ}C$ until required.

3.22. Gas-liquid chromatography

The analyses of the purity of methyl esters of oleic acids were

performed by using a Varian Aerograph (Model 1520) chromatograph on a 5' x $\frac{1}{4}$" stainless steel column with 12% diethylene glycol succinate (DEGS) on chromosorb W (60 - 80 mesh) at 165°C. A N_2 flow rate of 60 ml/min. was used. For the analyses of fatty acid composition of monogalactosyldiglyceride, the same chromatograph was used with a 6' x $\frac{1}{8}$" column of 12% DEGS on chromosorb W at a temperature of 150°C. Planimetry was used to estimate the proportions of methyl esters in the total sample. Each peak area was measured three times and the average value taken.

3.23. Determination of fatty acid composition of monogalactosyldiglyceride

Saponification of monogalactosyldiglyceride and subsequent extraction of fatty acids was carried out as described in section 3.20. Fatty acids were methylated with diazomethane (section 3.21), purified with thin-layer chromatography (subsection 3.11.2) and analysed by gas-liquid chromatography (section 3.22).

TABLE 5

Gas-liquid chromatographic analyses of the fatty acids of monogalactosyldiglyceride isolated from ryegrass

Fatty acids	Percentage composition
14:0	trace
16:0	2.89
Δ^9 16:1	trace
Δ^9 18:1	trace
$\Delta^{9,12}$ 18:2	3.46
$\Delta^{9,12,15}$ 18:3	84.42
Unidentified	9.24

3.24. Synthesis of radioactive triglyceride

3.24.1. Distillation of glycerol

Glycerol (M & B) was distilled under reduced pressure. The first and last 15% of the distillate were rejected (Mattson and Volpenhein, 1962).

3.24.2. Synthesis of glyceryl 1- palmitate

Glyceryl 1- palmitate was synthesised according to the method of Hartman (1960). The crystalline glyceryl 1- palmitate was checked for purity by thin-layer chromatography (sub-section 3.11.2).

3.24.3. Preparation of oleic acid

Purification of methyl esters of oleic acid from mixed fatty acid esters was carried out by fractional distillation under reduced pressure. A fractionating column (66.0 cm x 2.85 cm internal diameter) packed with glass helixes to a height of 56 cm was used. Fractions collected were checked by gas-liquid chromatography (section 3.22) for their purity. Oleic acid was derived from the methyl esters of oleic acid as described in section 3.20.

3.24.4. Preparation of oleyl chloride

Oxalyl chloride was used to prepare oleyl chloride from oleic acid according to Mattson and Volpenhein (1962).

3.24.5. Synthesis of glyceryl 1- palmitate - 2 - oleate and glyceryl 1- palmitate - 3 - oleate

Glyceryl 1- palmitate was acylated with oleyl chloride to get a mixture of glyceryl 1- palmitate - 2 - oleate and glyceryl 1- palmitate - 3 - oleate along with other reaction products. Mattson and

Volpenhein (1962).

3.24.6. Purification of diglyceride isomers

A mixture of free fatty acids, mono-, di- and triglycerides resulting from acylation of glyceryl 1- palmitate were chromatographed on acid-washed florisil which was prepared according to Carroll (1963) and activated at 110°C overnight before use. Eluting solvents were dried over anhydrous sodium sulphate and then dried over sodium wire for at least 24 hours. A standard glass Quickfit column (41.5 cm x 1.8 cm internal diameter) was packed with a slurry of 40 gm of florisil in hexane, which gave a height of about 35 cm. on settling. The crude mixture of free fatty acids, mono-, di- and triglycerides was dissolved in hexane:diethylether (19:1, $^{V}/V$) and was applied to the column using a Pasteur pipette. The flow rate of the elution solvent was about 200 - 300 ml /hour. Effluents were collected in 15 ml fractions and thin-layer chromatography was used to identify the eluted fractions. A mixture of 1,2 - diglycerides and 1,3 - diglycerides containing a slight amount of free fatty acid was obtained from the fractions containing 10 to 15% ether in hexane. The free fatty acids were removed from the diglyceride by washing with 5% sodium carbonate solution and washed with distilled water, until neutralised. The mixture of 1,2 - and 1,3 - diglycerides was applied to the second column in hexane:diethylether (19:1, $^{V}/V$). 1,3 - diglyceride was obtained from fraction no. 8 to 19 with 7% ether in hexane as eluting solvent but mixed isomers were obtained from fraction nos. 20 to 43 with a gradual increase in 1,2 - diglyceride. This 1,2 - diglyceride was purified by thin-layer chromatography.

3.24.7. Synthesis of triglycerides:

Linolenyl chloride prepared from linolenic acid and oxalyl chloride according to Mattson and Volpenhein (1962) was equilibriated with 1 - ^{14}C - linolenic acid in chloroform for 2 hours at room temperature (Bergstrom and Krabisch, 1963). The diglyceride isomers were acylated with the resulting 1 - ^{14}C - linolenyl chloride using a 50% molar excess of 1 - ^{14}C - linolenyl chloride with a reaction time of 72 hours at room temperature (Mattson and Volpenhein, 1962). The crude triglyceride isomers were purified on an acid-washed florisil column and stored at -20°C.

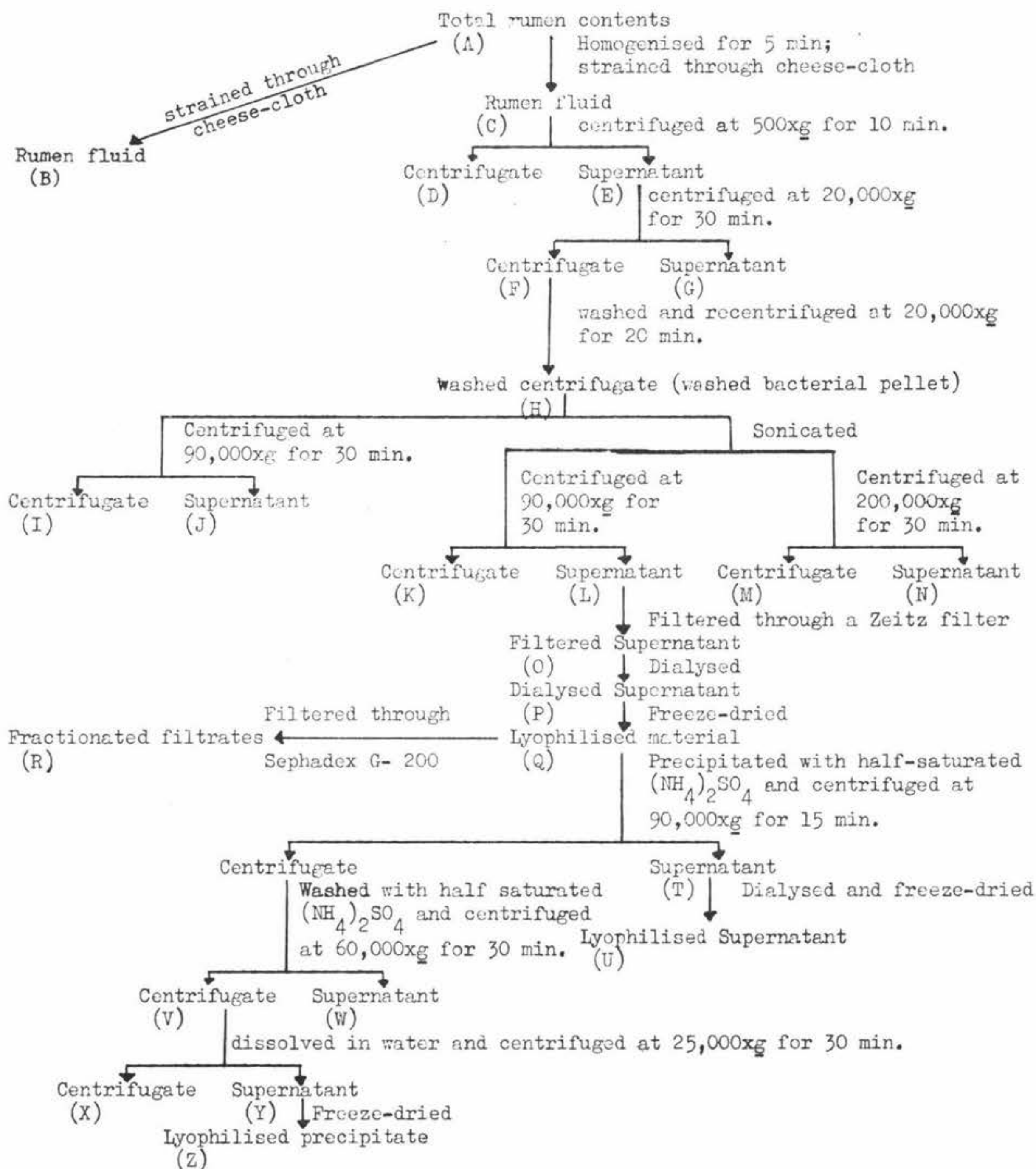

FIGURE 8. Steps in the purification of rumen lipase.

CHAPTER 4

RESULTS

4.1. Effect of homogenisation of total rumen contents and the lipolytic activity of the 20,000xg centrifugate

An outline of the procedures adopted to isolate lipolytic fractions from the rumen contents is given in fig. 8. To study the effect of homogenisation on the lipolytic activity of rumen fluid the latter was prepared from total rumen contents with or without prior homogenisation. When 5ml aliquots of each of these rumen fluids (Frac. B and C in fig. 8) were incubated with ^{14}C- triolein, it was found that the lipase activity of the rumen fluid prepared from homogenised rumen contents was approximately twice that of the unhomogenised rumen fluid during a 90 min. incubation.

TABLE 6

Lipolysis of ^{14}C- triolein by rumen fluid before and after homogenisation and by a 20,000xg centrifugate prepared from homogenised rumen fluid. Incubation conditions: 5ml of each fraction; glyceryl tri (oleate-1-^{14}C) (2.2×10^6 dpm) in 20μl hexane; 90 min. incubation at 39°C in presence of N_2 with constant shaking. Radioactive products of incubation were measured by radiochromatogram scans.

		Radioactivity in each lipid fraction (%).			
Fractions	Description	Triglyceride	Fatty acid	Diglyceride	Monoglyceride
B	Rumen fluid (unhomogenised)	55.0	25.4	7.0	12.6
C	Rumen fluid (homogenised)	44.9	48.6	3.4	3.1
H	20,000xg centrifugate prepared from fraction C	37.5	54.2	5.4	2.9

Furthermore, less substrate remained as mono- and diglyceride in the homogenised preparation (3.1 and 3.4% respectively compared with 7.0 and 12.6% in the unhomogenised preparation). When fraction C was centrifuged at 20,000xg approximately 54% of the ^{14}C- triglyceride fatty acids were removed by the centrifugate (Frac. H) under the same incubation condition (Table 6).

4.2. Effect of sonication on the lipolytic activity of the washed bacterial fraction

A two min. sonication of the bacterial pellet (Frac. H in fig. 8) resulted in a slightly increased lipase activity in the 90,000xg supernatant fraction (Frac. L) and a reduced lipolytic activity in the 90,000xg centrifugate (Fr.K)

TABLE 7

Lipolysis of ^{14}C- triolein by fractions prepared from the bacterial pellet and sonicated bacterial pellet.

Reaction mixture: 5 ml of each fraction; glyceryl tri (oleate-1-^{14}C) (1.1 x 10^6 dpm) in 10 μl hexane; 90 min. incubation at 39°C in presence of N_2 with constant shaking.

Radioactive products of incubation were measured by radiochromatogram scans.

		Radioactivity in each lipid component (%)			
Fractions	Descriptions	Triglyceride	Fatty acid	Diglyceride	Monoglyceride
I	Control 90,000xg centrifugate	12.8	77.0	2.8	7.4
J	Control 90,000xg supernatant	45.6	31.5	13.3	9.6
K	Sonicated 90,000xg centrifugate	27.8	59.9	7.0	5.3
L	Sonicated 90,000xg supernatant	39.5	31.1	16.8	12.6

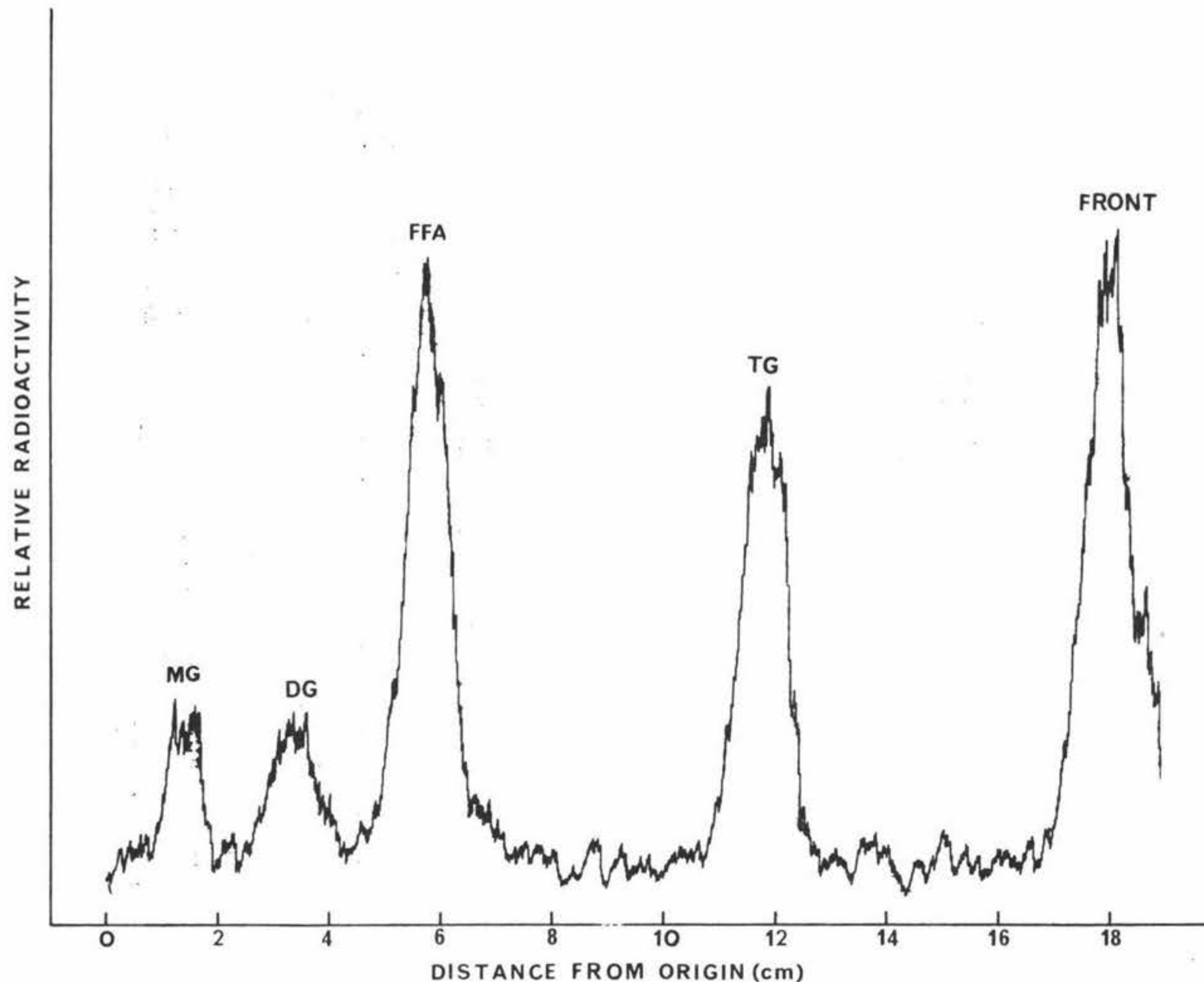

Figure 9. Radiochromatogram scan of the products of hydrolysis of ^{14}C- triolein separated by thin-layer chromatography.
Conditions of incubation: 10 μl hexane containing glyceryl tri (oleate-1-^{14}C) (1.1×10^6 dpm) was added to 5 ml of 90,000xg supernatant prepared from bacterial pellet without sonication. Incubation was carried out for 5 hours at 39°C in presence of N_2 with constant shaking.
Key: FFA, MG, DG and TG represents free fatty acids, mono-, di- and triglycerides respectively.

compared with the nonsonicated control. The results in table 7 show that the percentage recovery of the free fatty acids from both the supernatant fractions (Frac. J and L) is almost equal but radioactivity in both mono- and diglycerides was slightly higher in the sonicated supernatant resulting in a lower percentage of triglyceride remaining unhydrolysed.

The lipolytic activity of the two supernatant fractions (Frac. J and L in fig. 8) was investigated more fully by following the release of the fatty acids, mono- and diglycerides from ^{14}C- triolein with time of incubation. Approximately 42% of the radioactivity of the ^{14}C- triolein was recovered in the free fatty acid fraction after 5 hours incubation with the 90,000xg supernatant (Frac. J in fig. 8) prepared without sonication. An almost linear increase in the formation of fatty acids with time was apparent. There was a corresponding decrease in the triglyceride over the same time interval. The formation of monoglyceride reached a maximum of 12.3% after 3 hours. 11.8% diglyceride was obtained after 1 hour and the level remained fairly constant for the remainder of the incubation (Fig. 10). A control incubation consisting of 5 ml 0.06 M phosphate buffer with added ^{14}C- substrate showed no lipolytic activity.

Corresponding incubations using the 90,000xg supernatant (Frac. L in fig. 8) prepared from sonicated bacteria gave 15.4% free fatty acids after 1 hour and double this value after 2 hours. No further increase occurred after three hours. However, the percentage of triglyceride continued to decrease over the three hours period of incubation due to the continued formation of mono- and diglycerides (Fig. 11).

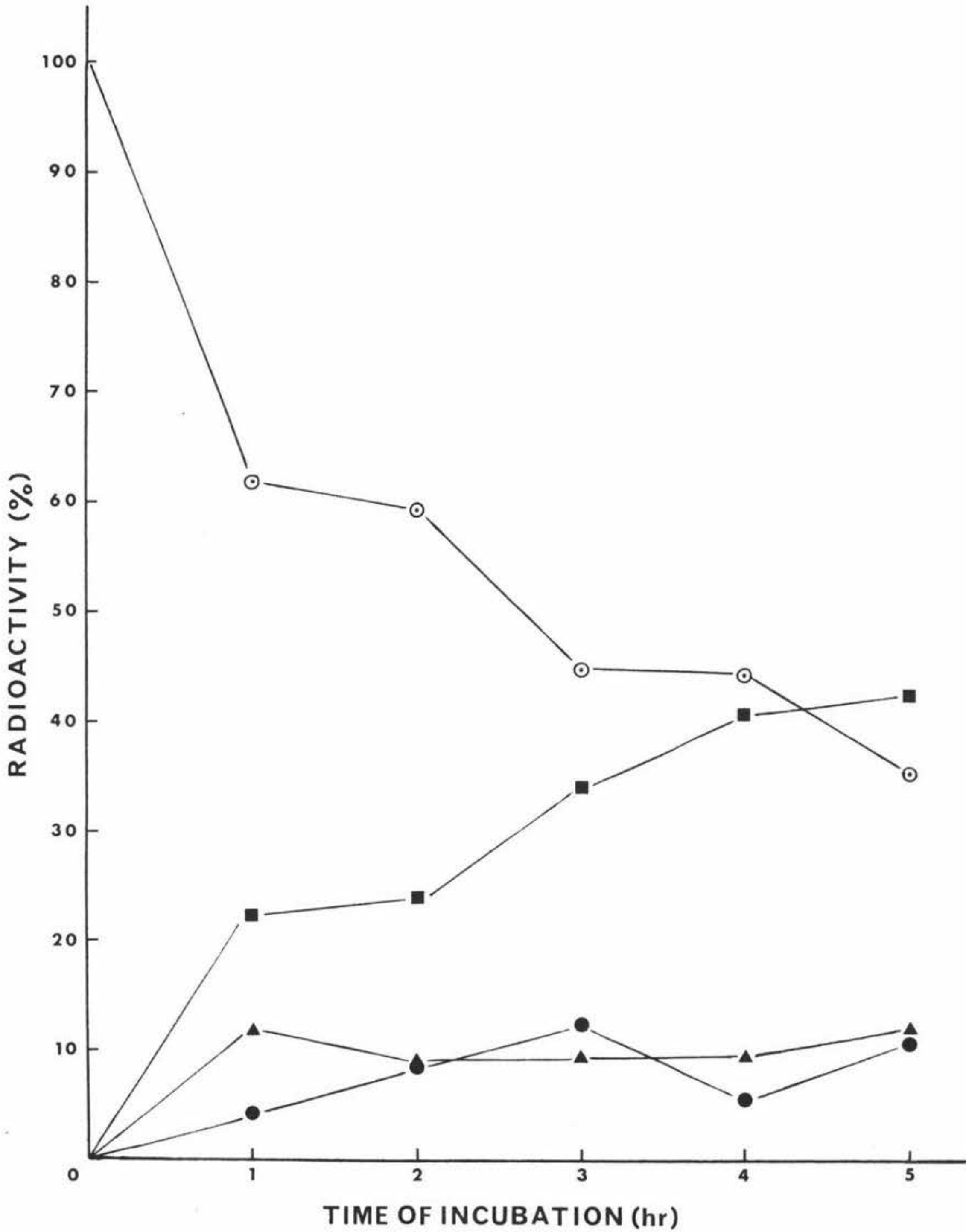

Figure 10. Lipolytic activity of 90,000xg supernatant prepared without sonication of bacterial pellet.
Key: ■, ● , ▲, and ⊙ represents free fatty acids, mono-, di- and tri-glycerides respectively.
Conditions of incubation: For each incubation 10 μl hexane containing glyceryl tri (oleate-1-^{14}C) (1.1 x 10^6 dpm) was added to 5 ml of supernatant solution and incubated at 39°C in presence of N_2 with constant shaking. Radioactive products of incubation were measured by radiochromatogram scans.

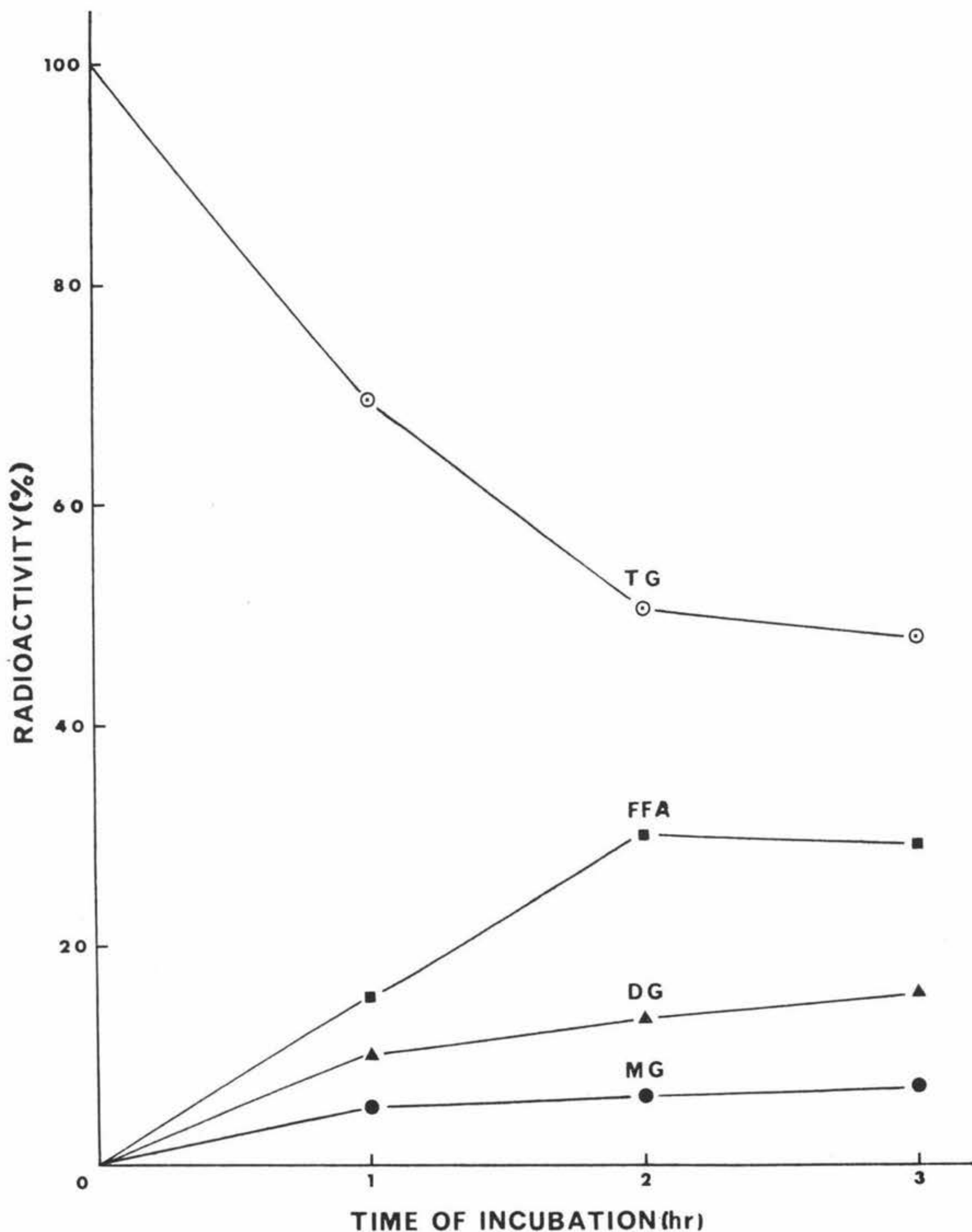

Figure 11. Hydrolysis of ^{14}C- triolein by 90,000xg supernatant prepared from sonicated bacterial pellet.
Key: FFA, MG, DG and TG represent free fatty acids, mono-, di- and tri-glycerides respectively.
Reaction mixture: 5 ml of supernatant solution was added to 20 μl hexane containing glyceryl tri (oleate-1-^{14}C) (2.2 x 10^6 dpm) and incubated at 39°C with constant shaking under N_2.
Radioactive products of incubation were measured by radiochromatogram scans.

4.3 Lipolytic activities of 90,000xg centrifugate and supernatant and 200,000xg centrifugate and supernatant prepared from a sonicated bacterial pellet

Enzymatic activity remained in the 200,000xg supernatant fraction (Frac. N) although this was lower than the 90,000xg supernatant (Frac. L). As expected, the 200,000xg centrifugate (Frac. M) possessed a greater lipolytic activity than 90,000xg centrifugate (Frac. K) - 67.8% free fatty acids being formed in two hours compared with 60% (Table 8).

TABLE 8

Lipolytic activities of centrifugates and supernatants obtained after centrifugation of sonicated bacterial pellet at 90,000xg and 200,000xg.

Reaction mixture: 10 µl hexane containing glyceryl tri (oleate-1-^{14}C) (1.1 x 10^6 dpm); 5 ml of each fraction; 2 hours incubation at 39°C in presence of N_2 with constant shaking.

Radioactivity of lipid was measured after separation by thin-layer chromatography and removal of zones.

		Radioactivity in each lipid component (%)			
Fractions	Description	Triglyceride	Fatty acid	Diglyceride	Monoglyceride
K	Sonicated 90,000xg centrifugate	32.1	60.0	5.3	2.6
L	Sonicated 90,000xg supernatant	41.3	45.6	8.6	4.5
M	Sonicated 200,000xg centrifugate	24.5	67.8	4.8	2.9
N	Sonicated 200,000xg supernatant	50.4	33.9	9.0	6.7

Conversely, lipolytic activity of the two corresponding supernatants was in the reverse order - 45.6% free fatty acids was formed by the 90,000xg

supernatant and 33.9% free fatty acids by the 200,000xg supernatant. The formation of both mono- and diglycerides are higher from the supernatant fractions than from the corresponding centrifugate fractions (Table 8).

4.4. Stability of soluble lipolytic fractions

A comparison of the stability of lipase activity of sonicated 90,000xg supernatant (Frac. L in fig. 8), dialysed supernatant (Frac. P in fig. 8) and lyophilised material (Frac. Q in fig. 8) were made by storing them at 4°C for 5, 4 and 4 days (approximately) respectively.

TABLE 9

Lipolysis of ^{14}C- triolein by soluble lipolytic fractions (after storing at 4°C for approximately 4-5 days) prepared from sonicated bacterial pellet.

Reaction mixture: 2 µl hexane containing glyceryl tri (oleate-1-^{14}C) (2.49 x 10^6 dpm); 2 ml from each of 90,000xg supernatant (Frac. L) and dialysed supernatant (Frac. P); 7.5 mg lyophilised material (Frac. Q) (equivalent to 2 ml of dialysed supernatant) dissolved in 2 ml 0.06 M phosphate buffer; 2 hours incubation at 39°C in presence of nitrogen with constant shaking.

Radioactivity of lipid was measured after separation by thin-layer chromatography and removal of zones.

		Radioactivity in each lipid component (%)			
Fractions	Description	Triglyceride	Fatty acid	Diglyceride	Monoglyceride
L	Sonicated 90,000xg supernatant	69.1	20.5	7.1	3.3
P	Dialysed supernatant	70.2	20.5	6.3	3.0
Q	Lyophilised material	43.4	39.7	11.9	5.0

39.7% of the ^{14}C- triolein was recovered in the free fatty acid fraction from the lyophilised material (Frac. Q) whereas the formation of free fatty acid was approximately half this value without freeze-drying in both

sonicated and dialysed supernatant (Frac. L and P). In the 3 fractions tested, the formation of mono- and diglycerides was higher from the lyophilised material (Table 9). It would seem from Table 9, that lipases are more stable in the lyophilised condition than in an aqueous solution with or without dialysis.

4.5. Lipolytic activity of lyophilised material

The formation of ^{14}C- labelled hydrolysis products from ^{14}C- triolein by lyophilised material (Frac. Q in fig 8) was determined by individual incubation of 15 to 75 min. under the conditions described in fig. 12. 7.6% of the radioactivity was recovered in the free fatty acid fraction after a 15 min. incubation and the level reached to a maximum of 19% after a 75 min. incubation. Over this period of incubation an approximately linear formation of free fatty acids was observed. No significant difference in the formation of mono- and diglyceride was apparent at the different incubation times (Fig. 12).

4.6 Fractionation of lyophilised material on a column of Sephadex G-200

By applying 20 mg protein (35.5 mg lyophilised material - frac. Q in fig. 8) to a column of Sephadex G-200 (as described in sub-section 3.9.8.), the elution pattern indicated the appearance of 3 protein peaks of varying sizes (Fig. 13).

It is observed in Table 10 that lipolytic activity was not concentrated in a single peak. However, there is a possibility that lipases are to some extent concentrated in the region of fraction numbers 33, 36, 40 and 42. In control incubation over two hours using 1 ml of distilled water in place of the column eluant it appeared that 4.7% of the radio-

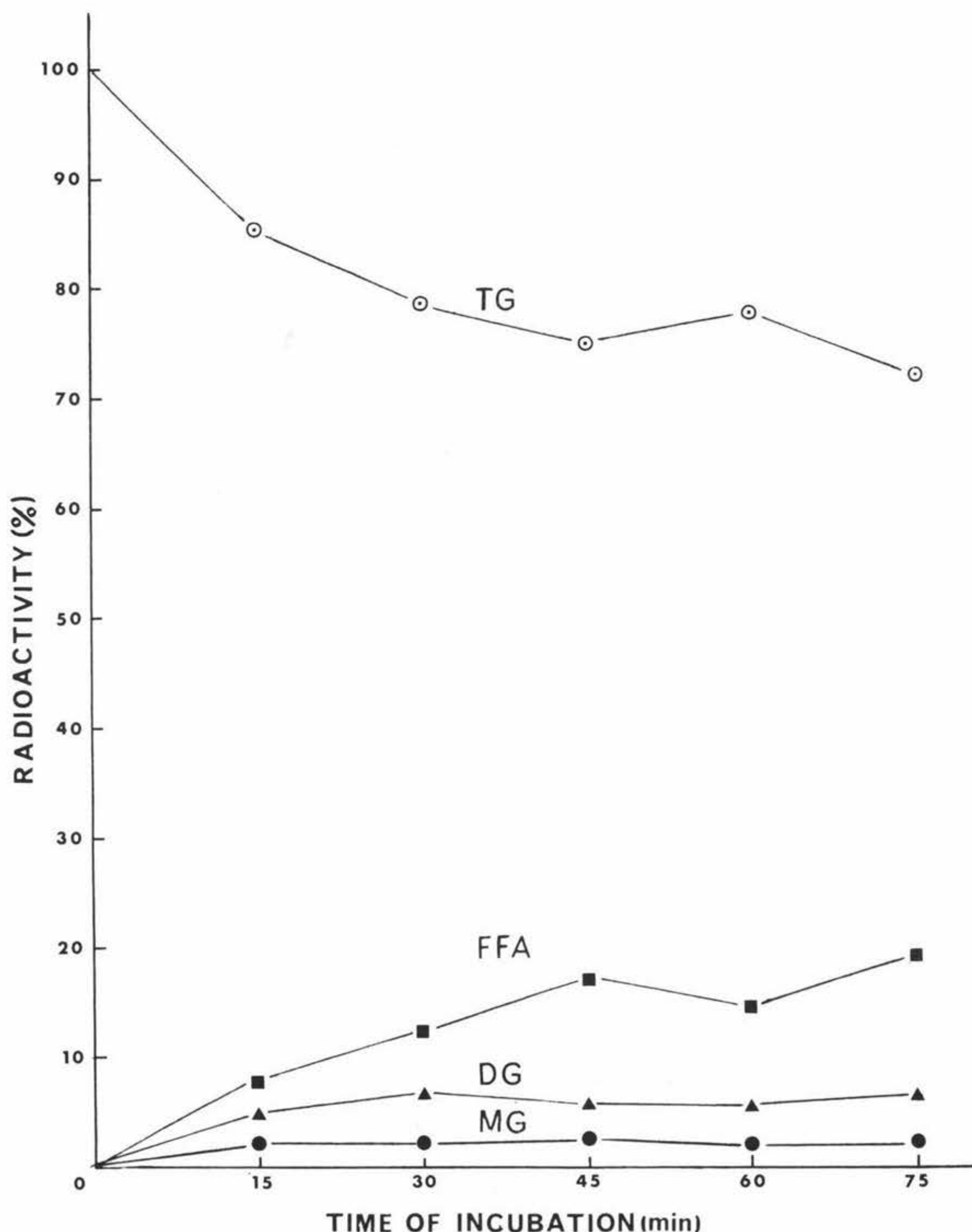

Figure 12. Hydrolysis of ^{14}C- triolein by lyophilised material as prepared from 90,000xg supernatant of sonicated bacterial pellet.
Key: FFA, MG, DG and TG represent free fatty acid, mono-, di- and tri-glycerides respectively.
Incubation conditions: 1 ml of 0.06M phosphate buffer containing 3 mg of lyophilised material (1.8 mg protein); glyceryl tri (oleate-1-^{14}C) (2.498 x 10^6 dpm) in 2 μl hexane; incubation was carried out in presence of nitrogen at 39°C with constant shaking.
Radioactivity of lipid was measured after separation by thin-layer chromatography and removal of zones.

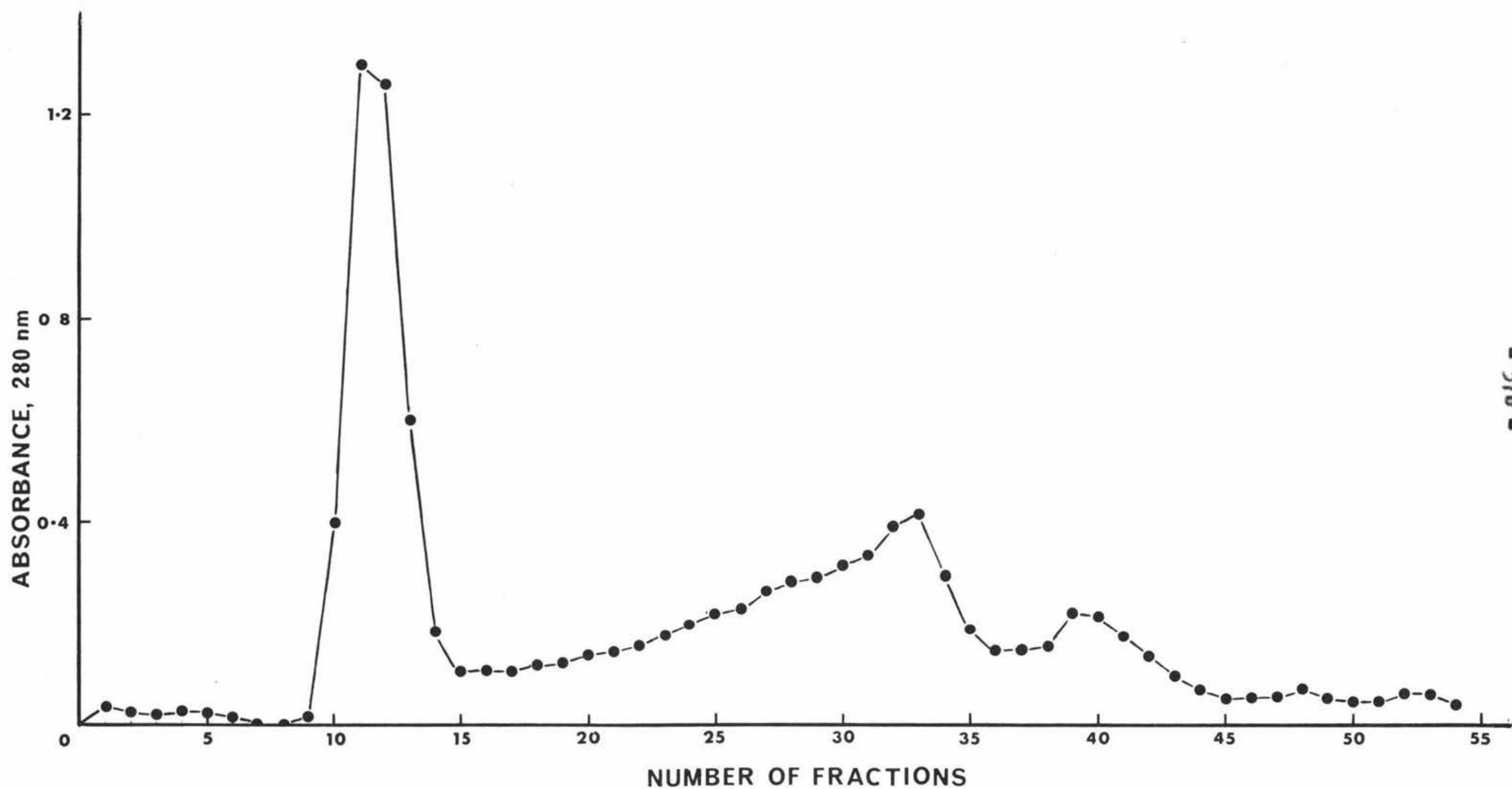

Figure 13. Fractionation of lyophilised material (35.5 mg containing 20 mg protein dissolved in 1.5 ml of 0.15 M-NaCl solution) prepared from sonicated bacterial pellet on Sephadex G- 200. Column, 71 cm x 1.8 cm internal diameter; eluant, 0.15 M-NaCl; fractions, approximately 4 ml; flow rate 12 ml to 18 ml / hour; protein determined at 280 nm.

activity was incorporated into the free fatty acid fraction (Table 10).

TABLE 10

Lipolytic activity of fractions obtained from a Sephadex G-200 column.

Reaction mixture: 0.5 μl of hexane containing glyceryl tri (oleate-1-^{14}C) (0.6425 x 10^6 dpm); 1.0 or 0.2 ml of each fraction; 2 hours incubation at 39°C with constant shaking.

Radioactivity of lipid was measured after separation by thin-layer chromatography and removal of zones.

			% of radioactivity in each lipid component			
Fraction number	Absorbance at 280 nm	Quantity in ml	Triglyceride	Fatty acid	Diglyceride	Mono-glyceride
11	1.3	1.0	76.5	11.6	7.7	4.2
14	0.185	0.2	74.0	11.7	9.5	4.8
19	0.12	0.2	73.8	10.4	11.9	3.9
24	0.196	0.2	79.2	7.8	11.6	1.4
29	0.292	0.2	80.3	9.6	8.6	1.5
33	0.41	1.0	64.3	19.1	11.6	5.0
36	0.142	0.2	67.6	12.3	16.2	3.9
40	0.215	1.0	66.2	11.9	12.3	9.6
42	0.133	0.2	64.8	11.7	20.2	3.3
48	0.071	0.2	84.4	7.9	6.3	1.4
Blank		1 ml distilled water	91.1	4.7	3.8	0.4

4.7 Lipolytic activity of lyophilised half-saturated $(NH_4)_2SO_4$ precipitate

Details of the $(NH_4)_2SO_4$ fractionation have been described in sub-section 3.9.9. A suitable aliquot of peanut oil emulsion containing approximately 10 mg of the oil with the amount of each fraction (Frac. U and Z in fig. 8) equivalent to 10 mg of protein was dissolved in 5 ml of 0.06 M phosphate buffer. The incubation was carried out for 4 hours under

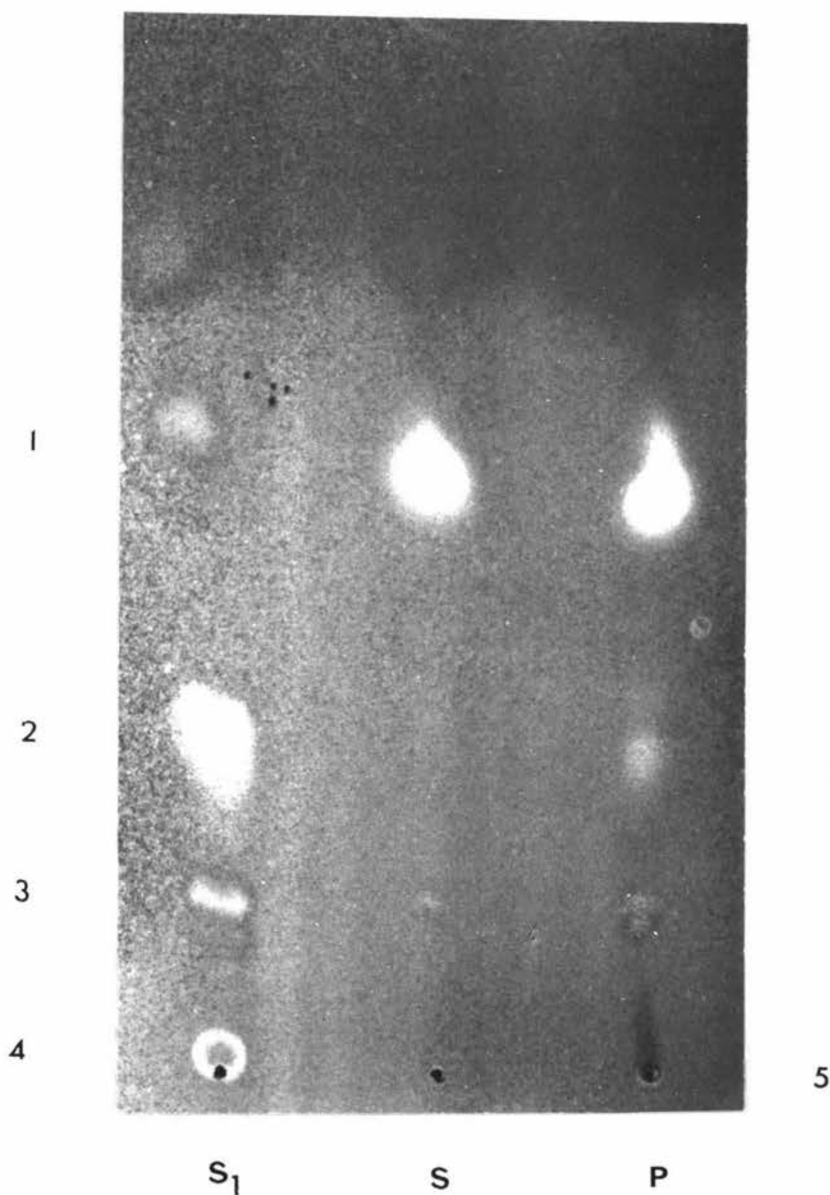

Fig. 14. Demonstration of lipolytic activity of half-saturated $(NH_4)_2SO_4$ precipitate as prepared from lyophilised material.
Key: 1, triglyceride; 2, free fatty acid ; 3, diglyceride; 4, monoglyceride; 5, origin; S_1, standard lipids; S&P, the lipids extracted from the supernatant and precipitate respectively after incubation of emulsified peanut oil for 4 hours (For details of incubation conditions see section 4.7.).

nitrogen with constant shaking. Approximately equivalent aliquots of the lipids extracted from the incubation products were separated on thin layers of silica gel, as shown in fig. 14. It was observed that the $(NH_4)_2SO_4$ precipitate possessed lipolytic activity whereas the supernatant fraction appeared to be inactive. The trace of diglyceride, which appeared in the reaction products of both the precipitate and the supernatant, was due to an impurity in the peanut oil (Fig. 14).

4.8. Effect of pH on enzyme activity

The effect of pH on the lipolytic activity of lyophilised material (Frac. Q in fig. 8) towards emulsified triolein was investigated by the use of a radiometer titrator as described in section 3.19. No activity was found at pH 6.5 (Fig. 15). Nonenzymatic hydrolysis of the substrate was observed only at the alkaline side of neutrality (Fig. 16). When corrections for nonenzymatic hydrolysis were made, it is apparent that as the pH was raised, the activity of lipase increased to reach a maximal value between pH 8.0 and 8.5. Further increases in pH decreased the activity (Fig. 17).

When a similar study was carried out using emulsified tributyrin as the substrate, the rate of lipolysis increased above pH 4.5, reaching a maximum value at pH 7.0. Further increase in pH up to pH 9.5, decreased the lipolytic activity (Fig. 20). Again corrections were made for some nonenzymatic breakdown of emulsified tributyrin which occurred on the alkaline side of neutrality and in addition at the lower pH values (Fig. 19).

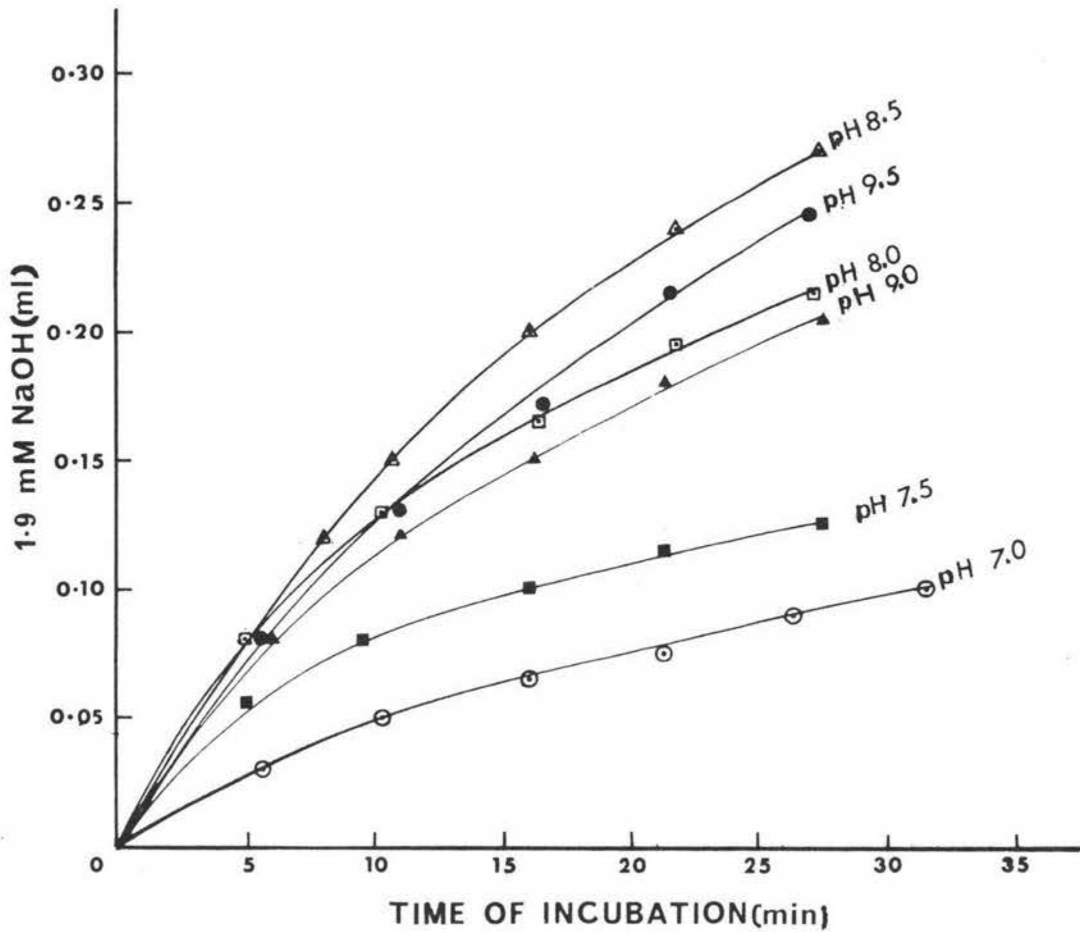

Figure 15. Effect of pH on the rate of hydrolysis of emulsified triolein by lyophilised material as prepared from 90,000xg supernatant of sonicated bacterial pellet.
Reaction mixture: Assays were performed by titration of the liberated acid after mixing 2 ml of lyophilised material (10.4 mg protein) with 2 ml triolein emulsion (triolein:water = 1:100, $^V/V$) at 39°C under N_2 with continuous mixing of the assay mixture.

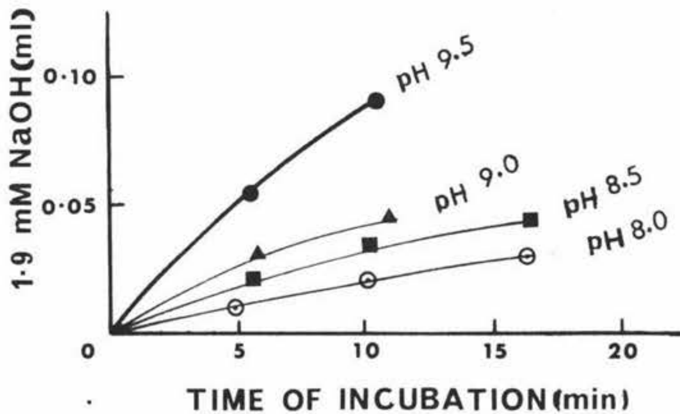

Figure 16. Effect of pH on the rate of non-enzymatic hydrolysis of emulsified triolein.
Reaction mixture: Assays were performed by titration of the liberated acid after mixing 2 ml of water with 2 ml of triolein emulsion (triolein:water = 1:100, $^V/V$) at 39°C under N_2 with continuous mixing of the assay mixture.

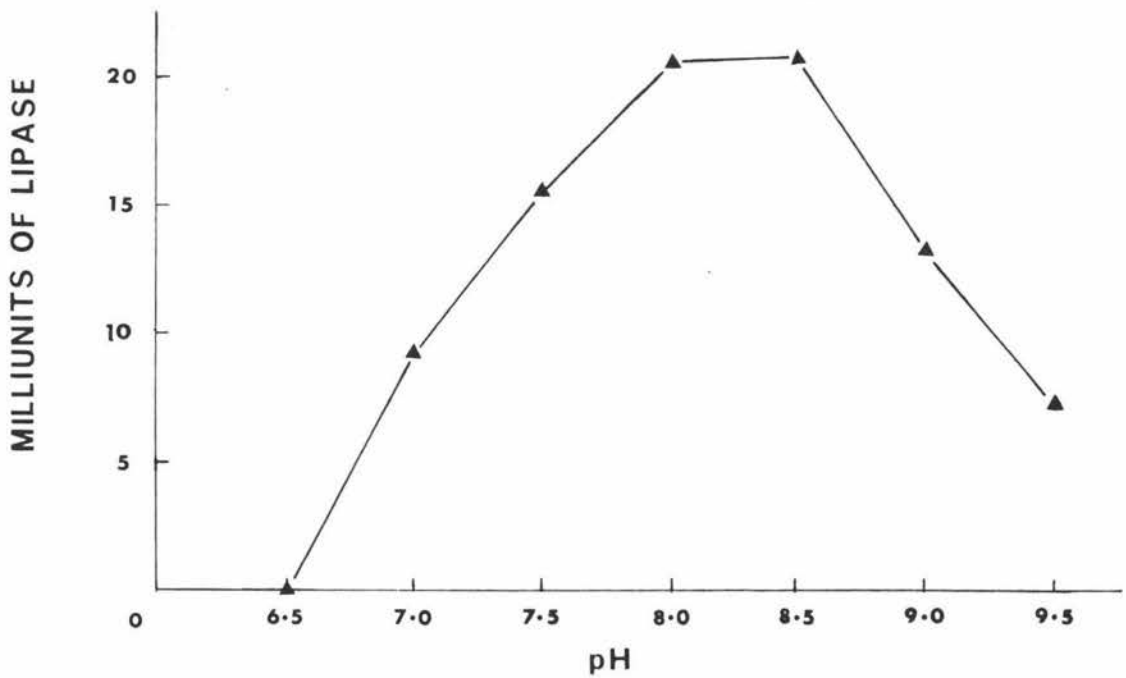

Figure 17. pH activity relationship for the hydrolysis of emulsified triolein by lyophilised lipase prepared from 90,000x$\underline{g}$ supernatant of sonicated bacterial pellet.
Titration values obtained from figure 15 at 10 min. incubation after correction for blank titre.

1.9 mM NaOH (ml)

pH 7.0
pH 6.5
pH 8.0
pH 6.0
pH 7.5
pH 9.5
pH 9.0
pH 5.5
pH 5.0
pH 4.5

TIME OF INCUBATION (min)

Figure 18. Effect of pH on the rate of hydrolysis of emulsified tributyrin by lyophilised material as prepared from 90,000xg supernatant of sonicated bacterial pellet. Reaction mixture: 0.5 ml of lyophilised material (2.6 mg protein) was mixed with 2 ml of tributyrin emulsion (tributyrin:water = 1:100, V/V). Water was added to give a final volume of 4 ml. Assays were performed by titration at 39°C under N_2 with constant mixing of the assay mixture.

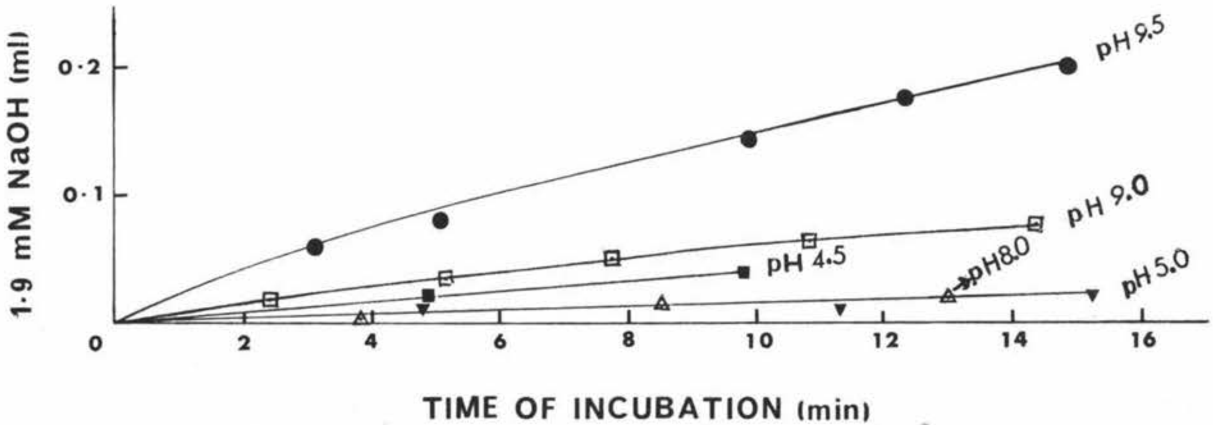

Figure 19. Effect of pH on the non-enzymatic hydrolysis of emulsified tributyrin. Reaction mixture: Assays were carried out by titration of the liberated acid after mixing 2 ml of tributyrin emulsion (tributyrin:water = 1:100, V/V) with 2 ml of water at 39°C in presence of nitrogen with constant mixing of the assay mixture.

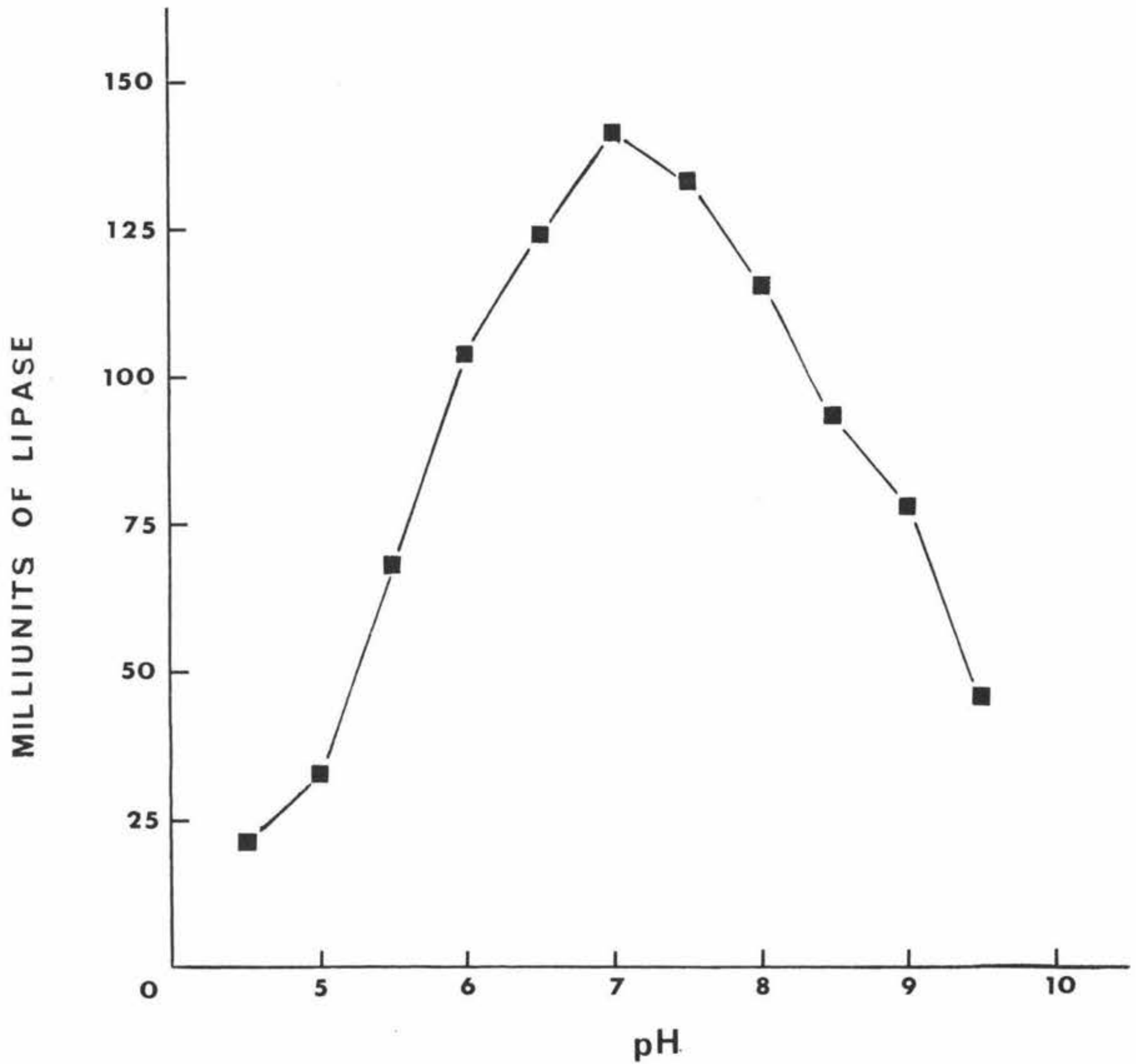

Figure 20. pH-activity relationship for the hydrolysis of emulsified tributyrin by lyophilised material prepared from 90,000xg supernatant of sonicated bacterial pellet.
Titration values obtained from fig. 18 at 5 min. incubation after correction for blank titre.

4.9 Relationship between enzyme concentration and reaction velocity

The effect of different concentrations of lyophilised material (Frac. Q in fig. 8) on the rate of fatty acid production from emulsified tributyrin was studied at pH 7.0 with aid of a radiometer titrator as described in section 3.19. A gradual increase in lipase activity was observed with the increase in enzyme concentration. The slopes of the straight lines are approximately proportional to the amount of the enzyme (Fig. 21). However, an approximately linear relationship was obtained when the amount of protein used in each assay was drawn against the milliunits of lipase (Fig. 22).

4.10. Counts of tributyrin - hydrolysing bacteria

Counts of tributyrin - splitting bacteria from fresh rumen fluid and bacterial pellets prepared from the rumen contents (sub-section 3.9.3.) were made. A few colonies with clear zones around them were noticed after 21 hours incubation. Most of the tributyrin - splitting colonies appeared between 20 and 40 hours after incubation began and produced macroscopic zones of clearing on tributyrin layer. In some cases zones of clearing appeared before visible colonies had developed. After 48 hours incubation it was possible to make a total viable count and a total count of tributyrin - splitting colonies. A 10^{-6} dilution of rumen fluid contained 148 colonies, of which 68 (46%), were surrounded by zones of clearing. This represents a total count of 1.4×10^{7}/ml of rumen fluid. When the organisms were derived from the bacterial fraction of rumen content, a 10^{-6} dilution contained 107 colonies of which 52 (48.6%) were surrounded by clear zones. The total count was 1.0×10^{7}/ml of rumen fluid. On prolonged incubation the colonies and the clear zones became larger and the zones tended to become confluent. Colonies which had not

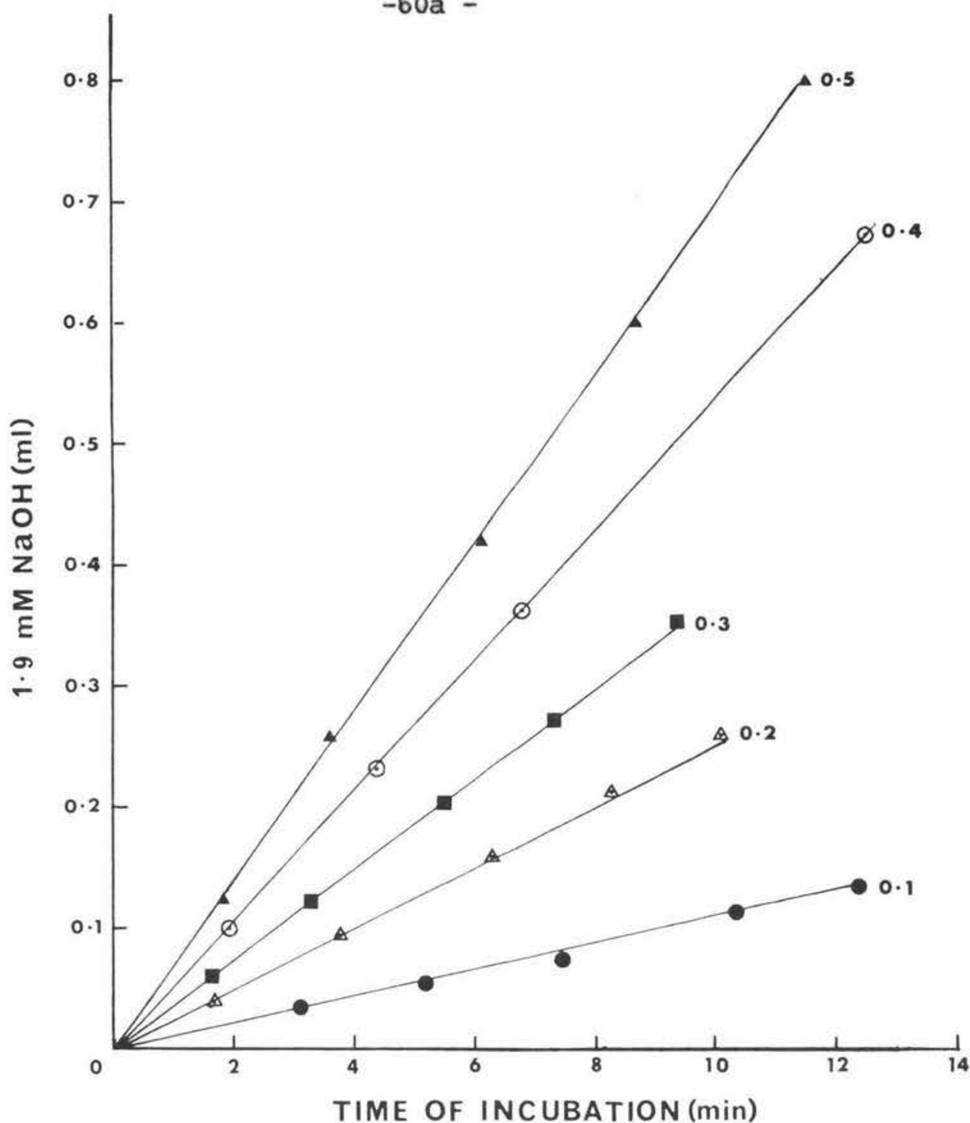

Figure 21. Hydrolysis of tributyrin by lyophilised material. The figures corresponding to each curve represents the relative amounts of lipase solution used in each assay.
Reaction conditions: Assays were carried out by mixing 2 ml of tributyrin emulsion (tributyrin:water = 1:100, V/V) 0.1, 0.2, 0.3, 0.4 or 0.5 ml (containing 0.52, 1.04, 1.56, 2.08 or 2.6 mg of protein respectively) of enzyme solution made to 4 ml with water.

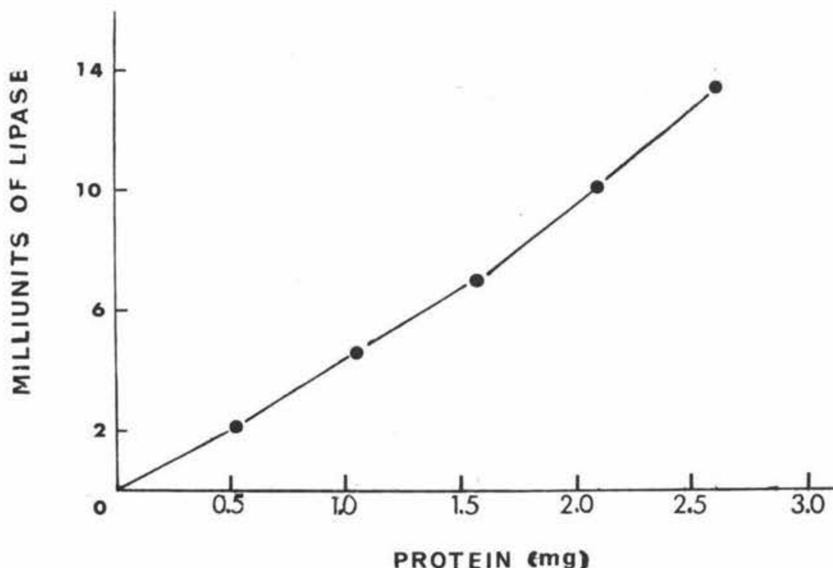

Figure 22. The relationship between lipase activity and the amount of protein in enzyme solution.
Titration values obtained from fig. 21 at 5 min. incubation.

produced zones of clearing within 48 hours usually failed to do so on prolonged incubation.

4.11. Isolation of tributyrin-splitting bacteria in pure culture

Three different types of tributyrin - hydrolysing colonies were isolated. The isolated colonies were selected because of their rapidity with which they produced zones of clearing in comparison with other tributyrin-splitting colonies. Table 11 shows the morphological characteristics of the 3 different types of colonies. The highly branching nature of strain 1 helped to distinguish it from others. This was observed on one occasion that strain 1 seemed to represent about 10% of the total tributyrin-splitting bacteria in the rumen.

4.12. Effect of time on the production of area of clearing of tributyrin by the tributyrin - splitting bacteria

A relationship between the area of clearing of 0.2% $(\frac{V}{V})$ emulsified tributyrin with the time of incubation by the 3 isolated tributyrin - hydrolysing bacteria was investigated. The double layer technique described in section 3.15. was used. Starting from the beginning of incubation, the diameter of the zones of clearing was recorded after every 24 hours. It is apparent from figures 27, 28 and 29, that the increase in the area of lipolysis is not linear with respect to time. It is also evident that strain 1 is most efficient in the hydrolysis of tributyrin and that the activity of the other strains decreased in the following order; strain 1 , strain 3 , strain 2.

TABLE 11

Cultural and morphological characteristics of bacteria

Strain	Colour	Shape	Edge	Density	Any distinctive feature	Gram staining study	
						Gram-negative or Gram-positive	Shape
1	white	Filamentous	Filamentous	Opaque at the centre	Highly branched	Gram-negative	Rod
2	white	circular	undulate	Opaque centre surrounded by inner transferent and outer translucent edges	Viscous	Gram-negative	Rod
3	white	circular	slightly undulate	Transferent with a slight translucent at the centre	-	Gram-negative	Rod

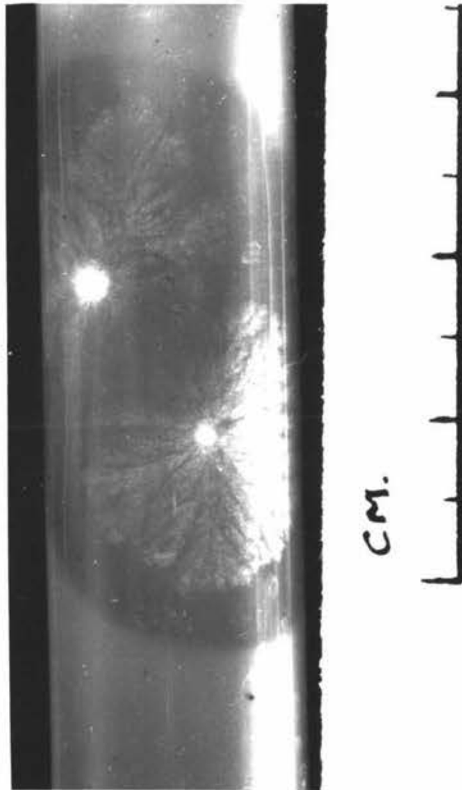

Fig. 23. Colony characteristics of Strain 1 growing in media A with the area of clearing of 0.2% tributyrin.

Electron micrographs of tributyrin-splitting bacteria negatively stained with 1% phosphotungstic acid.

Fig. 24: Strain 1 - magnification, 19,000 x

Fig. 24a: Strain 1 - magnification, 19,000 x

Fig. 25: Strain 2 - magnification, 52,000 x

Fig. 25a: Strain 2 - magnification, 68,000 x

Fig. 26: Strain 3 - magnification, 19,000 x

Fig. 26a: Strain 3 - magnification, 62,000 x

Fig. 24

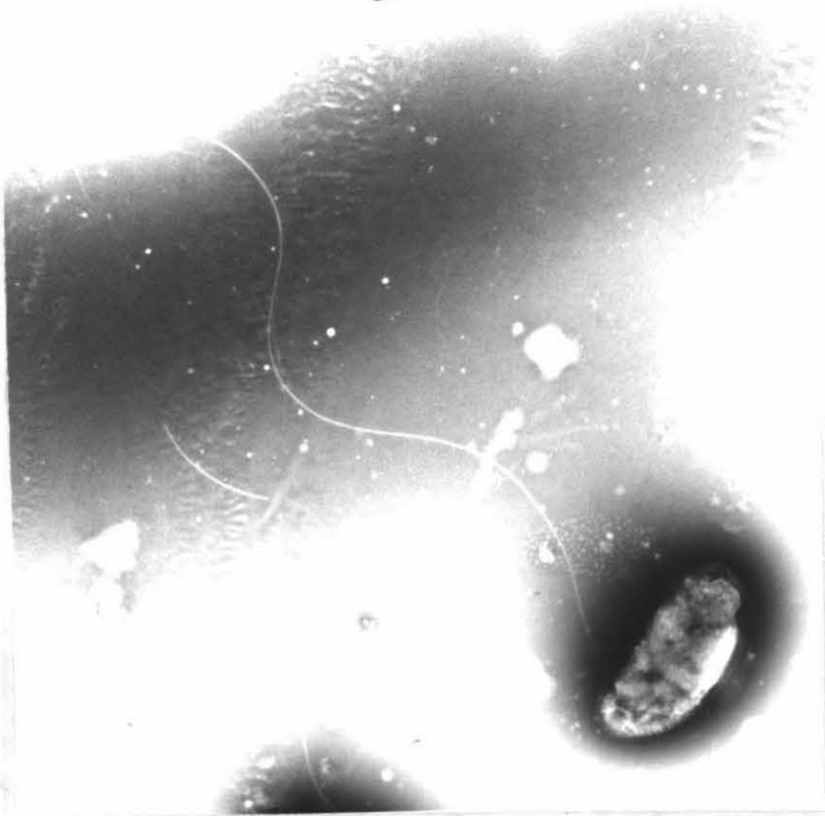

Fig. 24a

Fig. 25

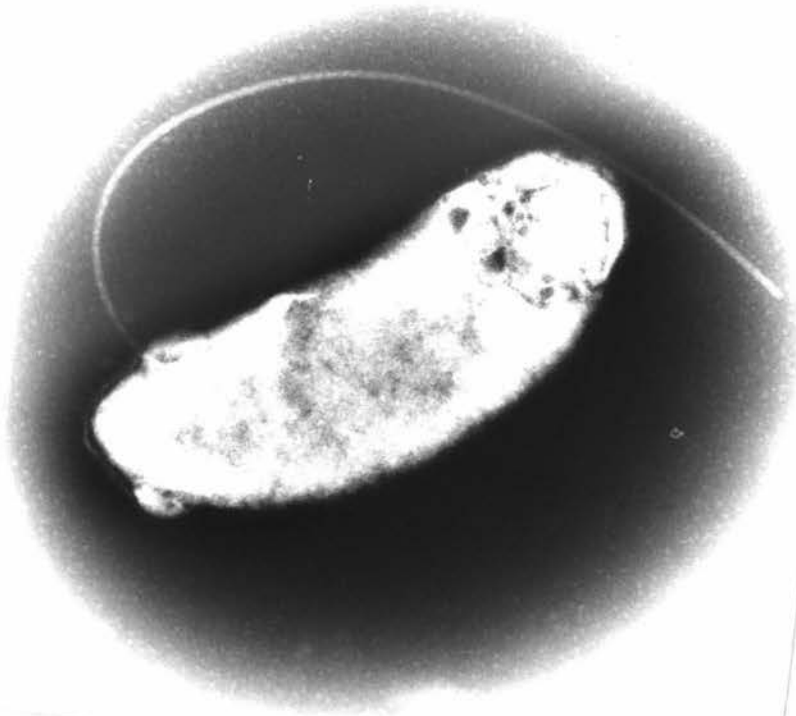

Fig. 25a

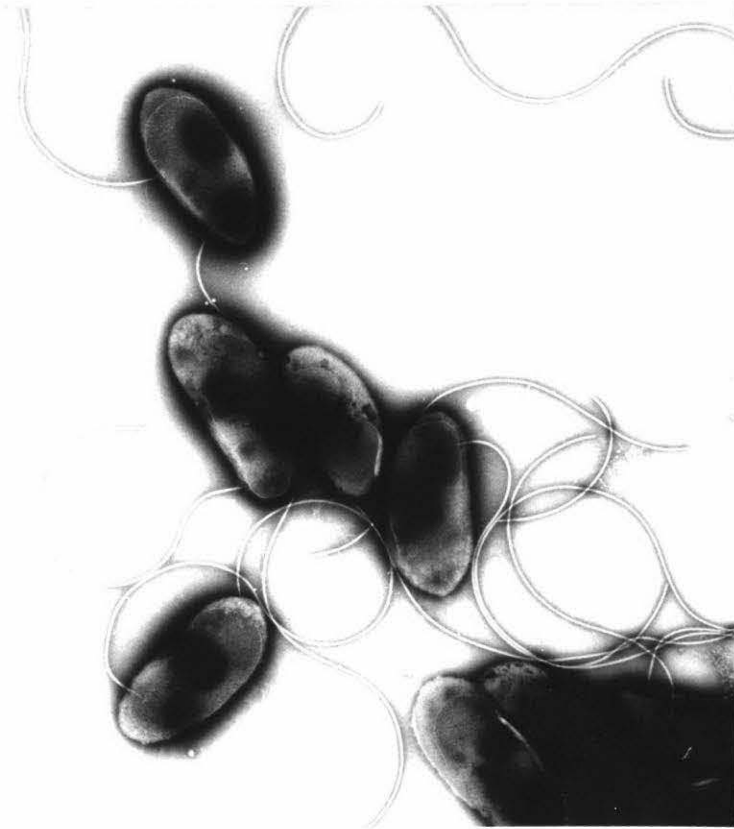

Fig. 26

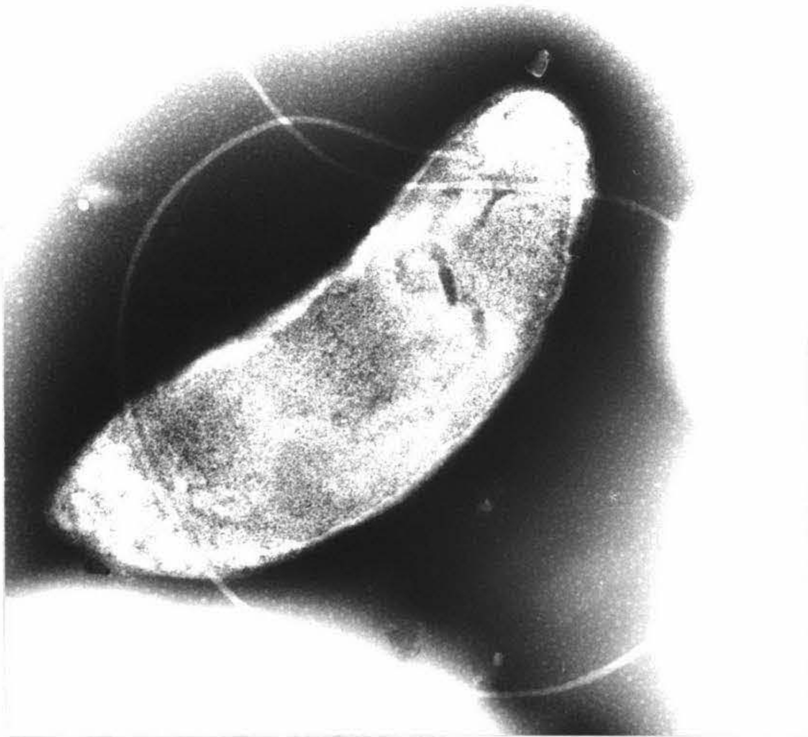

Fig. 26a

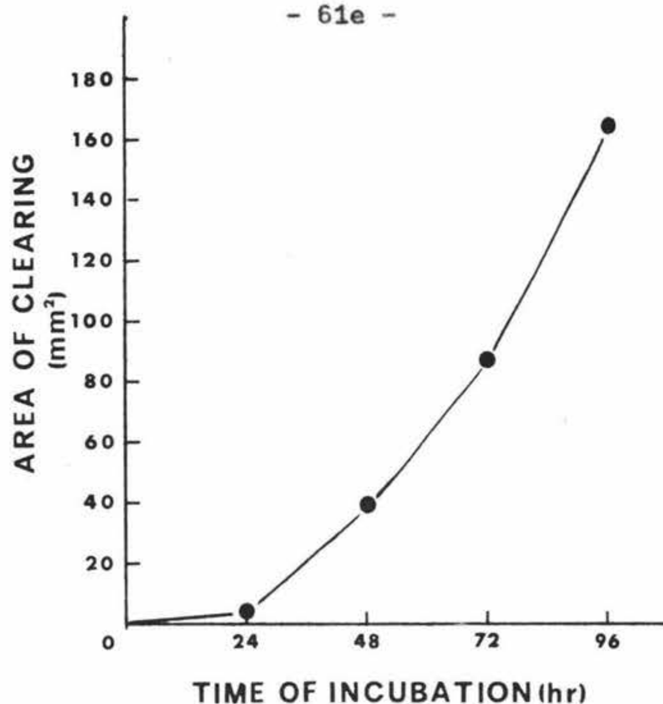

FIGURE 27

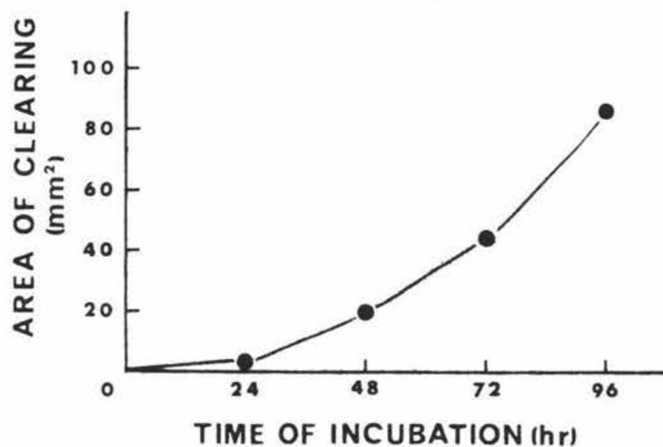

FIGURE 28

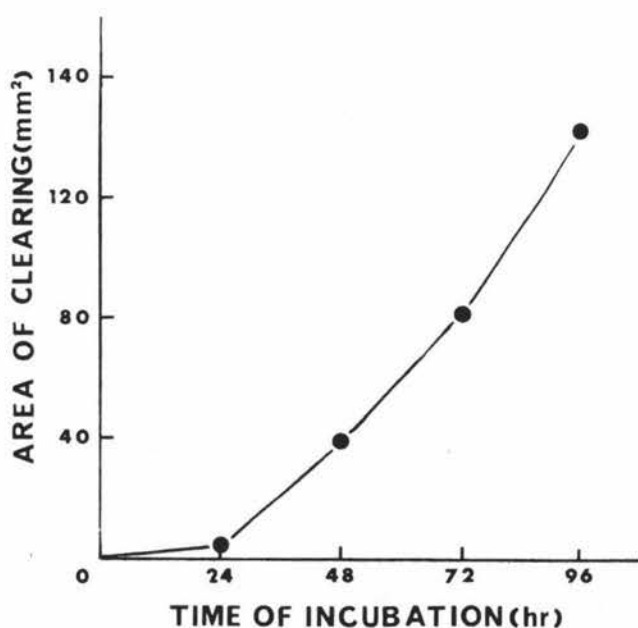

FIGURE 29

Relationship between the area of clearing of 0.2% tributyrin and time for B_1 (Fig.27), B_2 (Fig.28) and B_3 (Fig.29). B_1, B_2 and B_3 represent strains 1, 2 and 3 respectively.

4.13. Study of the lipolytic activity of tributyrin- splitting bacteria towards peanut oil

In an investigation of the lipolysis of peanut oil by the isolated tributyrin-hydrolysing bacteria (B_1, B_2 and B_3) double layer technique as described in section 3.15.,was used. After 72 hours incubation at $39^{\circ}C$, only very narrow zones of clearing were observed microscopically. These narrow zones of clearing did not increase in size even on prolonged incubation.

4.14. Activity of tributyrin - splitting bacteria in the hydrolysis of grass-galactolipid

A comparison of the hydrolysing capacity of the 3 strains of bacteria (B_1, B_2 and B_3) towards galactolipids was made by following the formation of free fatty acids colorimetrically (section 3.18) within 25 hours incubation time. A control experiment at zero hour showed 411 m μ moles of free fatty acids and the level reaches 462.5 after 25 hours incubation. A slight nonenzymatic hydrolysis of the substrate was apparent under the conditions of incubation (Table 12). Strain 1 was found to be most efficient in the hydrolysis of grass-galactolipid. However, it is apparent from table 12 that all 3 strains showed the same relative lipolytic activity toward galactolipid as they did toward tributyrin (section 4.12) i.e. the activity decreased in the following order: B_1 , B_3 , B_2.

TABLE 12

Hydrolysis of monogalactosyldiglyceride by tributyrin - splitting bacteria.

Sample *	Fatty acids in $m\mu$ moles	B_{25} less fatty acids in $m\mu$ moles	+ % of breakdown of total galactolipid
B_0	411.0	-	-
B_{25}	462.5	-	-
B_1	1316.2	853.7	6.53
B_2	630.0	168.5	1.3
B_3	961.0	498.5	3.81

* B_0 and B_{25} are the control experiments at zero hours and 25 hours respectively. B_1, B_2 and B_3 are strain no. 1, 2, and 3 respectively for which 25 hours incubation was carried out using 1 ml of galactolipid emulsion containing 5 mg of the substrate.

+ These figures were calculated on the assumption that linolenic acid was the only fatty acid associated with the monogalactosyldiglyceride.
For details of incubation conditions see sub-section 3.16.1.

4.15. Effect of time in the hydrolysis of monogalactosyldiglyceride by bacterium strain 1

Among the 3 types of bacteria studied, strain 1 showed the highest activity towards galactolipid (section 4.14). This was the reason it was selected to determine its activity against galactolipid with time. Formation of free fatty acids was followed by their measurement colorimetrically (section 3.18). Control experiment at 16 hours incubation time liberated 285.0 $m\mu$ moles free fatty acids. However, there is an increase in the concentration of liberated free fatty acid with the increase in time reaching a maximum value 451.2 $m\mu$ moles at the end of 16 hours (Fig. 30).

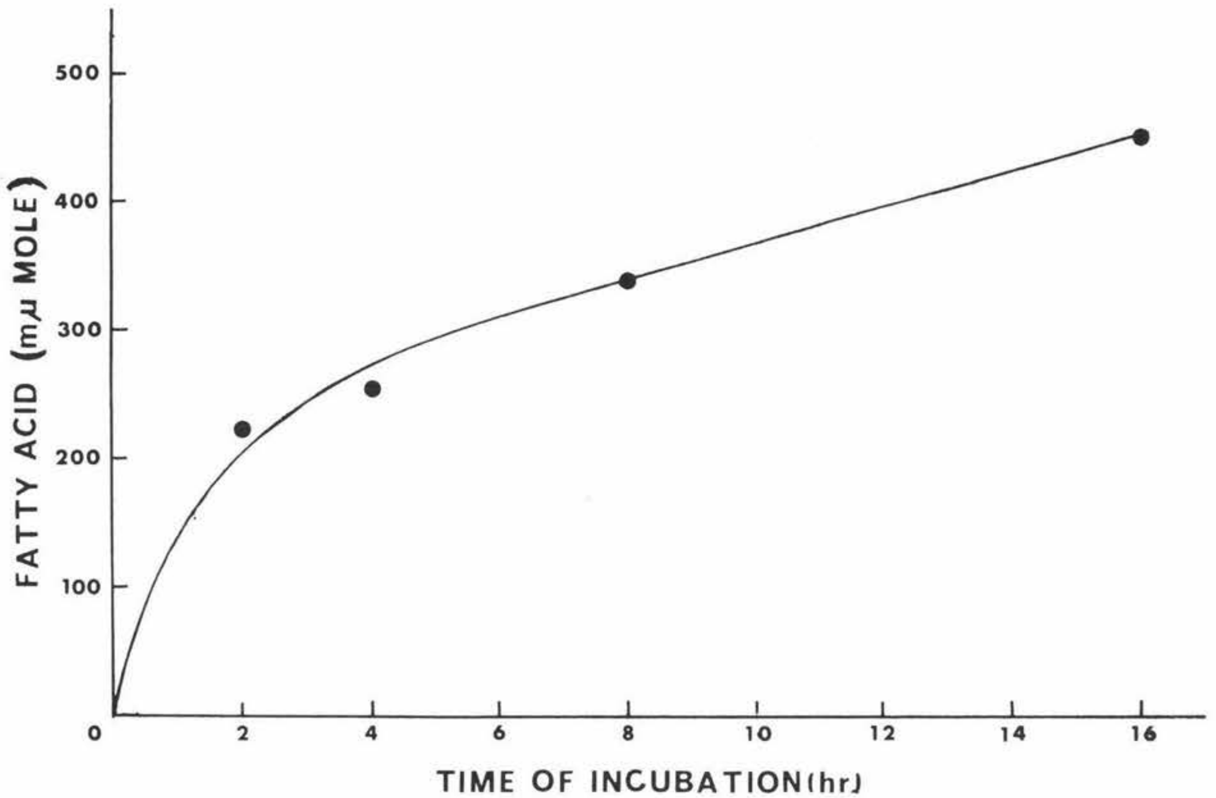

Figure 30. Effect of time on the formation of free fatty acids from monogalactosyldiglyceride by strain 1.
Reaction conditions: Incubations were carried out for 2, 4, 8 and 16 hours using 1 ml of galactolipid emulsion containing 1.69 mg of the substrate. Incubation of the control experiment was carried out for 16 hours (see subsection 3.16.2).

CHAPTER 5

DISCUSSION

5.1. Purification and characterisation of lipases from mixed rumen bacteria

Assay of lipolytic activity was carried out by continuous titration of liberated fatty acids using a radiometer. This permitted the measurement of the initial velocity of lipolysis at a constant pH. More sensitive radiochemical techniques (Clarke and Hawke, 1970) were used to follow the rate of lipolysis. It was confirmed that well separated radioactive lipids of the reaction mixture on a thin-layer chromatographic plate could be directly scanned or alternatively, by transfer of the appropriate areas of lipids from the plate into vials followed by the determination of radioactivity in a liquid scintillation spectrometer. The formation of mono- and diglyceride during the reaction could be followed using this technique. Finally, estimation of free fatty acids was carried out by a colorimetric method (Mahadevan *et al.*, 1968).

The attachment of cellulolytic microorganism of the rumen to plant particles in the digesta has been established by a number of workers (King, 1959; Hungate, 1966). It appears that there is a similar attachment of lipolytic microorganisms to the particulate material (Clarke and Hawke, 1970). The apparent effect of homogenisation is to detach some of the lipolytic microorganisms from their natural site in the aqueous media which results in a greater lipolytic activity (Table 6). In spite of this close resemblance between the cellulolytic and lipolytic bacteria on the point of their attachment to the plant particles, further investigations on their differences are necessary. Whether lipolytic bacteria are cellulolytic form to be followed by more precise details of the

relationship between the two groups of bacteria, have yet to be studied. Garton et al. (1961) did not detect mono- or diglycerides in the rumen contents after adding triglyceride. The presence of these compounds has been demonstrated by Hawke and Robertson (1964), Silcock (1968) and Clarke (1969). The present experiments further confirm that there is some accumulation of mono- and diglyceride. It is apparent that both mono- and diglyceride are formed in the hydrolysis of ^{14}C- triolein by strained rumen fluid (table 6). The percentage of radioactivity in the monoglyceride fraction appeared to be approximately half of the diglyceride level throughout the period of incubation (Fig. 11 and Fig. 12). The lipase activity in the rumen does not lead to a type of breakdown (Garton et al., 1961; Clarke and Hawke, 1970) as with pancreatic lipase (Savary and Desnuelle, 1956). This would hardly be expected because the lipases prepared from rumen contents are likely to be derived from many different bacterial sources. Whether or not there are specific lipases present, they cannot be described until the characteristics of the lipases of a single bacterial species have been determined.

High frequency sonication of bacterial preparation from the rumen resulted in the transfer of lipolytic activity to the 90,000xg supernatant (Clarke and Hawke, 1970). The results presented in Table 7 confirm the earlier work. The lower activity in the 90,000xg supernatant prepared from bacteria without prior sonication could be explained by some lysis in the 0.06 M phosphate buffer (Table 7).

High speed centrifugation was used for the preparation of lipases. Milk lipases were prepared from the supernatant fractions obtained after centrifuging skim milk (containing 0.75 M NaCl) at 80,000xg for 2 hours

(Downey and Andrews, 1969). A 60 min. centrifugation of pig pancreas homogenates at 100,000x$\underline{g}$ in 9 volume of water was carried out to get a clear extract which was used as the source of lipase for the next purification step (Sarda et al., 1964). However, in this study the supernatant fraction (Frac. L in fig. 8) obtained after centrifuging sonicated bacteria at 90,000x$\underline{g}$ for 30 min. was used in order to achieve a further purification. An appreciable lipolytic activity remained in the supernatant fraction (Frac. N in Fig. 8) after centrifugation of the sonicated bacterial pellet at 200,000x$\underline{g}$ for 30 min. Furthermore, the 200,000x$\underline{g}$ centrifugate had greater activity than that obtained at 90,000x$\underline{g}$ (Table 8). This seems to indicate that some lipolytic bacteria or particles with lipolytic activity remain suspended in the 90,000x$\underline{g}$ supernatant which are then removed by higher centrifugal force. When the latter was filtered through a Zeitz filter the bacteria-free filtrate showed slightly lower lipolytic activity than the non-filtered supernatant. The presence of lipolytic activity in the 200,000x$\underline{g}$ supernatant (Table 8) is also good evidence for the existence of soluble lipases.

Lipases from Fusarium lini Bolley was found to be very stable in dry state but unstable in aqueous solutions (Fiore and Nord, 1950). Electrophoretic studies provide evidence that the enzyme destruction in aqueous solutions resulted from proteolysis. In the present study, from a comparison of the stability of soluble lipases in aqueous solutions and in lyophilised material, it is apparent that rumen microbial lipases are unstable in aqueous solution. However, this could not provide information of the factors involved in the inactivation of lipases. On the other hand, lyophilised material is fairly stable. It also appears from the results presented in Table 9, that dialysis of the enzyme extract does

not inactivate the lipases.

The use of Sephadex columns seems to be one of the most important steps in the purification of microbial lipases. This technique successfully purified many microbial lipases such as Micrococcus and Pseudomonal lipase (Lawrence et al. 1967b), Staphylococcal lipase (Tirunarayanan and Lundbeck, 1968), and Pseudomonal lipase (Lu and Liska, 1969a). Substantial purification of pancreatic lipase was achieved when the former was filtered through Sephadex G- 200 (Benzonana et al., 1964; Sarda et al., 1964). However, in the present study, Sephadex G- 200 used to purify lyophilised lipase preparation (Frac. Q in fig. 8) did not result in a useful concentration of lipase activity. Although three different protein peaks (Fig. 13) were obtained, the lipolytic activity was distributed over all the peaks (Table 10). The main problem in applying this purifiaction procedure to the lyophilised material was the low specific activity of the lipase which meant that the appropriate protein loading (section 4.6) for the column was low in total lipase activity.

A 50% $(NH_4)_2SO_4$ precipitate of the lyophilised material showed lipolytic activity towards emulsified peanut oil leaving the corresponding supernatant, inactive (section 4.7). It is suggested that 50% $(NH_4)_2SO_4$ is sufficient to precipitate all the lipases including other protein impurities. Perhaps it would be a significant step in the purification of rumen microbial lipases. In this connection, lysolecithinase, an enzyme isolated from the rumen bacteria, was purified by $(NH_4)_2SO_4$ precipitation and adsorption on calcium phosphate gel (Dawson, 1959). $(NH_4)_2SO_4$ fractionation of culture supernatant purified Pseudomonas lipase (Lu and Liska, 1969a). Half-saturated $(NH_4)_2SO_4$ was used for the purifiaction of pan-

creatic lipase (Benzonana *et al*., 1964; Sarda *et al*., 1964).

It has been observed by some investigators that optimal pH of a lipase depends on the nature of the substrate it hydrolyses. *Mycoplasma Galliscepticum* lipase showed a pH optima 7.5 for the hydrolysis of mono-, di-, and tributyrin but it showed maximum activity at pH 8.0 for the hydrolysis of trilaurin and triolein (Rottem and Razin, 1964). Rat serum lipase showed pH optimas 7.2, 7.6 and 8.05 for the hydrolysis of ethyl butyrate, tripalmitin and tributyrin (Tuba and Hoare, 1950) respectively. During this study on rumen lipase, lyophilised material (Frac. Q in fig. 8) was used as the source of enzyme for the determination of its pH optimum towards triolein and tributyrin. The pH of the rumen ingesta is slightly acid and the bacteria are adapted to live between pH 5.5 to 7.0 (Hungate, 1966). However, the crude lipase preparation showed an optimum between pH 8.0 to pH 8.5 for the hydrolysis of triolein when assayed in the range of pH 6.5 to pH 9.5 (Fig. 17); this also showed maximum activity at pH 7.0 towards tributyrin when assayed in the range of pH 4.5 to pH 9.5 (Fig. 20) suggesting the possibility of more than one lipase.

Microbial lipase show a greater activity towards glycerides containing short-chain fatty acids than towards high molecular weight fatty acids. When Tirunarayanan and Lundbeck (1968) compared the activity of staphylococcal lipase towards a number of different triglycerides they found the rate of hydrolysis of tributyrin was the highest and triolein the lowest. Maximum hydrolysis was observed by Micrococcal lipase when tributyrin or tripalmitin was used as substrate (Rottem and Razin, 1964; Shah and Wilson, 1965). Hydrolysis of tributyrin was more rapid than any other triglyceride by human milk lipase

and pancreatic lipase (Schønheyder and Volqvartz, 1944). An approximately similar phenomenon was achieved when triolein and tributyrin was used in the lyophilised lipase preparation. The lipolytic activity was many times higher towards tributyrin than triolein. When tributyrin was used as substrate at pH 7.0, 0.054 unit of activity per mg of protein was achieved (Fig. 20). By comparison, when triolein was used as substrate at pH 8.5, only 0.002 unit of activity per mg of protein was acheived (Fig. 17).

5.2 Isolation and characterisation of tributyrin-splitting bacteria

Hobson and Mann (1961) detected lipolytic microorganisms by growing the isolates in media containing linseed oil and determining the total acidity in an acid-ether extract. It had been reported some years earlier that bacterial cultures contain ether-soluble acids originating from the metabolism of protein or carbohydrate (Lubert et al., 1949). Consequently, if the technique is to be used, the level of available protein and carbohydrate must be kept low. In the present study, pure culture of tributyrin-splitting bacteria was tested for their lipolytic activity using galactolipid as substrate (sections 4.14 and 4.15) since galactolipids are of quantitative importance as a class of dietary lipids in the grazing ruminants. The liberated free fatty acids were determined colorimetrically (section 3.18) after extraction with hexane. Since the colorimetric assay is specific for long-chain fatty acids, contamination of the extracts with alkaline or acid degradation products (Lawrence et al., 1967a) from the growing cultures would not interfere with the colorimetric estimation.

Clear zones around the colonies grown in linseed oil-agar or tributyrin-agar roll tube were taken to indicate lipolysis or esterase

activity (Hobson and Mann, 1961). In this study the double layer technique (section 3.15) was used for the detection of lipolytic bacteria. Since the colonies were grown separately from the site of lipase action, the technique avoided the possible inhibition of liberated fatty acids towards the organisms. Approximately 1.4 x 10^7 tributyrin-splitting bacteria (section 4.10) were found to be present /ml of rumen fluid.

Three strains of tributyrin-splitting bacteria were isolated in pure culture. The isolates were rapid in their action toward tributyrin (Figs. 27, 28 and 29). However, when the lipolytic activity of these three strains was investigated using peanut oil, only very narrow zones of clearing were observed microscopically after 72 hours incubation. On prolonged incubation, these zones of clearing did not increase in size (section 4.13). On the other hand, clear zones around colonies in contact with tributyrin-agar were much more obvious (section 4.10; fig. 23). In this connection, it should be noted that hydrolysis of tributyrin was found to be more rapid than triolein by lyophilised lipase preparation from mixed rumen bacteria (Fig. 17; Fig. 20). Oleic acid has been found to inhibit the lipolytic activity of various microorganisms such as Pseudomonas aeruginosa (Sierra, 1957), Pseudomonas fragi (Smith and Alford, 1966) and Micrococcus freudenreichii (Lawrence et al., 1967b). The growth of Streptococcus cremoris was markedly inhibited by oleic acid (Anders Jago, 1964). Using overnight broth cultures (in media A) of the three isolates (B_1, B_2 and B_3) their lipolytic activity towards emulsified ^{14}C- triolein with unlabelled carrier, was investigated. Apparently, they could not hydrolyse triolein within 24 hours incubation but further studies will be required to comment on this point. However, free fatty acids appeared in the incubation products when monogalactosyldiglyceride

was incubated with these three strains (Table 12; Fig. 30). Unfortunately, control experiments using culture of standard turbidity without added monogalactosyldiglyceride, were not carried out. So, it is inconclusive whether the liberated fatty acids are of bacterial or galactolipid origin. Further studies will be required to provide information on this point. The lack of activity when ^{14}C- triolein was used as substrate could possible be due to the inhibitory effect of oleic acid on the growth of the organisms or on their lipase activity. In this connection it should be noted that the grass-galactolipid used as substrate contained only a trace of oleic acid (Table 5).

The formation of lipase by some microorganisms are inducible e.g. lipases are synthesised in Candida paralipolytica under the influence of lipids and related substances (Ota et al., 1968). Addition of fats and oils to the base medium, increased the lipase production by Torulopsis ernobii (Toshida et al., 1968). However, it is apparent from fig. 27, 28 and 29, that the production of the area of clearing by tributyrin-splitting bacteria with respect to time is not linear. It is suggested that this may indicate a lag phase during which the tributyrin-splitting enzymes are induced.

The highly branching nature of colonies of B_1 (Fig. 23) made it easy to distinguish from other tributyrin-splitting bacterial colonies (Table 11). It was observed on one occasion that B_1 represents about 10% of the total tributyrin-splitting bacteria in the rumen. Approximately 2 months after the first isolation of B_1, B_2 and B_3, colonies similar to B_1 were again observed in isolates from rumen fluid. This may indicate that this bacterium is the normal inhabitant of the rumen of the

cow grazing on fresh pastures. It was not possible to make the same visual observation for B_2 and B_3 because they did not possess such a distinguished property, i.e. branching in the colony (Table 11).

In cellulose-agar medium colonies of Butyrivibrio vary in shape from lens-shaped to triangular or highly branched (Hungate, 1966). Lipolytic bacteria isolated by Hobson and Mann (1961) were vibrioshaped. In the present study, the isolated tributyrin-splitting bacteria have not been named. Nevertheless, the highly branching nature of B_1 (Fig. 23) perhaps gave an indication that this particular strain may be a strain of Butyrivibrio.

All the way through, medium A (section 3.4) with agar was used for the storage of the 3 groups of bacteria. When colonies of the three isolates has been transferred to medium B(section 3.4) with agar, a very good growth of all three isolates has been observed within an incubation period of 18 hours at 39°C. When the colonies of the 3 strains were transferred to media C (Table 4), a period of 96 hours was required for adaptation before any of the isolates could grow freely.

CHAPTER 6

SUMMARY

1. Soluble lipase fractions with lipolytic activity have been prepared from rumen bacteria which had been subjected to sonication. 90,000xg centrifugation, ultra-filtration, dialysis, freeze-drying and $(NH_4)_2SO_4$ precipitation was used for the preparation of lipase. Attempts to apply gel-filtration to purification were not successful.

2. Lipolytic activity has been assayed by radiochemical, colorimetric and titrimetric methods. The lipases were more stable in lyophilised state than in aqueous solution.

3. The activity of lyophilised lipase showed a pH optimum between pH 8.0 - 8.5 and at pH 7.0 for the hydrolysis of triolein and tributyrin respectively. Hydrolysis of tributyrin was found to be more rapid than triolein.

4. When tributyrin was used as the substrate, it was found that the reaction velocity was approximately proportional to the amount of protein in the lyophilised enzyme.

5. A double layer anaerobic technique was used for the detection and isolation of tributyrin-splitting bacteria from the rumen of a cow.

6. Total number of tributyrin-splitting bacteria in fresh rumen fluid from a grass fed cow was found to be approximately 1.4×10^7/ml.

7. Three strains of tributyrin-splitting bacteria were isolated. The isolated organisms were strictly anaerobic Gram-negative rods which were capable of growth in three different synthetic and semi-synthetic media. Investigations of the lipolytic activity of these strains was carried out using monogalactosyldiglyceride, peanut oil and ^{14}C- triolein.

8. One of these strains was considered to be a normal inhabitant of the cow rumen since it appeared to represent approximately 10% of the total tributyrin-splitting bacteria in the rumen.

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