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 micro-organisms 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 micro-equivalent 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

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.

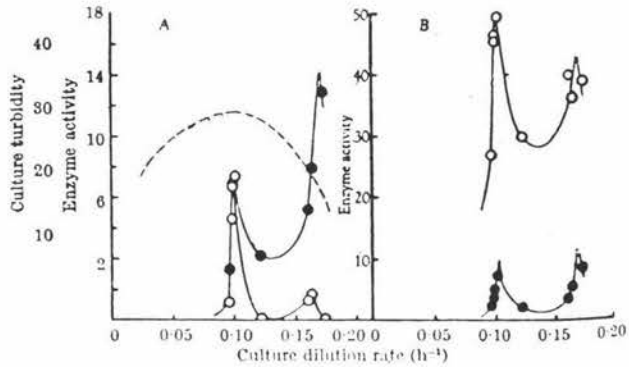

Figure 1. The lipase (A) and esterase (B) activities of cells (O) and supernatants (●)from a continuous culture growing at different rates. Substrates, naphthyl stearate (A), naphthylacetate(B). Superimposed on A is a graph of the steady-state cell concentrations at different growth rates (Hobson & Summers, 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 electrophoretically (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	25×10^5	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).

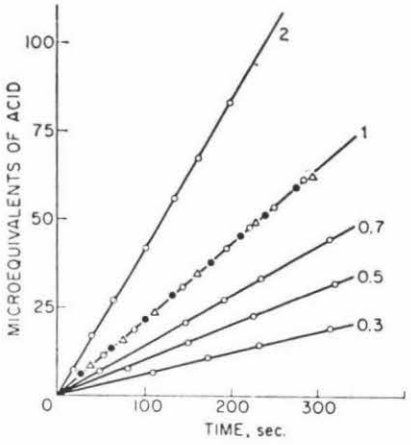

Figure 2. Potentiometric test for the determination of lipase activity. The figures indicate the relative amounts of lipase used in each assay (From Desnuelle, 1961).

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

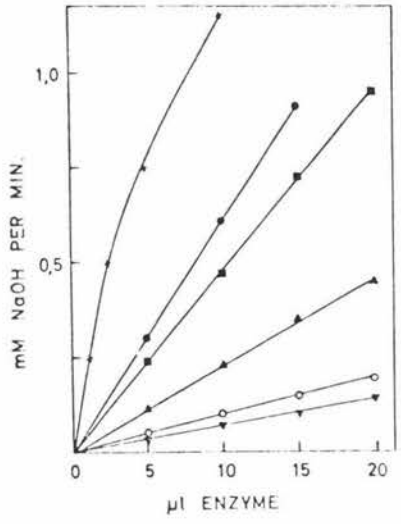

Figure 3. Hydrolysis of triglyceride by Staphylococcal lipase (Tirunarayanan & Lundbeck, 1968).

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

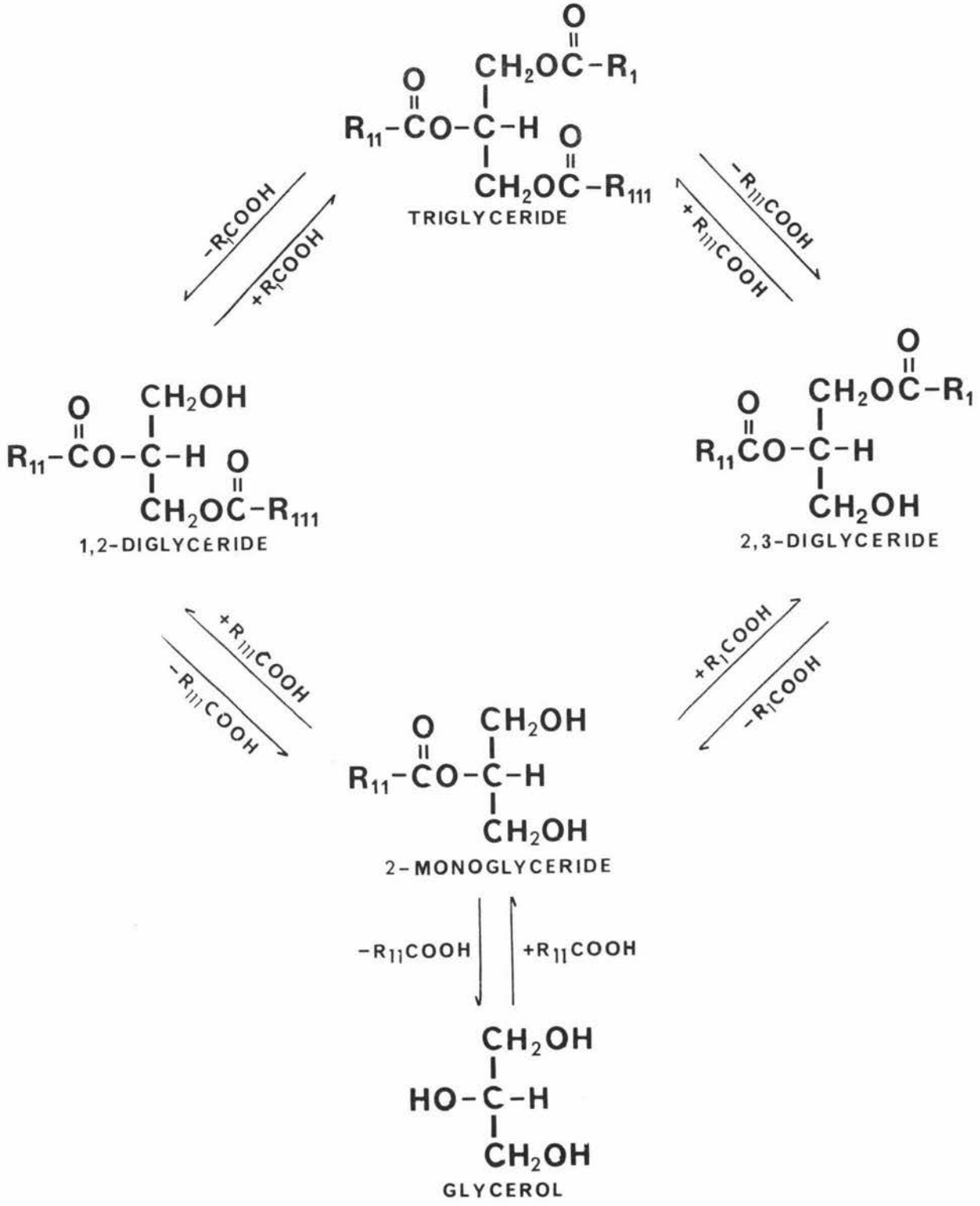

Figure 4. Major pathway for the hydrolysis of triglyceride by pancreatic lipase. Complete hydrolysis of triglyceride proceeds with the rapid splitting of the 1 & 3 linkages followed by slow hydrolysis of the 2- monoglyceride. R_1, R_{11}, R_{111} represent fatty acids of the same or different structure (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 (Prculx 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 Pseudo-monas fragi, Staphylococcus aureus, Geotrichum candidum, Candida paralipo-lytica 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).

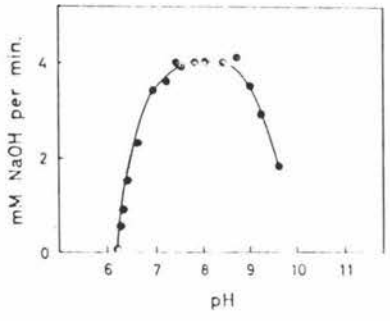

Figure 5. pH-activity relationship for the hydrolysis of tributyrin by Staphylococcal lipase (Tirunarayanan & Lundbeck, 1968).

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, Asperigillus niger lipase was found to be inactive in an emulsified system but activity was obtained simply by shaking the reaction mixture (Iwai et al., 1964).

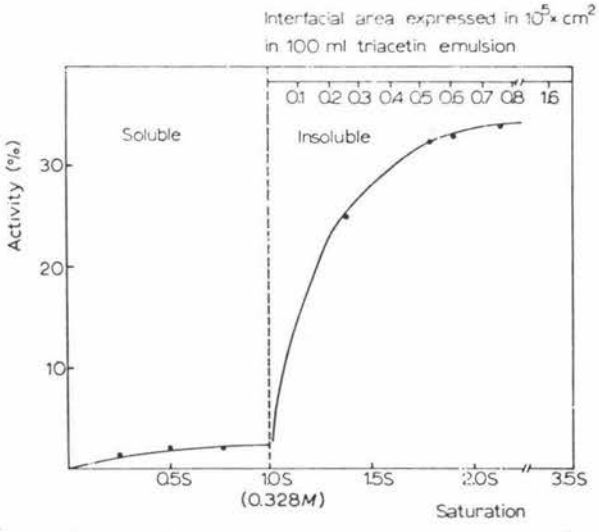

Figure 6. The influence of saturation on the hydrolysis of triacetin by lipase from swine pancreas. The numbers at the upper borderline give the interfacial area expressed in 10^5 x cm^2 in 100 ml triacetin emulsion (From Oosterbaan & Jansz, 1965).

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).