AN INVESTIGATION OF LIPOLYSIS

IN THE BOVINE RUMEN

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INTRODUCTION

1.1. Lipases

1.1.1. Terminology

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

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

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

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

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

1.1.2. Historical

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

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

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

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

1.1.3. Methods of detection

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

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

(a) Measurement of rate of disappearance of triglyceride

The rate of disappearance of triglyceride can be measured by

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

(b) Measurement of rate of fatty acid production

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

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

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

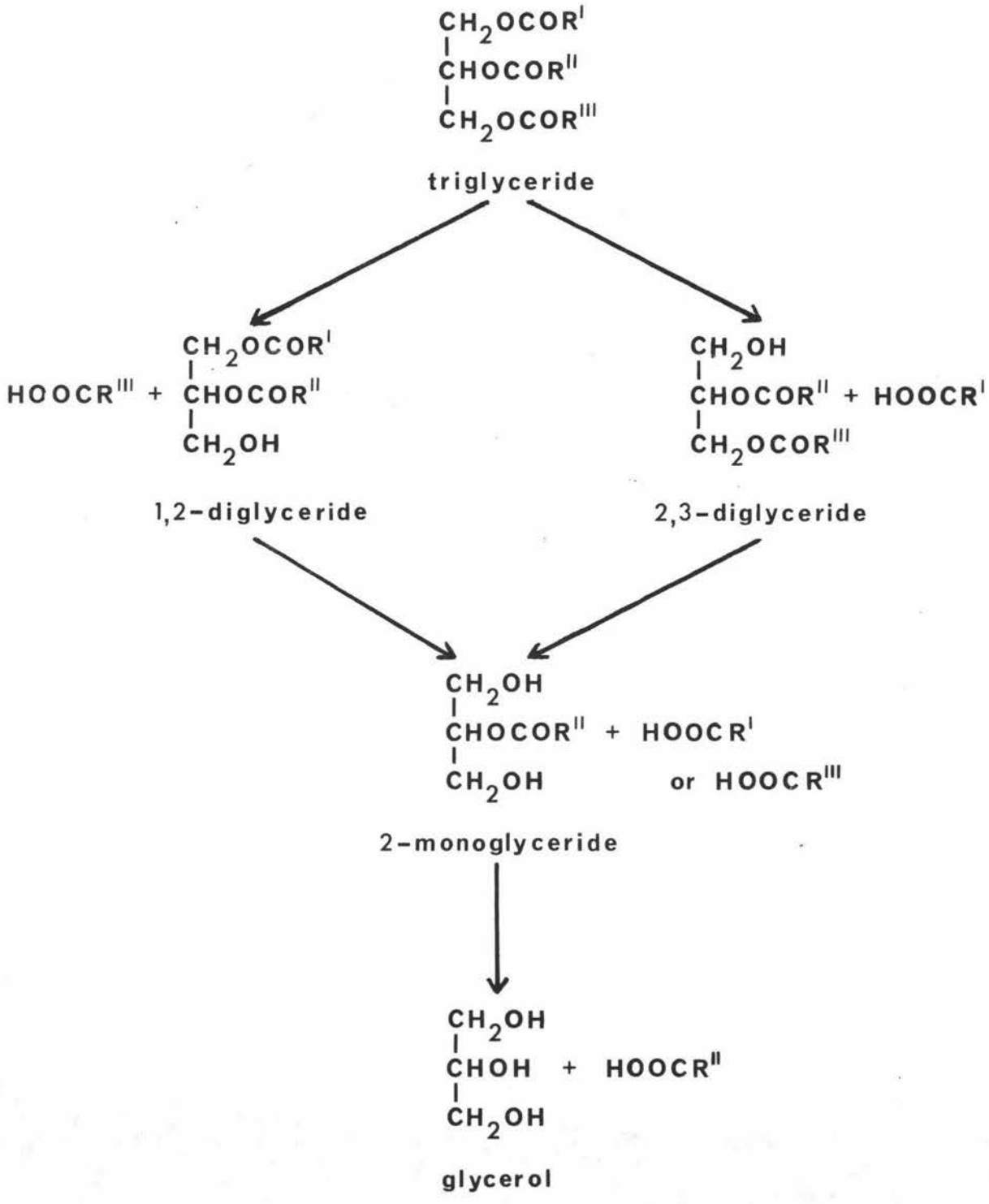

Figure 1. The major pathway for the hydrolysis of triglyceride by lipase. (R', R'', R''' represent fatty acids of the same or different structure).

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

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

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

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

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

1.1.4. Purification

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

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

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

1.1.5. Factors affecting velocity of hydrolysis

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

(a) Effect of emulsification of substrate

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

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

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

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

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

(b) Effect of pH

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

(c) Effect of temperature

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

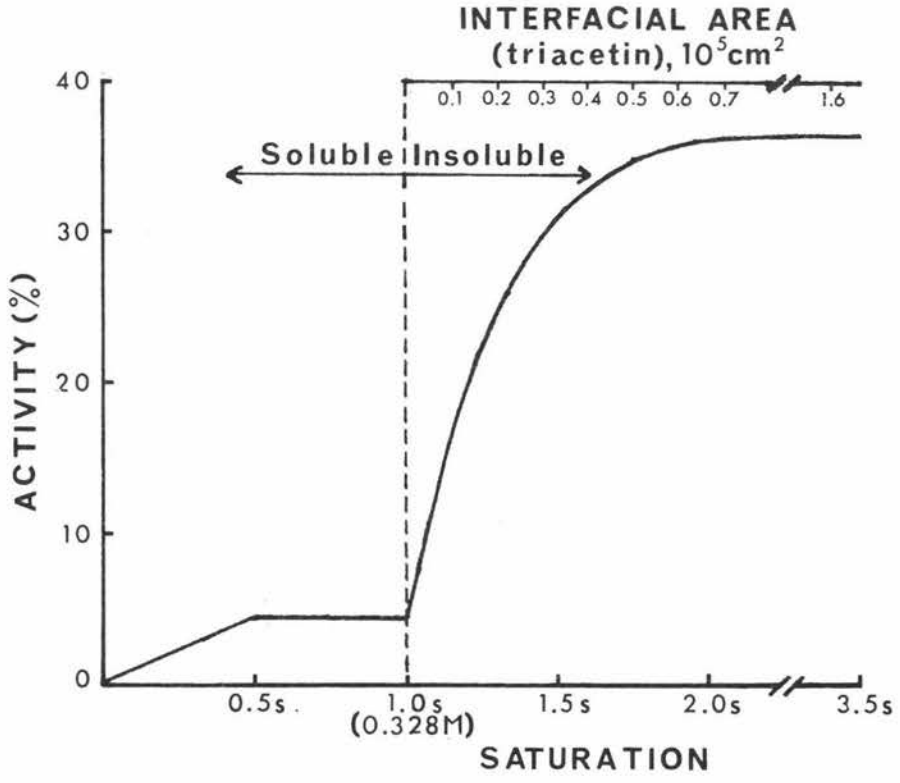

Figure 2. The influence of the saturation concentration on the rate of hydrolysis of triacetin by pancreatic lipase. The substrate concentration is expressed as a fraction of the saturation concentration. (from Sarda and Desnuelle, 1958).

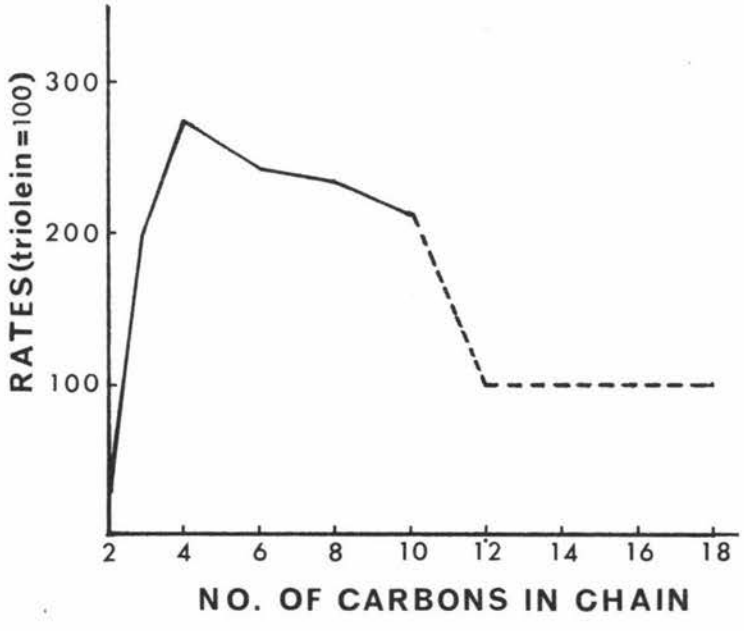

Figure 3. Variation of the rate of hydrolysis of simple glycerides with the chain length of the fatty acid. Hydrolysis rates are expressed as a percentage of the rate of hydrolysis of triolein. (from Entressangles et al, 1961).

1.1.6. Substrate specificity

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

(a) Alcohol moiety

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

(b) Fatty acid moiety

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

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

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

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

(c) Positional specificity of lipases

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

isomers were 1-monoglycerides.

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

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

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

1.2. Dietary Lipids of Ruminants

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

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

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

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

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

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

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

1.3. Modification of Dietary Lipids by Rumen Microorganisms

Studies during the last decade have shown that rumen microorganisms effect extensive changes in the dietary lipid. The earlier observations which made it apparent that there were differences between the lipid metabolism of ruminants and other mammals were:

(a) the depot fats of ruminants contained a high proportion of stearic acid (Hilditch, 1956),

(b) the depot fats of ruminants contained a high proportion of trans-acids (Swern, Knight and Eddy, 1952 and Hartman, Shorland and McDonald, 1954),

(c) dietary unsaturated fatty acids known to be readily assimilated into the depot fats of non-ruminants did not appear to be present in the ruminant tissue lipids (Garton, 1964),

(d) depot fats and milk fats of ruminants contained a complex mixture of unsaturated fatty acids (especially isomeric forms of octadecenoic and octadecadienoic acids) not found in the fats of other herbivores (Hilditch, 1956).

TABLE 1

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

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

(1) Shorland et al, 1955

(2) Garton, 1960

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

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

(a) hydrolytic release of esterified fatty acids,

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

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

1.3.1. Hydrolysis of dietary lipids in the rumen

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

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

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

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

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

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

TABLE 2

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

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

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

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

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

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

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

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

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

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

1.3.2. Hydrogenation of unsaturated fatty acids by rumen fluid

Reiser (1951) first demonstrated that rumen fluid possessed

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

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

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

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

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

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

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

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

1.3.3. Fermentation of glycerol and galactose

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

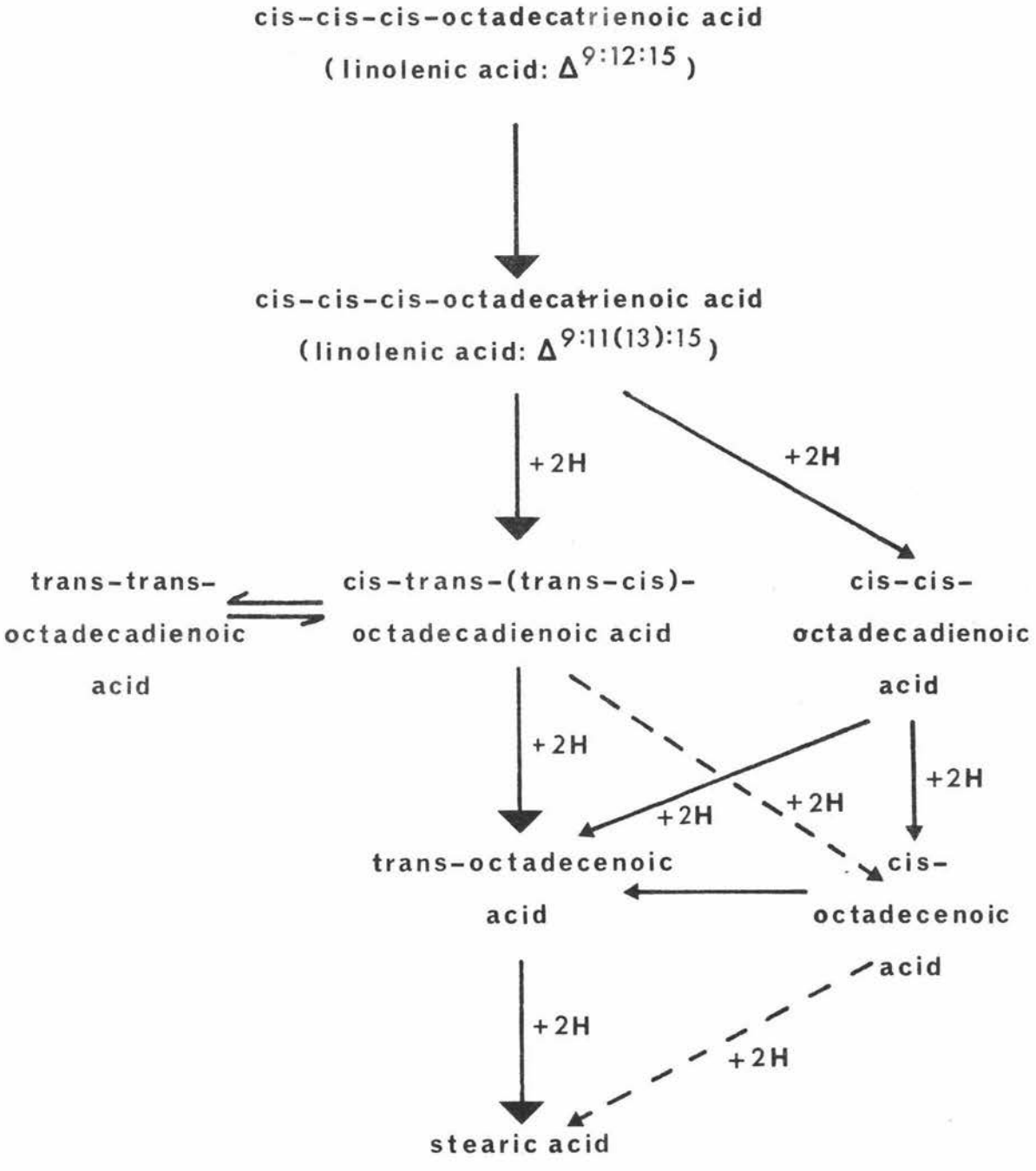

Figure 4. Pathway for the hydrogenation of linolenic acid by rumen microorganisms. (from Wilde and Dawson, 1966).

Hobson and Mann (1961) isolated Selenomonas ruminantium var lactilyticus from sheep rumen contents and found it to be capable of fermenting glycerol to propionic acid. It is agreed that propionic acid is the main product of glycerol fermentation (Johns, 1953; Hobson and Mann, 1961; Garton et al, 1961).

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

1.4. Significance of Hydrolysis in the Rumen

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

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

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

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

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