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RELATIONSHIPS BETWEEN HYDROGENATION AND HYDROLYSIS OF DIETARY FAT IN THE BOVINE RUMEN

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

by

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INTRODUCTION

1.1. Introduction

One of the earliest detailed comparisons of the fatty acid and glyceride composition of the depot fats of ruminants and non-ruminants in relation to the diet fed may be obtained from the work of Banks and Hilditch (1931, 1932). These studies involved beef tallows and sow depot fats respectively, and were part of an investigation of the fatty characteristics of market meat. It had been known for some time that very soft body fats, which have always been undesirable from the consumer's point of view, were produced when pigs were fed diets containing high levels of unsaturated fats. Banks and Hilditch (1932) found that the characteristic fatty acids of animal and vegetable fats when fed appeared in sow depot fats. In this case, the sow was fed a diet containing 7% of fish meal, which contains some 20% of unsaturated C20 and C22 fatty acids together with a similar amount of linoleic acid. All these fatty acids appeared in significant amounts in the depot fats of the sow.

In contrast, Banks and Hilditch (1931) found that beef tallows contained much less oleic acid and linoleic acid than was present in the diet. Other studies carried out by Thomas, Culbertson and Beard (1934) and by Edwards and Holley (1939) also showed that the type of fatty diet fed to cattle had little effect on the characteristics of the depot fats. Edwards and Holley (1939) note in their paper however, that a liberal allowance of oil in the diet will tend to soften the depot fat of cattle slightly if fed for a long enough period of time.

Although the differing effects of dietary fat on ruminant and non-ruminant depot fats were known at this time, there was little understanding of the processes of digestion of dietary fats, and the subsequent re-assembly of component fatty acids of the diet into depot fats. Banks and Hilditch (1931) observed that the component fatty acids of beef tallows all occurred in the diet, and, if in fact obtained directly from this source, after formation of the depot fats there would be left an excess of oleic and linoleic acids, as these were not present in the tallows to the extent to which they were present in the diet, especially linoleic acid. Banks and Hilditch (1931) proposed that this excess of oleic and linoleic acids might be removed by oxidation. Noting also the increased levels of stearic acid in the depot fats in comparison with the diet, Banks and Hilditch (1931) suggested that stearic acid is synthesised either from corresponding unsaturated fatty acids (i.e. by hydrogenation) or from non-fatty material. Following their study of pig depot fats, Banks and Hilditch (1932) suggested that the composition of storage fats indicated hydrogenation of pre-formed glycerides rather than of free fatty acids, but could not suggest where such hydrogenation might take place.

During a study of ultra-violet absorption by trans-fatty acids, Moore (1939) found that "Since the acids of grass were found to be in the spectroscopically 'pro-absorptive' form it is clear that absorptive acids found in the fats of herbivorous animals must be changed into this condition in the animal."

As late as 1950, Brooker and Shorland (1950), in a study of horse oil composition, suggested that linolenic acid from the diet may be destroyed in the rumen during prolonged digestion. However, the possibility also of some linolenic acid reaching the bloodstream is noted (Kelsey and Longenecker, 1941), and Brooker and Shorland (1950) concluded that linolenic

acid is either metabolised or hydrogenated in the body after absorption into the bloodstream.

It was not until 1951, when Reiser (1951), noting that the depot fats of sheep and cattle contain only small amounts of linolenic acid, even when being fed on a grass diet containing high levels of this acid, and that horses on the same diet accumulate significant amounts of linolenic acid in their depot fats, suggested that this difference may be due to hydrogenation of linolenic acid by rumen bacteria. Reiser incubated linseed oil emulsions with rumen contents and found that after four days, the linolenic acid content of the incubation mixture was reduced from 30% to 5%. As linoleic acid increased proportionately, and autoclaved controls were not hydrogenated, Reiser inferred that linolenic acid was being hydrogenated to linoleic acid in the rumen as a result of bacterial action.

Since 1951, there has been a fair advance in knowledge of ruminal modification involved in digestion of dietary fats in ruminants (Garton, 1965). Thus, the problem of explaining why ruminant depot fats contained higher levels of saturated fatty acids than did the dietary source (together with lower levels of the corresponding unsaturated fatty acids) was clarified, in that dietary unsaturated fatty acids were shown to be hydrogenated in the rumen. Studies involving infusion of diets rich in linolenic and linoleic acids into the duodenum of a sheep (Ogilvie, McClymont and Shorland, 1961) resulted in increased levels of unsaturated fatty acids in the perinephric depot fat of the sheep, indicating that when unsaturated fatty acids in the diet bypass the rumen, they are incorporated into depot fats unchanged.

It is generally accepted that in ruminants, as in monogastric animals, fat is absorbed from the small intestine and passes to the blood stream (Willey, Riggs, Colby, Butler, and Reiser, 1952), and from the blood stream

to depot or milk fat. Little is known in detail regarding these processes in ruminants, although it has been shown that cows fed a diet of iodinated triglycerides exhibited a rise in iodinated lipid in the blood. This rise reached a maximum in 1.5 to 2 days, and then dropped until after 5 days a negligible amount was present (Aylward, Blackwood, and Smith, 1937). From measurement of arterio-venous differences across the mammary gland, first of blood lipid content (Maynard, McKay, Ellis, Hodson, and Davis, 1938), and later of neutral fat glycerol (Voris, Ellis, and Maynard, 1940), it can be inferred that glycerides in blood plasma are an important source of lipids for milk fat synthesis.

With this knowledge, the consequences of intervention in the digestion of dietary fat by rumen microorganisms become clearer. Unsaturated fatty acids of the diet are hydrogenated in the rumen and the more saturated derivatives of these fatty acids pass into the ruminant blood stream either to form part of depot fat reserves, or, in lactating animals, milk fats. Thus, the "hardness" (as opposed to "softness") of ruminant depot fats depends on the degree of hydrogenation of unsaturated fatty acids of the diet taking place in the rumen.

Hydrogenation is the only important modification of long chain free fatty acids which occurs in the rumen, as was shown by Wood, Bell, Grainger, and Teekell (1963). In this study, wethers with ligated reticulo-omasal orifices had 1-^{14}C-linoleic acid placed in their rumens. Recovery of radioactive compounds from jugular blood, rumen mucosa, visceral organs, and the rumen itself showed that 85 - 96% of the added radioactive linoleic acid remained as long chain fatty acids in the rumen, but only 3 - 6% was unchanged linoleic acid, the rest being either hydrogenated to saturated acids (45%) or to octadecenoic acids (30 - 50%). Of the radioactivity not remaining as long chain fatty acids in the rumen, about 1% had been degraded

in the rumen, and approximately 9% had passed through the rumen wall into the blood stream. This 9% would include any volatile fatty acids produced after degradation of the 1-^{14}C-linoleic acid or its more saturated derivatives. Although the 1-^{14}C-linoleic acid was present for up to 48 hours in the rumen, in a normal animal long chain free fatty acids would pass more rapidly to the small intestine, thus allowing less hydrogenation and probably little or no degradation in, or absorption from, the rumen.

1.2. Hydrolysis Of Glycerides In Rumen Fluid

Very little information is available regarding hydrolysis of glycerides added to rumen fluid, especially with respect to specificity.

In a study made by Garton, Lough, and Vioque (1961), three natural triglycerides differing in degree of saturation were incubated with rumen fluid obtained from sheep fed on hay and concentrates. The results are summarised in Table 1.

TABLE 1

The effect of triglyceride composition on degree of hydrolysis in rumen fluid

Triglyceride	% saturated fatty acids	% octadecenoic fatty acids	% octadecadienoic and octadecatrienoic fatty acids	% hydrolysis of triglyceride
linseed oil	11.4	21.6	67.0	95
olive oil	13.9	80.7	5.4	68
cocoa butter	58.6	38.1	3.1	40

Garton *et al* (1961) note that the more saturated triglycerides were harder to emulsify, and therefore may not have been as readily available for hydrolysis. It was concluded that rumen bacteria produce a lipase.

Garton et al (1961) also carried out partial hydrolyses of linseed oil in rumen fluid. After 20% hydrolysis, no change in composition of the "residual glycerides" was seen. However, after hydrolysis had been completed to the extent of 32%, changes were noted in the fatty acid composition of the "residual glycerides", and in the free fatty acid fraction. The results of the 32% hydrolysis are shown in Table 2.

TABLE 2

The fatty acid composition of the free fatty acid and "residual glyceride" fractions obtained following 32% hydrolysis of linseed oil in rumen fluid

type of fatty acid	fatty acid composition of (a) free fatty acid fraction	(b) "residual glycerides" fraction	(c) linseed oil (unhydrolysed)
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

The results shown in Table 2 led Garton et al (1961) to suggest that hydrogenation of unsaturated fatty acids free in the rumen proceeded more rapidly than hydrogenation of unsaturated fatty acids remaining in esterified form. Monoglycerides or diglycerides could not be isolated from the "residual glycerides", and Garton et al (1961) concluded that these intermediates, if formed at all, had a very transient existence, and that there was no specificity of hydrolysis for fatty acids esterified at either the

primary or secondary hydroxyl groups of the glycerol molecule.

In an *in vivo* study by Hawke and Robertson (1964), monoglycerides and diglycerides were isolated from the rumen after infusion of linseed oil, and diglycerides were recovered from the rumen of a cow fed on fresh pasture alone. As Garton *et al* (1961) did not find these intermediates, Hawke and Robertson (1964) concluded that metabolism may differ in the rumen of animals fed on fresh pasture and on hay and concentrates.

Hawke and Robertson (1964) suggested that the 1- and 2-galactosidases present in rumen microorganisms (Conchie and Levvy, 1957) may lead to formation of diglycerides in the rumen of animals fed on grasses and clovers.

1.3. Hydrogenation Of Unsaturated Fatty Acids in Rumen Fluid

It has been well proven since the work of Reiser (1951) that unsaturated fatty acids of the diet are hydrogenated by rumen microorganisms, making available more saturated fatty acids than are present in the diet for incorporation into the depot and milk fats of ruminant animals.

That this hydrogenation takes place in the rumen itself was demonstrated by incubation of unsaturated fats with rumen fluid and isolation of the products, the latter being generally much more saturated (Garton, Hobson, and Lough, 1958). Boiling of rumen fluid before such an incubation resulted in no increase in saturation, and the same result was obtained using rumen fluid from which the microorganisms had been removed by ultra-centrifugation (Garton *et al*, 1961). The work of Ogilvie *et al* (1961) also supports the hypothesis that hydrogenation takes place in the rumen.

The first detailed study of the intermediates of hydrogenation by rumen fluid was carried out by Shorland, Weenink, Johns, and McDonald (1957), who incubated 5 gm. of unsaturated fatty acid with 700 ml. of rumen fluid (obtained from sheep after a 6-hour fast following grazing on ryegrass-white clover pasture) for 48 hours at 37°. The pH of the incubation medium was maintained

TABLE 3

Fatty acids formed following incubation of unsaturated fatty acids with sheep-rumen contents

fatty acid added	fatty acid (% by weight)							
	palmitic acid	stearic acid	octadecenoic acids	octadecadienoic acids		octadecatrienoic acids		trans-acids
				n-c	c	n-c	c	
none (control)	14	51	29	trace	trace	trace	trace	-
oleic acid	-	23	59	1	1	1	1	17
linoleic acid	-	16	45	19	15	trace	trace	48
linolenic acid	-	17	72	trace	1	1	trace	67

n-c = non-conjugated

c = conjugated

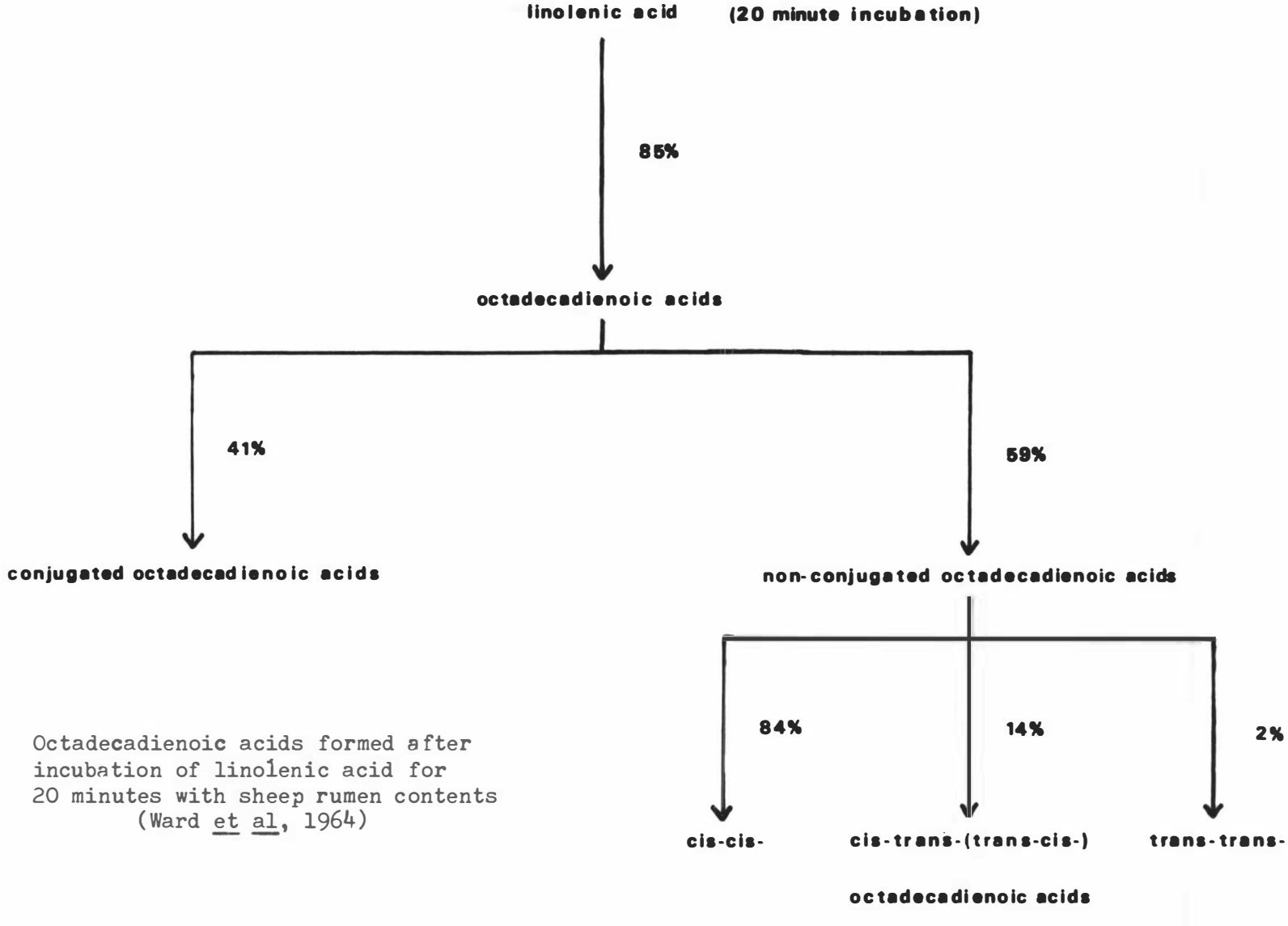
between 5.8 and 6.5 by addition of solid sodium bicarbonate as required.

The results of this work are summarised in Table 3, and show the formation of two types of fatty acid which are unique in ruminant depot and milk fat amongst herbivores: trans-unsaturated fatty acids, and conjugated octadecadienoic fatty acids (Hartman, Shorland, and McDonald, 1954; Hartman, Shorland, and McDonald, 1955; Hansen, 1963). During hydrogenation in rumen fluid, linolenic acid underwent more geometric isomerisation to trans-acids (67%) than linoleic acid (48%), which in turn showed a higher formation of trans-acids than oleic acid (17%). Shorland et al (1957) found that the dienoic acid arising from initial hydrogenation of linolenic acid is a non-conjugatable octadeca-11, 15-dienoic acid. The term non-conjugatable implies that a further migration of either double bond in either direction would still not result in a conjugated double bond system. Non-conjugatable fatty acids therefore, such as those formed during hydrogenation of linolenic acid to a dienoic acid, would have given rise to fatty acids available for hydrogenation. It was also found (Shorland et al, 1957) that conjugated acids appeared resistant to further hydrogenation.

Ward, Scott, and Dawson (1964) incubated radioactive fatty acids in an artificial rumen consisting of a dialysis sac containing 60 ml. of rumen fluid (obtained from Clun Forest sheep fed on a 5:1 (w:w) diet of hay : oats at 1200 gm. per day) around which flowed the supernatant obtained after centrifugation of rumen fluid from the same source. The apparatus was kept at a temperature of 39°, and was maintained in an anaerobic state by continuous gassing with either N_2:CO_2:CH_4 (19:11:10 by volume) or N_2:CO_2 (19:1 by volume). The pH was kept relatively constant with removal by dialysis of volatile fatty acids produced by microorganism metabolism.

In this study (Ward et al, 1964), much smaller amounts of fatty acid were incubated in comparison with the work of Shorland et al (1957). This

FIGURE 1



Octadecadienoic acids formed after incubation of linolenic acid for 20 minutes with sheep rumen contents (Ward et al, 1964)

difference had a marked effect on the times taken for hydrogenation in the two studies. Shorland et al (1957) found 25 micromoles of linolenic acid per ml. of rumen fluid to be converted to stearic acid and octadecenoic acid to the extent of 89% after 48 hours, whereas Ward et al (1964) found that 1 micromole per ml. was completely converted to stearic acid and octadecenoic acid after 4 hours.

The time-course of hydrogenation of U-^{14}C-linolenic acid in the artificial rumen used by Ward et al (1964) is summarised in Table 4.

TABLE 4

The radioactive fatty acids obtained during incubation of U-^{14}C-linolenic acid in rumen fluid

Acid in which radio-activity is recovered	Time of incubation (minutes)		
	20	180	240
octadecanoic acid	1	39	60
octadecenoic acid	2	33	40
octadecadienoic acid	84	14	0
octadecatrienoic acid	13	14	0
	100%	100%	100%

Of the octadecadienoic acids formed after 20 minutes, Ward et al (1964) found that 41% were probably conjugated, the remainder non-conjugated. Of the latter, 84% were cis-cis-octadecadienoic acids, 14% were cis-trans- (or trans-cis-) octadecadienoic acids, and 2% were trans-trans-octadecadienoic acids. This is summarised in Figure 1.

The positions of the double bonds in the non-conjugated octadecadienoic acids were investigated by oxidation with periodate and permanganate, the fatty acids resulting being separated by gas-liquid chromatography. The results are shown in Table 5.

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TABLE 5

The products of oxidation of the non-conjugated acids obtained after incubation of U-^{14}C-linolenic acid with rumen fluid

monocarboxylic acids	% present	short-chain dicarboxylic acids	% present	long chain dicarboxylic acids	% present
acetic	55	malonic	6	azelaic	15
propionic	40	succinic	57	sebacic	8
butyric	3	glutaric	25	undecanedioic	44
valeric	1	adipic	12	dodecanedioic	26
hexanoic	1			tridecanedioic	5
				tetradecanedioic	2

There was a majority of double bonds at the positions 11 or 12 and 15 or 16. Shorland et al (1957) found that the major non-conjugated dienoic acid arising from hydrogenation of linolenic acid was an octadeca-11,15-dienoic acid.

Further information was obtained from analysis of the octadecenoic acids produced, using permanganate-periodate oxidation. This process forms monocarboxylic acids from the methyl end of the oxidised octadecenoic acid. The results are shown in Table 6.

TABLE 6

The products of oxidation of the octadecenoic acids obtained after incubation of U-^{14}C-linolenic acid with rumen fluid

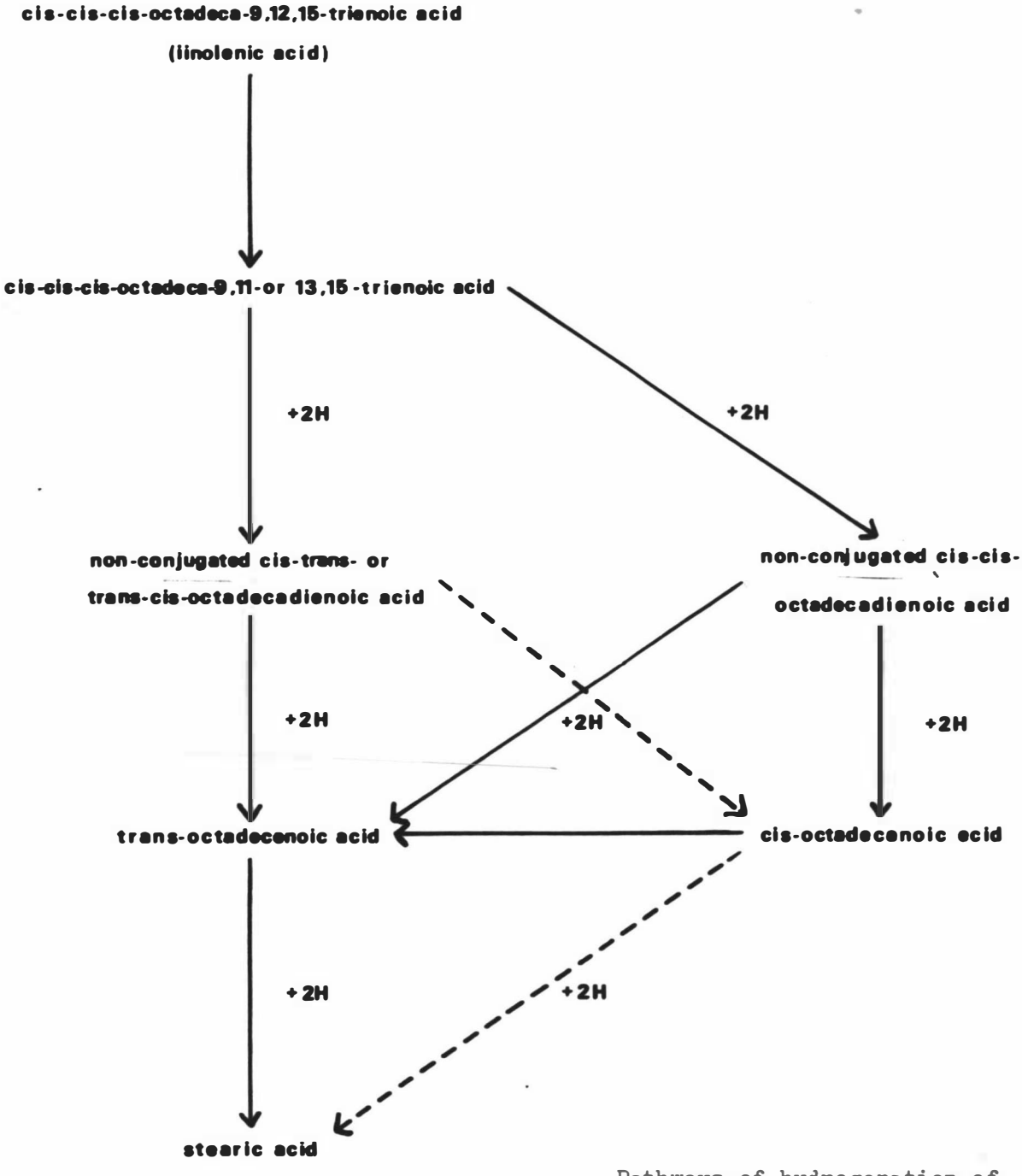
monocarboxylic acids	% present
formic	2
acetic	6
propionic	10
butyric	21
valeric	22
hexanoic	14
heptanoic	12
octanoic	6
pelargonic	7

The majority of octadecenoic acids are octadec-13- and 14-enoic acids, with a range from octadec-9- to 17-enoic acids. This result may be compared with that of Shorland *et al* (1957), who found that the main octadecenoic acids formed on hydrogenation of linolenic acid were octadec-11-, 12-, and 13-enoic acids.

Both Shorland *et al* (1957) and Ward *et al* (1964) found that the octadecenoic acids resulting from linolenic acid hydrogenation were mainly *trans*-octadecenoic acids. Ward *et al* (1964) showed that oleic acid isomerises to a *trans*-isomer, which is then hydrogenated to stearic acid, and concluded that a major pathway of hydrogenation *via* *trans*-octadecenoic acids existed, with any *cis*-octadecenoic acids formed isomerising to *trans*-octadecenoic acids before hydrogenation.

Wilde and Dawson (1966) reported further studies on hydrogenation of linolenic acid by rumen fluid, in which a detailed characterisation of the

FIGURE 2



Pathways of hydrogenation of linolenic acid by rumen microorganisms (Wilde and Dawson, 1966)

intermediates was carried out. Small amounts of U-^{14}C-linolenic acid were used, a typical incubation involving 0.5 gm. of linolenic acid and 4 ml. of rumen contents (2 micromoles of linolenic acid per ml., which is not much higher than the loadings of Ward et al (1964)).

Results of this work were similar to those obtained by Ward et al (1964), except that the major pathway involved cis-trans- (or trans-cis-) octadecadienoic acids, instead of cis-cis-octadecadienoic acids. The detailed characterisation of intermediates showed that the first product of hydrogenation of linolenic acid was an octadecatrienoic acid with two double bonds conjugated. Wilde and Dawson (1966) suggested that this acid was either octadeca-9,11,15-trienoic acid or octadeca-9,13,15-trienoic acid with an all-cis geometric structure. Kepler and Tove (1967) isolated a linoleate cis-12,trans-11-isomerase from the cell envelope of Butyrivibrio fibrisolvens, a bacterium common in the rumen. This enzyme was shown to catalyse the isomerisation of linolenic acid to a tentatively identified octadeca-cis-9-trans-11-cis-15-trienoic acid. Shorland et al (1957) found that the major dienoic acid formed on hydrogenation of linolenic acid in rumen fluid was an octadeca-11,15-dienoic acid, and a similar conclusion was reached by Kepler and Tove (1967). Such an acid would be formed by hydrogenation of an octadeca-9,11,15-trienoic acid at the 9-position.

Wilde and Dawson (1966) suggested a scheme for hydrogenation of linolenic acid, shown in Figure 2. The major pathway involved trans-octadecenoic acids, and non-conjugated cis-cis-octadecadienoic and cis-octadecenoic acids were converted by isomerisation, and by isomerisation and hydrogenation, respectively, to trans-octadecenoic acids.

Polan, McNeill, and Tove (1964) showed that B. fibrisolvens was able to hydrogenate linoleic acid to an octadecenoic acid, but not to stearic acid. Kepler, Hirons, McNeill, and Tove (1966) found that B. fibrisolvens

could hydrogenate a mixture of cis-9-trans-11-octadecadienoic acid and trans-10-cis-12-octadecadienoic acid as readily as linoleic acid itself, whereas trans-9-trans-12-octadecadienoic acid, cis-9-trans-12-octadecadienoic acid, and trans-9-cis-12-octadecadienoic acid were not hydrogenated. Kepler et al (1966) suggested that the two conjugated cis-trans-octadecadienoic acids might be intermediates in linoleic acid hydrogenation by B. fibrisolvens, and this was shown by incubation of 60 mg. of linoleic acid in a 20 ml. suspension of B. fibrisolvens for 2.5 hours. Gas-liquid chromatography showed that the methyl esters of the products consisted of 6% linoleic acid and 4% octadecenoic acid, with the remaining 90% a mixture of cis-trans- and trans-cis-octadecadienoic acids. Ozonolysis of these dienoic acids and gas-liquid chromatography of the products gave only a C7 aldehyde and a C9 aldehydo-ester, showing that there were no other dienoic acids formed than the 9,11-isomers.

This finding agrees with that of Shorland et al (1957) in that conjugated octadecadienoic acids were formed from linoleic acid, but differs in that no other octadecadienoic acids were formed. 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.

Characterisation of the octadecenoic acids formed on hydrogenation of either linoleic acid or the cis-trans- and trans-cis-octadecadienoic acid intermediates (Kepler et al, 1966) showed that the octadecenoic acids were all trans-acids, both trans-9-octadecenoic acid and trans-11-octadecenoic acid being present. Incubation of up to 24 hours showed that almost complete conversion of linoleic acid to trans-octadecenoic acids had occurred.

1.4. Factors Affecting The Degree Of Hydrogenation Of Unsaturated Fatty Acids Of The Diet Which Takes Place In The Rumen, With Respect To The Composition Of Depot And Milk Fats

Despite the fact that the composition of ruminant fats is less dependent on the diet than that of non-ruminant fats, several studies have indicated that the degree of saturation of depot and milk fats of ruminant animals may change in response to the diet.

Hydrogenation of unsaturated fatty acids of the diet is carried out by microorganisms in the rumen (Reiser, 1951), and so the nature and state of the population of hydrogenating microorganisms present in the rumen will determine the extent to which hydrogenation of dietary lipid will take place. There is however, some evidence (Garton et al, 1961; Patton and Kesler, 1967) that hydrolysis of dietary glycerides is a prerequisite for hydrogenation of dietary fatty acids, as esterified fatty acids do not seem to undergo hydrogenation to the same extent. If this is so, hydrolysis may become a rate-limiting step preceding hydrogenation in the rumen. As hydrolysis is also carried out by microorganisms in the rumen (Garton et al, 1961), factors affecting hydrogenation will affect hydrolysis to a similar extent, provided that such factors exert their effects through interference (either favourable or unfavourable) with the microorganisms responsible.

Early studies of the effect of unsaturated fats of the diet on the composition and physical characteristics of depot fat of steers (Thomas et al, 1934) and sheep, goats, steers, and swine (Edwards and Holley, 1939) showed that no significant "softening" or increase in Iodine Number resulted in the depot fats of the ruminants studied. It was concluded that dietary fats had no noticeable effect on the composition of ruminant depot fats.

A study by Willey et al (1952) was based on four groups of steer calves divided according to high or low fat and high or low energy levels in the diet. Thus the four groups were classified as "low fat-low energy",

"low fat-high energy", "high fat-low energy", and "high fat-high energy". By "energy" was meant productive energy content in therms, calculated according to Fraps (1947).

The "low fat-low energy" and "low fat-high energy" groups of the trial of Willey et al (1952) are comparable to the two groups of a trial carried out by Shaw, Ensor, Tellechea, and Lee (1960) in terms of energy and fat content of the diets. Shaw et al (1960) used two groups of steer calves, one fed on a diet of chopped alfalfa hay, ground corn, and linseed oil meal, and the other (the "high energy" group) fed a similar diet, but with more easily digestible components substituted (ground and pelleted alfalfa hay, steamed flaked corn, together with linseed oil meal). The results of these trials are summarised in Table 7.

TABLE 7

The effect of diet on Iodine Number of subcutaneous, visceral, and body fats of steer calves

	diet	Iodine Number	% increase
subcutaneous fat*	normal	44.2	-
	high energy	46.6	0.54
visceral fat*	normal	35.3	-
	high energy	42.4	20.1
body fat+	low fat-low energy	42.48	-
	low fat-high energy	44.95	0.58
body fat+	low fat-low energy	42.48	-
	high fat-low energy	39.55	-6.9

* Shaw et al (1960)

\+ Willey et al (1952)

Contrary to the theory accepted by Garton (1960), based on the earlier findings of Thomas et al (1934) and Edwards and Holley (1939), the studies of Willey et al (1952) and Shaw et al (1960) indicated that under certain circumstances, dietary fats can affect the level of saturation of ruminant depot fats. It seems that from the study of Willey et al (1952) feeding high levels of unsaturated fats (other components of the diet remaining unchanged) may increase the saturation of depot fats, and that from the study of Shaw et al (1960) addition of easily digestible components to the diet (with no change in fat content) may decrease the saturation of depot fats.

It is difficult to predict with any certainty the effect of dietary fats on the composition of ruminant depot fats. Shaw et al (1960) explained a decreased saturation of visceral fats following addition of more easily digestible components to the diet of steer calves by suggesting a change in the type of microorganism population present in the rumen (see Leffel, Lakshmanan, Brown, and Shaw, 1956; Eusebio, Shaw, Leffel, Lakshmanan, and Doetsch, 1959). It follows that the effect of dietary fats on depot and milk fats of ruminants must be carefully interpreted because the type of digestion occurring in the rumen tends to vary with the diet being fed, as a result of altering microorganism populations. Thus, it could be concluded from the results of Shaw et al (1960) that addition of readily digestible foodstuffs to a diet containing unsaturated fats results in a changed microorganism population such that, although fermentation is increased in the rumen, hydrogenation of unsaturated fatty acids is detrimentally affected, resulting in lower levels of saturated fatty acids becoming available to the ruminant itself. Similarly, from the study of Willey et al (1952), the conclusion could be made that the foodstuffs fed with the high and low levels of fat in the diet were such that the population of hydrogenating microorganisms present in the rumen was little affected, resulting in the

usual saturated fatty acids being passed from the rumen to the small intestine. Addition of extra unsaturated fat (cottonseed oil) would lead to an increase in the levels of saturated fatty acids, especially stearic acid, becoming available for depot fat synthesis.

It is interesting in this connection to compare the productive energy content in therms (Fraps, 1947) of the foodstuffs used by Willey et al (1952) and Shaw et al (1960). The difference between the low and high energy diets used by Shaw et al (1960) was 46.3 against 59.8, or a change of 29.2%, which would be expected to have some effect on the type of microorganism population present in the rumen and consequently on the degree of hydrogenation occurring there. In ccntrast, the difference between the low fat-low energy and low fat-high energy diets in the study of Willey et al (1952) was 57.85 against 63.89, an increase of 10.4%, which would be expected to have a lesser effect on the type of microorganism population present in the rumen, allowing normal hydrogenation to continue.

There have been more studies carried out to investigate effects of dietary fat on the production and composition of milk fat in ruminants than on depot fat. This greater interest seems to have arisen from the finding that a large proportion of milk fatty acids are synthesised in the mammary gland from volatile fatty acid precursors (Popják, French, Hunter, and Martin, 1951) which result from fermentation of certain dietary constituents in the rumen (see Baker, Harriss, Phillipson, McNaught, Smith, Kon, and Porter, 1947-48; Oxford, 1955; Phillipson and Cuthbertson, 1956; Cuthbertson, 1958). The proportions of volatile fatty acids produced are greatly dependent on the type of diet (Balch and Rowland, 1957; Shaw, 1958; Ensor, Shaw, and Tellechea, 1959). The main effect of interest here is that an increase in the proportions of acetic acid in the rumen results in increased production of milk fat, whereas an increased proportion of propionic acid

depresses the secretion of milk fat. However, about 27% of milk fat is synthesised by direct incorporation of long chain fatty acids derived from the diet (Glascock, Duncombe, and Reinius, 1956). These may be completely saturated or less saturated according to the degree of hydrogenation occurring in the rumen. Thus, the composition of milk fat can be affected by the diet either by an increase or decrease in the level of hydrogenation of unsaturated fatty acids from the diet, or by an increase or decrease in the level of fermentation leading to acetic acid production. The final composition of milk fat is determined by the summation of these interacting effects.

The effects on milk fat of feeding diets containing certain added fats have been summarised by McDowall, Reid, and Patchell (1957). Feeding linseed oil, soyabean oil, and peanut oil tends to increase the Iodine Number of milk fat, with, in many cases, a decrease in the Reichert Number of the milk fat. In general, the Iodine Number of the milk fat tends toward that of the added fat in the examples quoted by McDowall *et al* (1957). A decrease in Reichert Number suggests a type of fermentation in the rumen resulting in decreased production of acetic acid. Thus, the mammary gland receives a reduced supply of the precursor for fatty acid synthesis, and, to maintain milk fat production, substitution is made with long chain fatty acids taken up from the blood stream, such fatty acids being derived either directly from the diet, or from the diet *via* depot fat reservoirs. A study of the fatty acid composition of the milk fat of identical twin dairy cows grazing on ryegrass at two stages of maturity was made by Hawke (1963). The ryegrass was shown to contain more lipid at the less mature stage of growth (8% against 5% on a dry weight basis), and also the less mature ryegrass contained significantly higher proportions of unsaturated fatty acids, mainly linolenic acid.

It was found (Hawke, 1963) that the milk fat of the cows grazing on the ryegrass with the higher lipid content contained increased levels of unsaturated C18 acids, decreased levels of saturated fatty acids from hexanoic to hexadecanoic acids, and increased levels of butyric acid. Hawke (1963) concluded that the higher unsaturated lipid content of the less mature ryegrass caused a decrease in hydrogenation of unsaturated fatty acids in the rumen, resulting in the increased levels of these acids in milk fat. This conclusion was based on the findings of Shaw et al (1960), leading Hawke (1963) to suggest that the less mature ryegrass was more readily digestible. Hawke (1963) was unable to differentiate between the effects of increased levels of unsaturated fat or the increased digestibility of the less mature ryegrass on the degree of hydrogenation occurring in the rumen, but suggested that these effects would be complementary in increasing the content of unsaturated fatty acids in milk fat.

It seems clear that the degree of hydrogenation in the rumen is influenced by several interrelated effects of the diet on the microorganism population present. The results of these effects on hydrogenation are reflected in changing long chain fatty acid composition of milk and depot fats in ruminants.

Chapter 2

THE AIM OF THE PRESENT STUDY

The process of hydrolysis of dietary fats in the rumen has not been studied in any great detail (Chapter 1, section 1.2.). It is known that dietary glycerides are hydrolysed in the rumen (Hawke and Robertson, 1964) and by rumen fluid *in vitro* (Dawson, 1959; Garton *et al*, 1961). There is some indication of the types of microorganism involved, in that Hobson and Mann (1961) isolated a lipolytic bacterium from the rumen, and Garton *et al* (1961) prepared a washed suspension of rumen microorganisms which exhibited lipolytic activity. However the enzyme(s) responsible have not been obtained, and the mechanism of the reaction is not known. Neither is it known whether any specificity of hydrolysis for component fatty acids or for fatty acids esterified to primary or secondary hydroxyl groups of the glycerol molecule occurs. The possibility of specificity was investigated in the present study by incubation of synthetic triglyceride isomers containing radioactive linolenic acid esterified to either the primary or secondary hydroxyl group of the glycerol molecule, and following the time-course of hydrolysis and liberation of component fatty acids.

Hydrogenation of unsaturated fatty acids in the rumen (Hawke and Robertson, 1964) and in rumen fluid (Shorland *et al*, 1957) is understood in some detail. Kepler *et al* (1966) showed that *B. fibrisolvens* was able to hydrogenate linoleic acid to octadecenoic acids, but no other hydrogenating microorganism has been isolated. To demonstrate in the present study that hydrogenation of unsaturated fats takes place in the rumen, freshly-cut pasture grass was fed to a dairy cow. That the results obtained could be reproduced *in vitro* was shown by incubation of dried perennial ryegrass

with rumen fluid in a non-permeable system.

As outlined by Patton and Kesler (1967), the relationship between hydrolysis and hydrogenation in the rumen is of particular interest. There is some evidence (Garton et al, 1961; Hawke and Robertson, 1964) that hydrolysis of dietary fats precedes hydrogenation, which may involve only unsaturated fatty acids free in the rumen, and not esterified fatty acids. In order to investigate the relationship between hydrogenation and hydrolysis, synthetic triglycerides (see above) were incubated with rumen fluid, special attention being paid to the stage of hydrogenation reached in partially hydrolysed glycerides and in the free fatty acids produced.

Chapter 3

MATERIALS AND METHODS

3.1. Solvents and Reagents

All solvents used were purified by distillation as described by Vogel (1956), and reagents were of "Analar" or "Technical" grade. U-^{14}C-linolenic acid and 1-^{14}C-linolenic acid were obtained from the Radiochemical Centre, Amersham, England.

3.2. Incubation Procedures

3.2.1. Method

A non-lactating Jersey x Aberdeen Angus cow was used for *in vitro* experiments. The cow was fed on grass and hay prior to the experiments, with a daily intake of approximately 15 lb of dry matter of which 80% was hay (Wilson, *pers. comm.*). Before experiments the cow was starved overnight and at the start of the sampling period was fed *ad lib.* a weighed quantity of freshly-cut pasture grass similar to that from which the cow was taken. Before each experiment the fatty acid composition of the grass was determined, and a sample of the rumen contents was taken to find the prefeeding level of unsaturated fatty acids. Sampling commenced one hour after the start of feeding and continued through the day to give a post-feeding period of up to 8 hours.

All *in vitro* experiments were carried out in 250 ml. glass conical flasks connected in series. The flasks were kept anaerobic by continuous steady passage of nitrogen. Boiling tubes were included in the system, one between each pair of conical flasks, as a safeguard against incubation medium passing from one flask to the next as a result of fluctuations of

gas pressure. Incubation was carried out by immersing the flasks to about three-quarters of their height in water in a large tank fitted with shakers. The temperature of the water was thermostatically controlled at 39°. Because of the impermeable nature of the system, incubations did not last longer than 4 hours. The incubation medium was rumen fluid obtained from a non-lactating dairy cow with a rumen fistula (sub-section 3.2.2.). For each incubation flask, 100 ml. of rumen fluid was used. The substrates for *in vitro* incubations were oven-dried perennial ryegrass harvested from a pure stand during spring of 1966, and synthetic triglycerides (sub-section 3.3.).

3.2.2. Sampling

Before sampling, the contents of the rumen were well mixed by hand. Crude rumen contents were obtained through a rumen fistula and strained through two layers of muslin cloth to remove large particles. The material remaining in the muslin was kept in some cases and its fatty acid composition determined.

Samples taken during *in vivo* experiments were transferred to a flask and an equal volume of ethanol added. The mixture was shaken and heated to boiling to stop any further reaction. After cooling, samples were stored at 5° until the fatty acids were extracted. The contents of *in vitro* flasks were treated in the same way.

3.2.3. Emulsification

It was necessary to emulsify radioactive triglycerides incubated *in vitro* in order to attain a reasonable rate of utilisation by rumen microorganisms. Garton *et al* (1961) found that incubation of cocoa butter or olive oil with rumen fluid presented similar difficulties. The emulsion consisted of equal parts of the triglyceride and water, with 1 part to 100 of Lissapol N-450 (Imperial Chemical Industries) as emulsifying agent. The emulsion was

prepared with a homogeniser, and pipetted into the incubation flasks immediately prior to incubation.

3.3. Synthesis of Radioactive Triglyceride

3.3.1. Synthesis of glyceryl 1-palmitate

Glyceryl 1-palmitate was prepared according to the method of Hartman (1960). The crystalline glyceryl 1-palmitate was checked for purity using thin layer chromatography (sub-section 3.5.2.).

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

Acylation of glyceryl 1-palmitate was carried out according to Mattson and Volpenhein (1962) with oleyl chloride, prepared as described by Mattson and Volpenhein (1962) from oxalyl chloride.

3.3.3. Isolation of the pure diglyceride isomers

The crude mixture resulting from acylation of glyceryl 1-palmitate was separated into triglyceride, free fatty acid, diglyceride, and monoglyceride fractions on acid-washed Florisil (Carroll, 1963). A standard glass Quickfit column with an inside diameter of 18 cm. was used. Acid-washed Florisil prepared according to Carroll (1963) was activated before use for 48 hours at 110°. It was found that a slurry of 40 gm. of acid-washed Florisil in hexane was sufficient to fill the column on settling to height of about 35 cm.

The crude mixture of 1,2-diglyceride and 1,3-diglyceride (sub-section 3.3.2.) was applied to the column in hexane : diethyl ether (19:1, v:v). The flow rate of the elution solvent was such that 200-300 ml. per hour was collected in 15 ml. fractions.

Separation of 1,2-diglyceride and 1,3-diglyceride was possible by slowly increasing the polarity of the elution solvent, as shown below:

compound	Elution Solvent (%) diethyl ether	hexane
glyceryl 1-palmitate-3-oleate	15-20	85-80
glyceryl 1-palmitate-2-oleate	20	80

There was very little overlap of the two diglyceride isomers, with about 10% of the mixture not being resolved. A typical separation resulted:

glyceryl 1-palmitate-3-oleate	60%	
mixed isomers	10%	
glyceryl 1-palmitate-2-oleate	30%	(by weight)

Thin layer chromatography as described in sub-section 3.5.2. was used to monitor the eluted fractions. The purified diglyceride isomers were stored at -20° until required.

3.3.4. Synthesis of Glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate and glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate

Acylation of the diglyceride isomers prepared as described in sub-section 3.3.2. was carried out using 1-^{14}C-linolenyl chloride obtained by equilibration of linolenyl chloride prepared according to Mattson and Volpenhein (1962) with 1-^{14}C-linolenic acid in hexane for two hours at room temperature (Borgstrom and Krabisch, 1963). A 50% molar excess of 1-^{14}C-linolenyl chloride was used, with a reaction time of 72 hours at room temperature (Mattson and Volpenhein, 1962).

The triglyceride isomers were purified on acid-washed Florisil columns (Carroll, 1963) and stored at -20° until required.

3.4. Extraction and Methylation of Fatty Acids

3.4.1. Extraction of Lipid

Samples obtained after *in vivo* and *in vitro* incubations were dispersed in an aqueous ethanol medium (sub-section 3.2.2.) which was evaporated under

vacuum at temperatures not exceeding 75°. The residue was extracted by refluxing with chloroform : methanol (2:1, v:v) on a sand bath for 2 hours. Samples containing radioactive material were extracted for two additional 1-hour periods. The extracts were combined, evaporated to dryness in a rotary evaporator, and the residue extracted with diethyl ether. The diethyl ether extract was filtered through a filter funnel plugged with cotton wool and the filtrate was stored at -20° until required.

3.4.2. Saponification and Extraction of Fatty Acids

Saponification of lipid was carried out by refluxing with an appropriate volume of 5% (w:v) potassium hydroxide in methanol on a sand bath for two hours.

The methanolic solution was then transferred to a separating funnel, and non-saponifiable material was extracted with diethyl ether. The aqueous fraction, which contained the potassium salts of the fatty acids, was acidified with 5N hydrochloric acid and the fatty acids formed extracted with diethyl ether. The diethyl ether extract was washed 3 times with water, dried over anhydrous sodium sulphate, and stored at 5° until required for methylation.

3.4.3. Methylation of Fatty Acids

Diazomethane was prepared from N-methyl-N-nitroso-toluene-p-sulphonamide by addition of 60% (w:v) potassium hydroxide and 2-methoxyethanol in a medium of diethyl ether. After standing for 30-40 minutes to allow complete reaction, the mixture was distilled in a fume cupboard and the diazomethane and diethyl ether collected in a boiling tube surrounded by ice. The diazomethane-diethyl ether mixture was added to the fatty acid sample using a Pasteur pipette fitted with a rubber bulb until yellow coloration due to excess diazomethane remained. Excess diazomethane and diethyl ether were removed by warming. The methyl esters of the fatty acids were redissolved in hexane and stored at 5°.

3.5. Thin Layer Chromatography

3.5.1. Preparation of Plates

The required amount of Silica Gel G (Merck Ag) was weighed out and placed in a screw-top jar with an appropriate volume of distilled water. The mixture was shaken into a slurry and poured into a Desaga applicator. Plates (full size = 20 cm. x 20 cm.) were spread in the usual manner and were activated in an oven at 110° for 30 - 60 minutes.

Both qualitative and preparative thin layer chromatography was used in the present study. Preparative plates were made 1 mm. thick, and qualitative plates 0.25 mm. thick. Preparative plates were activated for a longer period than qualitative plates.

3.5.2. Elution Solvents

Hexane and diethyl ether were dried over anhydrous sodium sulphate before mixing in the desired proportions. The solvent mixtures used were:

	Component (% by volume)		
purpose	hexane	diethyl ether	glacial acetic acid
separation of free fatty acids, triglycerides, diglycerides, and monoglycerides	70	30	1
purification of methyl esters of fatty acids	85	15	

3.5.3. Chromatographic Procedure

Glass capillary tubes were used to apply the samples to the chromatographic layer. Following development in chromatographic tanks lined with filter paper, plates were dried and sprayed with a 0.1% (w:v) solution of 2,7-dichlorofluorescein in ethanol and viewed under ultraviolet light.

Components were identified by comparison with known compounds chromatographed simultaneously on the same plate.

Prior to elution of components from preparative thin layer chromatographs, silica gel other than that on which the required components were adsorbed was scraped away. The remaining zones of silica gel were scraped into centrifuge tubes containing elution solvent. The tubes were shaken until solvent and silica gel were well mixed, centrifuged, and the supernatents decanted. The solvents used in elution of compounds from silica gel were:

compound	solvent
glycerides and free fatty acid	chloroform : methanol (2:1, v:v) with 1 drop of water
methyl esters of free fatty acids	diethyl ether

3.5.4. Scanning of Thin Layer Chromatograms containing Radioactive Components

To determine the distribution of radioactivity between components of a mixture of free fatty acid and glycerides, quarter size (20 cm. x 5 cm.) qualitative plates were used. The areas of radioactivity were determined by a Packard Model 7200 Radiochromatogram Scanner modified to take thin layer plates.

3.6. Gas-liquid Chromatography

3.6.1. Apparatus

Two gas-liquid chromatographs were used. For non-radioactive samples in earlier work, a Pye-Argon Chromatograph fitted with an argon ionisation detector containing a 20 millicurie strontium 90 radiation source (Lovelock, 1958) was used to separate methyl esters of fatty acids. In later work, a Varian Aerograph Model 1520 Chromatograph fitted with a hydrogen flame ionisation detector was used. For separation of methyl esters of radioactive fatty acids, the Aerograph Chromatograph was modified by addition of a stream splitter which passed 8-10% of the sample to the flame ionisation detector

and the remainder to a proportional counter (Nuclear Chicago) connected to a single-channel ratemeter (Nuclear Chicago). The best results were obtained at 2900 volts, a linear range of 30K, and a time constant of 1 second. Butane gas was passed through the proportional counter at a rate of 60 ml. per minute to give a carrier gas : butane ratio of approximately 1:1. Impulses were collected by the ratemeter and recorded on a dual channel Westronic recorder. The mass record of methyl esters of fatty acids was shown on the other channel of the recorder.

3.6.2. Columns

Columns in the Pye-Argon Chromatograph were made of glass, and were 1 metre in length with an internal diameter of 4 mm. Columns used in the Aerograph Chromatograph were made of stainless steel. In earlier work with the Aerograph Chromatograph 5' x $\frac{1}{8}$" columns were used, but, when it was found that the specific activity of the samples was low, 5' x $\frac{1}{4}$" columns were used to permit application of larger samples.

Column packings were either 20% DEGS on 85-100 mesh Celite 545 (Pye-Argon Chromatograph) or 12% DEGS on 60 - 80 mesh silanised Celite (Aerograph Chromatograph).

3.6.3. Operating Conditions

Operating conditions used for each chromatograph were as follows:

	Pye-Argon	Aerograph
Column temperature	148-165°	150-183°
Detector temperature	-	195-200°
Injector temperature	-	227-240°
Carrier gas	argon	helium
Flow rate of carrier gas	33-69 ml. min.$^{-1}$	30-44 ml. min.$^{-1}$
Detector voltage	1250 volts	-

Samples were introduced onto the column of the Pye-Argon Chromatograph using small lengths of capillary tube (approximately 1 mm.) with an internal diameter of 0.5 - 1.0 mm. The sample of methyl esters was dissolved in hexane and injected into one of these using a 10 microlitre syringe (Hamilton). The solvent was allowed to evaporate and the capillary tube containing the sample was introduced onto the top of the column by stopping the flow of carrier gas and opening the inlet. During this process a small volume of air entered the column, and after the gas flow was reconnected the negative air peak was used as a marker for measuring retention times of component methyl esters.

A 10 microlitre syringe (Hamilton) was used to introduce samples onto the column of the Aerograph Chromatograph by injection through a silicone rubber septum directly into the uninterrupted gas flow at a temperature which immediately vapourised the sample. The retention times of methyl esters were measured from the leading edge of the solvent peak.

All samples of methyl esters were purified by thin layer chromatography (sub-section 3.5.2.) before gas-liquid chromatography. This procedure resulted in level recorder baselines and absence of diffuse peaks due to unmethylated fatty acids.

3.6.4. Identification of Methyl Esters of Fatty Acids

Retention times of methyl esters were expressed as ratios of the retention time of methyl palmitate. These relative retention times were used to identify unknown methyl esters, either by comparison with known samples or by plotting the logarithm of base 10 of the relative retention time against the number of carbon atoms in the fatty acid (Hawke, Hansen, and Shorland, 1959). Methyl esters of radioactive fatty acids were similarly identified, and comparison with the simultaneous mass recording afforded a check on identification.

Planimetry was used to estimate the proportions of methyl esters in the sample. Each peak was measured three times and the average value taken. The area was expressed as a percentage of the total area of methyl esters present.

3.7. Radiochemical Methods

Radioactivity was measured by liquid scintillation counting using a vial head (Nuclear Enterprises Ltd.) connected to a pulse height analysing system (Philips).

The scintillation solution consisted of 0.6% (w:v) of 2,5-biphenyloxazole (PPO) and 0.05% (w:v) of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) dissolved in toluene.

A suitable aliquot of sample was placed in the counting vial using a 10 or 50 microlitre syringe (Hamilton). Scintillation fluid (5 ml.) was added after the solvent had been evaporated under a 250 watt heat lamp.

Before counting, the efficiency of the system was checked using a standard sample of U-^{14}C-hexadecane containing 1.29×10^{-2} microcuries of radioactivity.

TABLE 8

Fatty acid composition of samples obtained during *in vivo* hydrogenation of dietary lipid

Time after commencement of feeding (hours)	Trial G (25.5.67)							Trial J (6.7.67)						
	Amount of lipid in sample (g.)	Fatty acid (moles %)						Amount of lipid in sample (g.)	Fatty acid (moles %)					
		16:0	18:0	18:1	18:2	18:3			16:0	18:0	18:1	18:2	18:3	
0	0.67	39	28	23	3	7		0.15	40	20	14	8	18	
1	0.36	33	20	16	4	27		0.18	16	10	15	14	44	
2	0.39	30	27	22	9	12		0.20	22	16	19	15	28	
3	0.42	21	46	15	6	12		0.17	20	8	8	8	56	
4	0.27	34	20	15	8	23		0.32	25	13	16	12	35	
6	0.18	34	19	14	7	26		0.17	38	16	14	11	21	
8	0.20	35	18	16	9	22		0.14	33	21	15	11	19	
10	-	-	-	-	-	-		0.23	27	16	21	11	25	
pasture grass	(96.5 g. fed)	20	2	3	14	61		(70.8 g. fed)	10	2	2	12	74	

Chapter 4

RESULTS

4.1. In vivo Hydrogenation of Dietary Lipid

Before carrying out a study of the relationship between hydrogenation and hydrolysis of dietary lipid by rumen fluid, it was desirable to establish that hydrogenation of dietary lipid was occurring in the rumen under the conditions chosen for feeding. Fresh pasture grass was fed to a non-lactating cow in each of two trials, denoted G and J. The 28 lb. of pasture grass fed in trial G contained 0.76% of lipid (diethyl ether extract; wet weight basis), or a total of 96.5 g., and the 23 lb. fed in trial J contained 0.68% of lipid or a total of 70.8 g. The fatty acid composition, and the amount of lipid extracted, for each sample obtained in trials G and J is given in Table 8. The composition of the pasture grass fed in each trial is also given.

Although there appears to be no clear relationship between time and the proportions of the fatty acids present in trial G, a high positive correlation exists between the amount of lipid extracted and the proportion of stearic acid present. Similarly, a high negative correlation exists in each sample between the amount of lipid extracted and the proportion of linolenic acid. The possibility that increased amounts of lipid in the strained rumen fluid samples may give misleading results is discussed in Chapter 5.

With one exception the amounts of lipid extracted from each sample in trial J were within a relatively narrow range, and a relationship is seen to exist between time and the proportions of fatty acids present. One hour after the commencement of feeding, 44% of fatty acid was linolenic

TABLE 9

Comparison of the fatty acid composition of the residues and the fluid samples obtained by straining rumen contents (trial J)

Time after commencement of feeding (hours)	Residue after straining						Rumen fluid					
	Amount of lipid in sample (g.)	Fatty acid (moles %)					Amount of lipid in sample (g.)	Fatty acid (moles %)				
		16:0	18:0	18:1	18:2	18:3		16:0	18:0	18:1	18:2	18:3
0	0.32	28	4	24	10	34	0.15	40	20	14	8	18
1	0.83	24	35	11	8	22	0.18	16	10	15	14	44
10	0.74	23	42	20	5	10	0.23	27	16	21	11	25

acid. This proportion increased to 56% in 2 hours, then decreased to 25% after another 7 hours had elapsed. At the same time the proportions of stearic, oleic, and palmitic acids increased.

The rumen fluid samples of trials G and J were obtained by straining the crude rumen contents through muslin cloth. To determine whether the fatty acid composition of the strained fluid differed from that of the coarse material retained by the muslin cloth, the coarse material was recovered in three samples from trial J. The fatty acid composition of these three samples and of the fluid obtained from them is given in Table 9.

The two fractions differed markedly in fatty acid composition. The coarse material, after feeding, contained higher levels of saturated fatty acids. The relationship between this finding and the results obtained in trial G is discussed in Chapter 5.

4.2. In vitro Hydrogenation of Dietary Lipid

To establish the validity in the present study of *in vitro* incubation as a method of investigating hydrogenation of unsaturated fatty acids in the rumen, dried perennial ryegrass was incubated with rumen fluid. All incubation flasks were duplicated as a check on reproducibility. Results are shown in Table 10 together with the fatty acid composition of the dried perennial ryegrass and rumen fluid used.

The reproducibility of the results in Table 10 is not high, although where discrepancies occur there appears to be a positive correlation between the amount of lipid in the sample and the proportion of either stearic or oleic acid present. A similar correlation appeared in the results of trial G, and is discussed in Chapter 5. There was however, a discrepancy in the values obtained after 20 minutes, where similar amounts of lipid were extracted.

TABLE 10

Fatty acid composition of samples obtained during *in vitro* hydrogenation of dietary lipid (trial H)

Incubation time (minutes)	Incubation mixture		Amount of lipid in sample (g.)	Proportion of fatty acid in lipid (%)	Fatty acid (moles %)				
	rumen fluid	dried grass			16:0	18:0	18:1	18:2	18:3
0 a	35 ml.	1 g.	0.06	-	25	28	13	9	25
b	"	"	0.06	-	19	12	22	9	38
20 a	100 ml.	3 g.	0.20	30	22	38	15	8	17
b	"	"	0.21	29	21	12	25	15	27
60 a	"	"	0.33	24	25	45	17	6	8
b	"	"	0.18	22	24	32	23	8	13
120 a	"	"	0.22	23	23	19	33	10	15
b	"	"	0.31	13	18	9	50	13	11
180 a	"	"	0.31	29	19	41	22	8	10
b	"	"	0.24	17	17	20	26	24	13
rumen fluid a	50 ml.		0.02	-	38	20	19	7	16
b	"		0.04	-	35	15	18	8	24
dried grass	-		-	-	22	3	9	11	55

There was an overall trend of decreasing proportions of linolenic acid accompanied by increasing proportions of the more saturated C18 acids, especially oleic acid. Thus a similar result to that of trial J was obtained. In both trial H (*in vitro*) and trial J (*in vivo*) hydrogenation of linolenic acid occurred.

During the 3 hours of trial H there was no marked rise in the proportion of palmitic acid. After 3 hours, the proportions of stearic and oleic acids remained higher than at the commencement of the incubation.

4.3. *In vitro* Hydrolysis of Triglyceride

Five experiments were carried out, denoted 1, 2, 3, 4 and 5 in Table **11**. The triglyceride used in experiment 1 was glyceryl 1-palmitate-2-(U-^{14}C)-linolenate-3-oleate, in experiments 2 and 3 glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate, and in experiments 4 and 5 glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate. Incubation times are shown in Table 11 for experiments 1, 2, and 4. Little hydrolysis of triglyceride occurred in experiments 2 and 4, and, as the substrate triglyceride was difficult to synthesise, and therefore valuable, lipid was extracted from these experiments and reincubated to constitute experiments 3 and 5 respectively. The total incubation times for experiments 3 and 5 are shown in Table 11.

The synthetic triglyceride to be used in experiment 1 was purified on an acid-washed Florisil column (Carroll, 1963) but still contained 23% of free U-^{14}C-linolenic acid, as shown in Table 11 (0 minutes). In experiment 1 there was a steady loss of radioactivity from the triglyceride fraction after each incubation period, indicating hydrolysis of the triglyceride. The radioactivity appeared subsequently in the free fatty acid and monoglyceride fractions. After 3 hours 48% of the radioactivity was present as free fatty acid and 23% as monoglyceride. At no stage did any

TABLE 11

Percentage distribution of radioactivity during hydrolysis by rumen fluid of synthetic radioactive triglyceride

Incubation Time (minutes)	Fraction			
	Triglyceride	Diglyceride	Monoglyceride	Free Fatty Acid
	Experiment 1			
0	77	0	0	23
20	56	0	22	22
40	48	0	24	28
80	55	0	17	28
120	35	0	27	39
180	28	0	23	48
	Experiment 2			
0	90	3	7	0
20	78	13	9	0
60	73	14	13	0
120	73	11	16	0
240	80	7	13	0
	Experiment 3			
20+ 60	70	6	9	15
120+180	56	3	9	32
240+240	72	2	5	21
	Experiment 4			
0	100	0	0	0
60	100	0	0	0
120	100	0	0	0
	Experiment 5			
20+ 60	89	7	4	0
60+120	50	18	16	16
120+240	30	0	2	68

radioactivity appear in diglyceride.

The radioactive triglyceride to be used in experiment 2 was contaminated with small amounts of radioactive diglyceride (3% of total radioactivity) and monoglyceride (7%). Each incubation period of experiment 2 resulted in a relatively small loss of radioactivity from the triglyceride fraction by comparison with experiment 1. The greatest decrease was from 90% to 73% after both 60 and 120 minutes. No radioactivity appeared in free fatty acid, but was distributed between diglyceride and monoglyceride. Maximum radioactivity appeared in diglyceride after incubation for 60 minutes (14%) and in monoglyceride after 120 minutes (16%).

Experiment 3 consisted of reincubation of lipid extracted from flasks of experiment 2. No fractionation of this lipid was carried out, and the reincubation substrate therefore contained radioactive triglycerides, diglycerides, and monoglycerides. The results of experiment 3 (Table 11) show marked increases in radioactive free fatty acid. These increases were contributed to by radioactive free fatty acid derived from diglycerides and monoglycerides, in which the proportion of radioactivity decreased by 8% and 7% respectively, and from triglycerides, in which reincubation for 180 minutes resulted in a 17% decrease in the proportion of radioactivity (from 73% to 56%).

Results of experiment 4 indicate no loss of radioactivity from triglyceride after 120 minutes.

Significant hydrolysis occurred in experiment 5 after reincubation for 240 minutes, when triglyceride radioactivity decreased from 100% to 30%. A simultaneous increase in radioactive free fatty acid (0% to 68%) occurred, with a small amount of radioactivity remaining as monoglyceride (2%). Loss of radioactivity from triglycerides occurred to a lesser extent after reincubation for 60 or 120 minutes. After 120 minutes diglyceride,

TABLE 12

Radioactive fatty acids obtained after incubation of synthetic triglyceride

Reincubation time (minutes)	^{14}C-fatty acid			
	18:3	18:2	18:1	18:0
	experiment 3			
0	100	0	0	0
60	100	0	0	0
120	100	0	0	0
180	100	0	0	0
240	100	0	0	0
	experiment 5			
60	100	0	0	0
120	100	0	0	0
240	38	38	24	0

monoglyceride, and free fatty acid contained similar amounts of radioactivity (18%, 16%, and 16% respectively) whereas after 60 minutes only 11% of total radioactivity was not present in triglyceride. This radioactivity was localised in diglyceride (7%) and monoglyceride (4%).

4.4. Relationships between Hydrogenation and Hydrolysis of Unsaturated Triglyceride in Rumen Fluid

The radioactive fatty acids from each incubation flask of experiments 3 and 5 were identified by gas-liquid radiochromatography. The results are shown in Table 12.

No significant hydrogenation of 1-^{14}C-linolenic acid occurred except after reincubation for 240 minutes in experiment 5, where 62% of the linolenic acid was hydrogenated. Of this 62%, 38% was identified as octadecadienoic acids and 24% as octadecenoic acids. No other radioactive fatty acids were present. Significant hydrogenation occurred only where radioactive triglyceride had undergone a marked degree of hydrolysis (Table 11). This suggests that hydrogenation of unsaturated fatty acids free in the rumen may occur in preference to hydrogenation of unsaturated fatty acids in esterified form.

To investigate this process in more detail the radioactive fatty acids from the glyceride and free fatty acid fractions of experiment 5 were analysed by gas-liquid radiochromatography. The results are given in Table 13. There was insufficient radioactivity in the monoglyceride fractions to obtain satisfactory chromatographic recordings.

The results in Table 13 show that fatty acids free after reincubation were more saturated than those remaining in glyceride combination. After 80 minutes no hydrogenation of free 1-^{14}C-linolenic acid had taken place, suggesting that hydrogenation is specific for free fatty acids. After reincubation for 240 minutes, however, 30% of the radioactivity in the

TABLE 13

Radioactive fatty acids from free fatty acid and glyceride fractions after incubation of glyceryl 1-palmitate-2-oleate 3-(1-^{14}C)-linolenate (experiment 5)

Reincubation time (minutes)	^{14}C-fatty acids				
	18:3	18:2	18:1	18:0	other
	triglyceride				
60	100	0	0	0	0
120	100	0	0	0	0
240	100	0	0	0	0
	diglyceride				
60	100	0	0	0	0
120	100	0	0	0	0
240	70	23	7	0	0
	free fatty acid				
60	100	0	0	0	0
120	25	34	37	0	4*
240	8	42	50	0	0

* see text

diglyceride fatty acids was present as octadecadienoic acids (23%) and octadecenoic acids (7%), indicating that some hydrogenation of esterified fatty acids had occurred. Table 13 shows that after 180 minutes, 4% of the radioactivity in free fatty acids was other than linolenic, octadecadienoic, octadecenoic, or stearic acids. This radioactive fatty acid gave a single gas-liquid radiochromatogram peak with a retention time, relative to methyl palmitate, of 4.27. This may correspond to that of an octadecatrienoic acid with two conjugated double bonds, isolated by Wilde and Dawson (1966) after incubation of U-^{14}C-linolenic acid with rumen contents, which had an R_F on the same liquid phase of 4.39.

Although a high proportion (50%) of radioactive oleic acid was present in the free fatty acid fraction after reincubation for 240 minutes, no radioactive stearic acid was detected.

The total lipid obtained after reincubation for 240 minutes in experiment 3 was resolved by column chromatography using acid-washed Florisil (Carroll, 1963) into free fatty acid, triglyceride, diglyceride, and monoglyceride fractions. Of the fractions obtained, three contained sufficient radioactivity to allow separation by gas-liquid radiochromatography of the methyl esters of component fatty acids. These were triglyceride, diglyceride, and an unidentified fraction (5A). The R_F of fraction 5A on thin layer chromatography with hexane : diethyl ether : acetic acid (70:30:1 by volume) was slightly less than that of free fatty acid. After elution in chloroform negligible radioactivity was recovered from fraction 5A when shaken with 5% (w:v) sodium carbonate solution to remove contaminating free fatty acid. Saponification with 5% (w:v) potassium hydroxide in methanol yielded radioactive fatty acids which were separated by gas-liquid radiochromatography. The results are shown in Table 14 together with the radioactive fatty acid composition of the triglyceride and diglyceride fractions.

TABLE 14

Radioactive fatty acids of fractions isolated after reincubation of glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate for 240 minutes

fraction	^{14}C-fatty acids (moles %)				
	18:3	18:3c+	18:2	18:1*	16:0*
triglyceride	79	21	0	0	0
diglyceride	50	27	23	0	0
5A	39	0	0	22	39

* tentative identification

\+ conjugated octadecatrienoic acid

A high proportion of radioactivity was present as conjugated octadecatrienoic acid in the triglyceride and diglyceride fractions. Hydrogenation did not proceed to any great extent after reincubation for 240 minutes in experiment 3 (Table 11). However, the results show that what hydrogenation did take place proceeded further in the unsaturated fatty acids of the diglyceride fraction than of the triglyceride fraction.

It is possible that the radioactive peaks corresponding to the methyl esters of oleic and stearic acids on the gas-liquid chromatogram were due to aldehydo-esters resulting from oxidative breakdown of 1-^{14}C-linolenic acid on acid-washed Florisil (Chapter 5).

4.5. Fatty Acid Composition of the Total Lipid after Incubation of Synthetic Triglyceride

The fatty acids of the total lipid from experiments 3 and 5 were analysed by gas-liquid chromatography. The results are shown in Table 15.

In both experiments the proportions of linolenic acid decreased with time. Simultaneous increases of more saturated C18 acids indicate that hydrogenation

TABLE 15

Fatty acid composition (moles %) of the total lipid extracted after incubation in rumen fluid of synthetic triglyceride

Chemical Name of Fatty Acid	Shorthand Notation	Reincubation time (minutes)							
		Experiment 3					Experiment 5		
		0	60	120	180	240	60	120	240
n-decanoic	10:0						1.8		
n-decenoic	10:1?*	0.2					0.9		
n-undecanoic	11:0?	0.2				0.2	0.9		
n-dodecanoic	12:0	0.5		2.1	1.4	0.6	1.8		
n-dodenenoic	12:1?	0.2				0.4	0.9	1.9	2.2
n-tridecanoic	13:0								
n-tridecenoic	13:1?	0.2				2.4			
n-tetradecanoic	14:0	1.9	1.4	2.1	2.4		0.9		2.2
13-methyltetradecanoic	i.br.15:0	1.6	1.8	4.1	2.4	1.2	1.8	3.3	4.5
n-pentadecanoic	15:0	1.2	1.8	2.1	2.4	1.2	0.9	1.2	4.5
n-pentadecenoic	15:1								
n-hexadecanoic	16:0	29.1	22.1	24.9	21.9	32.3	18.3	26.1	13.3
n-hexadecenoic	16:1	1.9	1.8	0.6	1.4	8.4	1.8	1.2	18.3
15-methylhexadecanoic	i.br.17:0	8.5							
n-heptadecanoic	17:0	1.4							
n-heptadecenoic	17:1	1.2	1.4		1.4		3.7	1.7	1.1
n-octadecanoic	18:0	5.9	37.3	49.7	37.5	4.2	31.2	22.4	14.2
n-octadecenoic	18:1	26.9	19.4	14.5	19.8	23.9	19.3	28.5	23.1
17-methyloctadecanoic	i.br.19:0?								
n-octadecadienoic	18:2	6.9	4.6		6.3	3.0	5.5	5.4	7.3
n-octadecatrienoic	18:3	12.1	7.9		5.2	22.1	10.1	8.1	8.1
conjugated octadecatrienoic	18:3c								

* signifies tentative identification

occurred. The longest incubations in each experiment resulted in significant increases in palmitoleic acid and decreases in stearic acid. Levels of palmitic acid were relatively unchanged.

4.6. Fatty Acid Composition of the Glyceride and Free Fatty Acid Fractions after Incubation of Synthetic Triglyceride

After resolution of the total lipid extracted in experiment 5 into free fatty acids, triglycerides, diglycerides, and monoglycerides, the component fatty acids of each fraction were analysed by gas-liquid chromatography (Table 16).

Significant hydrogenation of fatty acids in triglyceride combination did not occur, as no decrease in theproportions of such fatty acids, especially linolenic acid, or corresponding increase in the proportions of oleic or stearic acids was detected. An increase in palmitic acid occurred, seemingly at the expense of oleic acid. In the triglyceride fractions there was no marked increase of fatty acids typical of the lipids of rumen bacteria.

Table 16 shows that hydrogenation of esterified 1-^{14}C-linolenic acid occurred in the diglyceride fraction after reincubation for 240 minutes. However, all C18 fatty acids were present in small proportions only, amounting to 19.1% of fatty acid present. There was 12.5% more palmitic acid present (43.6%) than at 60 minutes (31.1%). Linolenic acid, esterified in synthetic triglyceride at the 3-position, was present in diglycerides in significantly lower proportions than in the triglyceride fraction. The appearance in the diglyceride fraction of radioactive octadecadienoic acids indicates hydrogenation of esterified 1-^{14}C-linolenic acid. The diglyceride fractions contained small amounts of oleic acid, but the proportions of palmitic acid rose markedly. Proportions of stearic acid decreased by comparison with the triglyceride fractions. High proportions of fatty acids typical of rumen microorganism lipids, such as

TABLE 16

Fatty acid composition (moles %) of the glyceride and free fatty acid fractions obtained after incubation in rumen fluid of synthetic triglyceride* (experiment 5)

Chemical Name of Fatty Acid	Shorthand Notation	Reincubation time (minutes)											
		Triglyceride			Diglyceride			Monoglyceride			Free Fatty Acid		
		60	120	240	60	120	240	60	120	240	60	120	240
n-decanoic	10:0												
n-decenoic	10:1?												0.2
n-undecanoic	11:0?									3.4			
n-dodecanoic	12:0	1.2	0.5	1.5	2.1	1.0		0.9	0.7		1.2	1.0	0.4
n-dodecenoic	12:1?			0.1				0.6	0.3	1.3		0.4	0.5
n-tridecanoic	13:0			0.3					0.3	0.4		0.2	0.3
n-tridecenoic	13:1?			0.4	1.2	4.5	1.2	1.2		4.7	0.4	1.8	0.9
n-tetradecanoic	14:0	2.6	1.2	3.2	5.8	2.5		4.7	4.0	10.5	1.8	3.0	2.5
13-methyltetradecanoic	i.br.15:0	2.2	0.6	2.1	8.7	21.9		15.0	12.1	24.1	1.9	10.1	7.2
n-pentadecanoic	15:0	1.6	0.4	0.8	4.6	7.6	11.7	7.1	6.7	7.6	1.8	3.2	2.5
n-pentadecenoic	15:1			0.4			5.9	1.8	2.7	1.6	0.2	0.4	0.7
n-hexadecanoic	16:0	22.9	29.7	32.8	31.1	21.2	43.6	29.2	40.2	24.1	13.0	14.3	12.1
n-hexadecenoic	16:1	2.6	1.0	2.7			2.3	2.4	0.7	6.0	0.8		1.8
15-methylhexadecanoic	i.br.17:0				6.6						3.1	2.4	3.7
n-heptadecanoic	17:0		0.4				0.5					4.0	
n-heptadecenoic	17:1		0.3	0.8			15.7						
n-octadecanoic	18:0	18.1	4.3	11.0	21.1	19.7	2.3	18.5	15.1	4.2	51.5	20.8	22.3
n-octadecenoic	18:1	22.9	31.4	18.8	13.7	12.1	0.6 (7)	11.5	13.1	6.7	20.7	29.7(37)	32.5(50)
17-methyloctadecanoic	i.br.19:0?									1.3			
n-octadecadienoic	18:2	3.8	3.2	2.7	1.2	2.3	10.9(23)	3.7	1.3	4.7	2.4	9.5(34)	11.3(42)
n-octadecatrienoic	18:3	21.9(100)	27.1(100)	22.3(100)	3.7(100)	6.8(100)	5.3(70)	5.3	2.7	4.2	1.2(100)	0.5(25)	0.7 (8)
conjugated octadecatrienoic	18:3c												

* distribution of radioactivity amongst fatty acids in each fraction is shown in brackets beside the appropriate fatty acid.

13-methyltetradecanoic acid and palmitic acid, occurred in the diglyceride and monoglyceride fractions. Palmitic acid was present in the monoglycerides in smaller proportions than in the diglycerides.

Both the fatty acid composition and the distribution of radioactivity in the free fatty acid fractions are indicative of a high proportion of saturated free fatty acids. Linolenic acid was almost absent, while increases in both linoleic and oleic acids were detected (Table 16). There was a simultaneous decrease in the proportion of stearic acid, and the content of palmitic acid remained steady. There was a high proportion of free stearic acid early in the incubation period.

Chapter 5

DISCUSSION

5.1. Sampling of Rumen Contents

Results of trial G (Table 8) show a strong positive correlation between the proportions of stearic acid and the amount of lipid in each sample taken after feeding. A similar result was obtained in trial H (Table 10). The solid material retained after straining rumen samples was shown to contain a high proportion (35%) of stearic acid immediately after feeding (Table 9). This proportion increased to 42% 10 hours after feeding due to hydrogenation of unsaturated C18 fatty acids (Table 9). The fluid obtained by straining contained much lower proportions of stearic acid, with a maximum of 16% 10 hours after feeding (Table 9). If differing amounts of solid material were allowed into separate samples the amounts of lipid would have varied, with the higher proportions of stearic acid in the lipid of the solid material causing an upward bias in the estimated proportion of stearic acid in those fluid samples containing greater amounts of solid material. Hence the proportions of fatty acids in each sample of trial G would have depended more on the amount of lipid present than on the time which had elapsed since feeding, and any trends in these proportions, such as would be expected following hydrogenation of linolenic acid, would have been masked. Hawke and Robertson (1964) also obtained rumen fluid samples during *in vivo* hydrogenation studies by straining rumen contents through muslin cloth. These authors found similar marked variations in proportions of fatty acids. These variations were not clearly related to time elapsed since feeding (Hawke and Robertson, 1964: Table II). The amount of lipid present in each

rumen fluid sample was given (Hawke and Robertson, 1964: Table I), and a correlation between amount of lipid and the proportion of stearic acid present similar to that in trial G of the present study was recorded. However, when the rumen liquor samples obtained by straining were centrifuged for 30 minutes at 14,600 g (Hawke and Robertson, 1964), the fatty acid composition of the supernatent was clearly related to time elapsed after feeding. These results appear to confirm the suggestion that the presence of solid material in rumen fluid samples obtained by straining through muslin cloth can lead to difficulty in interpretation of fatty acid composition data.

In trial J of the present study the amounts of lipid in each sample were relatively similar, and a fairly clear relationship exists between the proportions of fatty acids and time elapsed since feeding.

After incubation for 20 minutes in trial H (Table 10) where similar amounts of lipid were present, a marked discrepancy occurred in theproportions of fatty acids in the duplicate samples. Higher proportions of linolenic and linoleic acids and a lower proportion of stearic acid indicate that less hydrogenation occurred in one of the flasks. That such a marked difference in metabolism could come about after 20 minutes is surprising, as all samples of rumen fluid used as incubation medium were treated alike. It seems unlikely that samples of dried grass could differ sufficiently in linolenic acid content to influence hydrogenation to the extent indicated.

5.2. *In vivo* Hydrogenation of Dietary Lipid

As discussed in the previous section, the results of trial G (Table 8) do not give a clear indication of the fate of unsaturated fatty acids during *in vivo* hydrogenation. The results of trial J, however, (Table 8) show that linolenic acid present in pasture grass is hydrogenated in the rumen. One hour after feeding, the proportion of linolenic acid rose from 18% to 44%.

and after 3 hours to 56%. There followed a steady decrease to 25% after 10 hours. Meanwhile the proportions of stearic and oleic acids increased slightly, while the proportion of palmitic acid rose markedly. Similar results were obtained in vivo by Hawke and Robertson (1964), who concluded that the changes in proportions of the fatty acids were consistent with known hydrogenation reactions in the rumen.

Hawke and Robertson (1964) also detected an increase in the proportion of palmitic acid present, and suggested that this increase may indicate a delay in release of plant lipid. Such a delay, however, would also be expected to result in a simultaneous increase in the proportion of linolenic acid. But it is clear from the results of trial J (Table 8) and from the results of Hawke and Robertson (1964) that the maximum proportion of linolenic acid occurred 2-4 hours before palmitic acid reached a maximum. Some evidence for a late release of linolenic acid may be derived from the results of trial G of the present study (Table 8) and from the results of Hawke and Robertson (1964: Tables II and III), although this is of doubtful validity in thelight of the discussion presented in the previous section of this chapter.

5.3. Formation of Palmitic Acid in the Rumen

An alternative suggestion for the observations discussed in the final paragraph of the previous section is that palmitic acid is formed in some way at the expense of C18 acids (Garton et al, 1961; Hawke and Robertson, 1964). Although work of Gray, Pilgrim, Rodda, and Weller (1952) does not provide support for synthesis of ^{14}C-palmitic acid from 1-^{14}C-acetic acid in the rumen, Hawke and Robertson (1964) noted that synthesis of palmitic acid from short chain fatty acids is a well established route in other biological systems (Wakil, 1961). Synthesis of palmitic acid from products of breakdown of C18 acids implies the occurrence of beta-oxidation or a

similar mechanism for fatty acid degradation in the rumen. The process of beta-oxidation is coupled to the mitochondrial cytochrome oxidase system in aerobic organisms and requires molecular oxygen as a final hydrogen acceptor. Certain omega-oxidation systems have also been shown to require molecular oxygen (Kusunose, Kusunose, and Coon, 1964). However, some anaerobic microorganisms can degrade fatty acids by a beta-oxidation system (Stadtman and Barker, 1950), presumably passing protons produced during desaturation to a hydrogen acceptor other than molecular oxygen (Peel and Barker, 1956). Thus it is possible that rumen microorganisms are able to degrade C18 fatty acids to acetyl coenzyme A by beta-oxidation. However, following the work of Gray *et al* (1952), it is unlikely that acetyl coenzyme A formed by beta-oxidation would contribute to synthesis of palmitic acid in the rumen. It is nevertheless possible that incomplete beta-oxidation of C18 acids may occur in the rumen. It has been shown by Elovson (1965) using fasted or fasted and refed rats that about 10% of the total stearic acid degraded in beta-oxidation can be identified as palmitic acid. If beta-oxidation of C18 acids is carried out by anaerobic rumen microorganisms, then it is possible that palmitic acid could accumulate at the expense of stearic acid as described by Elovson (1965).

5.4. *In vitro* Hydrogenation of Dietary Lipid

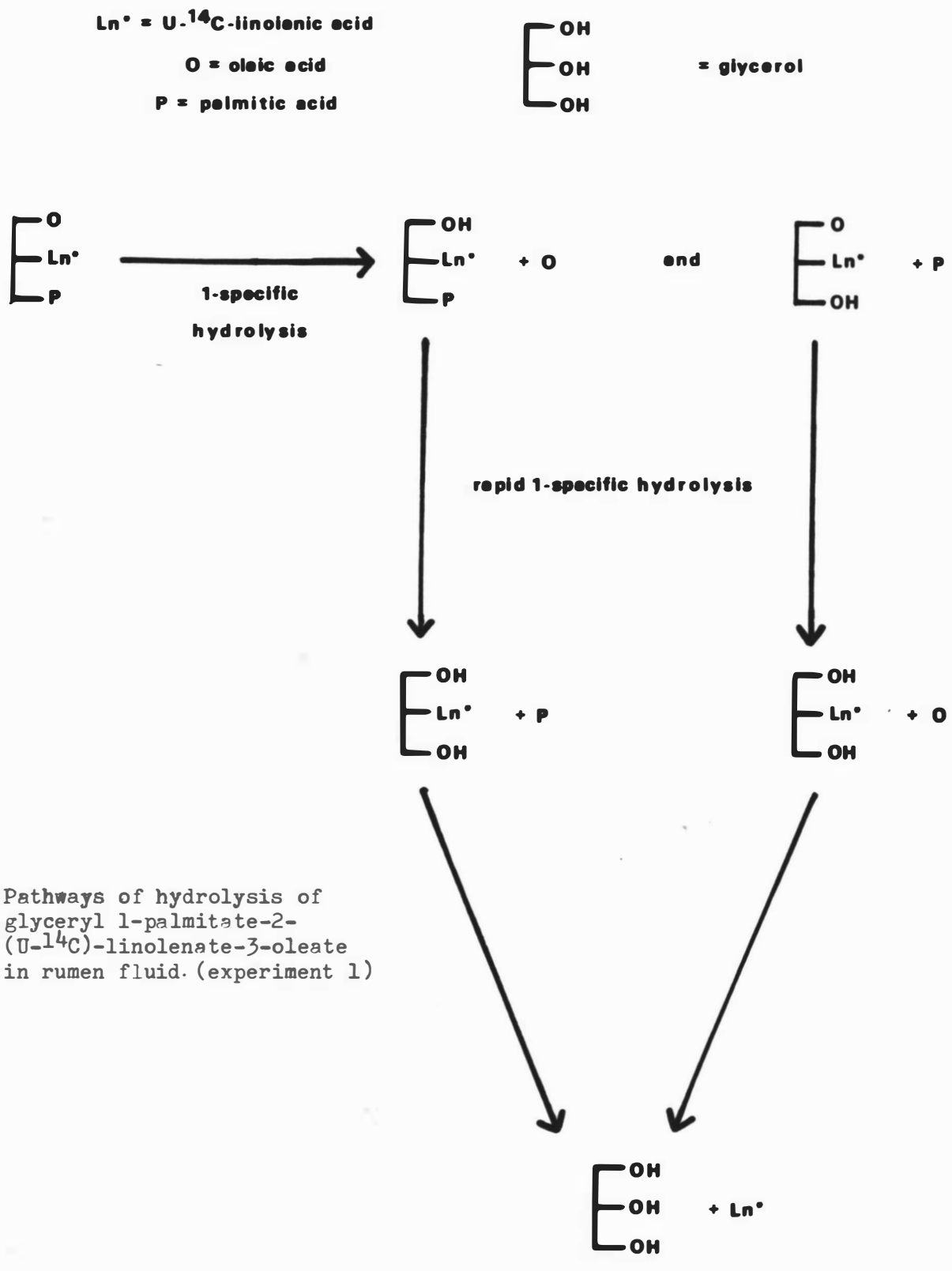
The results in Table 10 are similar to those obtained *in vivo* in trials G and J (Table 8). The proportions of stearic and oleic acids increased while the proportion of linolenic acid decreased, indicating that, at least in incubation of up to 3 hours, *in vitro* and *in vivo* hydrogenation followed the same course. It is concluded that results of *in vitro* experiments in the present study provided a valid indication of events occurring in the rumen.

5.5. Hydrolysis of Triglyceride in Rumen Fluid

The ester linkages of fatty acids to glycerol will be referred to as 1, 2, and 3 in this discussion. Thus, esterification of a fatty acid to a primary hydroxyl group occurs at the 1- and 3-positions, and to a secondary hydroxyl group at the 2-position. Isomerisation of fatty acids in diglycerides and monoglycerides is probably an important factor affecting the order in which fatty acids are released from these compounds. There is a tendency for fatty acids esterified at the 2-position to migrate to a vacant 1- or 3-position in both diglycerides and monoglycerides, especially so in monoglycerides, and more so in a monoglyceride containing an unsaturated fatty acid. Mattson and Volpenhein (1962) found that 80-90% of glyceryl 2-oleate shaken at 40° at pH 8 was converted to glyceryl 1-oleate after 30 minutes, and that glyceryl 2-stearate was almost unchanged. The difference was suggested to be due to the lower melting point and thus easier dispersal of glyceryl 1-oleate in an aqueous medium. Isomerisation of 1,2-diglyceride to 1,3-diglyceride is a slower process and not likely to occur in the conditions under which incubations were carried out in the present study. Mattson and Volpenhein (1962) showed that shaking glyceryl 1,2- or 1,3-dioleate with buffer solution at 40° for 30 minutes over a pH range of 6-8 caused little, if any, isomerisation.

The results of experiment 1 (Table 11) suggest that hydrolysis of diglyceride to monoglyceride was very rapid, as no radioactivity appeared in the diglyceride fraction. Because the original triglyceride contained 1-^{14}C-linolenic acid in the 2-position, radioactive diglyceride must have been an intermediate unless hydrolysis specific for the 2-position took place. Hydrolysis specific for the 2-position would have lead to a rapid increase in radioactivity in the free fatty acid fraction with no radioactivity in the diglyceride and monoglyceride fractions. As there were significant

FIGURE 3



Pathways of hydrolysis of glyceryl 1-palmitate-2-(U-^{14}C)-linolenate-3-oleate in rumen fluid. (experiment 1)

proportions of radioactivity in the monoglyceride fraction, such hydrolysis seems unlikely. Hydrolysis specific for the 1- or 3-positions would produce radioactive diglyceride and monoglyceride, with production of radioactive free fatty acid delayed while isomerisation of U-^{14}C-linolenic acid from the 2-position to the 1- or 3-positions took place. About 23% of the radioactivity was present in free fatty acid before incubation began, and this value did not rise significantly until the triglyceride had been hydrolysed to the extent where slightly more than half its radioactivity was liberated (40-80 minutes). This lag in production of free fatty acid supports the suggestion that hydrolysis specific for the 1- or 3-positions took place. Thus the results of experiment 1 suggest hydrolysis specific for the 1- or 3-positions of the triglyceride, followed by rapid hydrolysis of the resultant diglyceride. Hydrolysis to liberate the remaining esterified fatty acid appears to follow at a slower rate due to the necessity for isomerisation to occur before 1- or 3-specific hydrolysis is able to continue, resulting in accumulation of radioactive monoglyceride. This sequence may be summarised as shown in Figure 3.

Mattson and Volpenhein (1962) showed that 2-monoglycerides containing an unsaturated fatty acid isomerise more readily than those containing a saturated fatty acid. Hence, glyceryl 2-linolenate could be expected to isomerise quite readily in rumen fluid (Figure 3), although such isomerisation would take some minutes (Mattson and Volpenhein, 1962).

Thus, in experiment 1, hydrolysis of triglyceride at the 1- or 3-positions would lead to formation of radioactive 1,2-diglycerides, which appear to be rapidly hydrolysed to radioactive 2-monoglycerides. Accumulation of monoglycerides before subsequent hydrolysis is probably a consequence of the time required for isomerisation to 1-monoglycerides, a much slower process than hydrolysis.

Because of the rigid tetrahedral arrangement of the substituent groups around carbon atoms 1 and 3 of the glycerol molecule in a triglyceride, lipase may distinguish between the 1- and 3-ester linkages, as has been shown for the action of phospholipase A on beta-lecithins (van Deenen and de Haas, 1963). Thus, it is possible to envisage stereospecific removal of fatty acids at the 1-position or the 3-position of a triglyceride, as well as specificity of hydrolysis for ester linkages involving primary hydroxyl groups. Stereospecific removal of either palmitic acid or oleic acid from the triglyceride incubated in experiment 1 would result in accumulation of radioactive diglyceride, as the latter would not become available for further stereospecific hydrolysis until U-^{14}C-linolenic acid migrated to the vacated primary hydroxyl group. Subsequent removal of U-^{14}C-linolenic acid would lead to an increase in radioactive free fatty acid. No radioactivity would appear in the monoglyceride fraction. Unless two stereospecific enzymes were present, one specific for each of the 1- and 3-positions, accumulation of either glyceryl 1-palmitate or glyceryl 1-oleate would follow. Palmitic and oleic acids would be released slowly from these accumulated monoglycerides as a result of isomerisation to maintain the equilibrium of 88% 1-monoglyceride and 12% 2-monoglyceride (Mattson and Volpenhein, 1962). Once at the 2-position, further isomerisation to form the ester linkage being attacked by stereospecific lipase would result in removal of the fatty acid.

The distribution of radioactivity suggested by these considerations does not occur in experiment 1, and it seems unlikely therefore that stereospecific hydrolysis as described has taken place. Indeed, rapid hydrolysis of diglyceride suggests the absence of specificity of hydrolysis at one or other of the primary hydroxyl groups of the glycerol molecule. Similar considerations indicate that stereospecific hydrolysis is unlikely

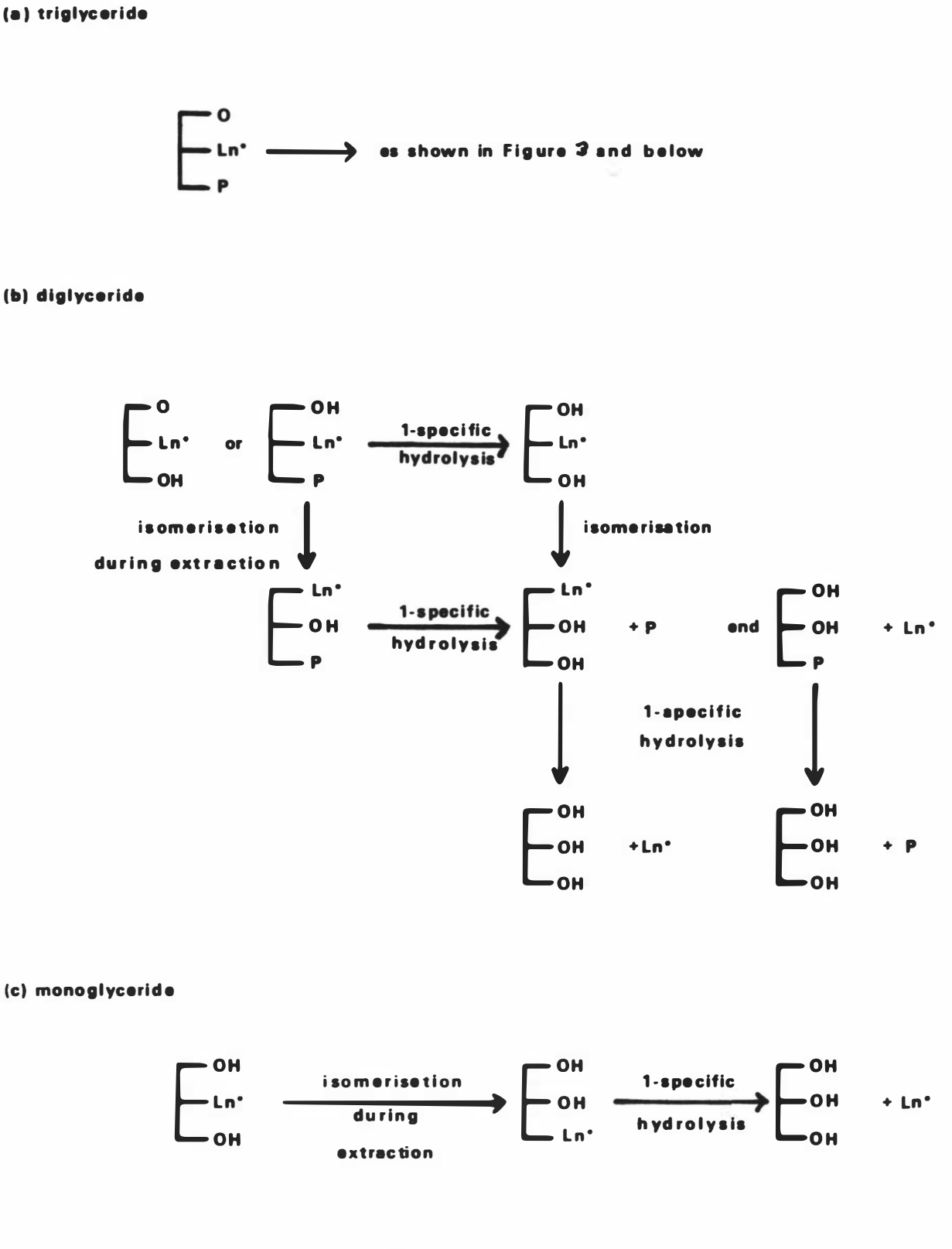
in experiment 3 (Table 11), although the results of experiment 5 (Table 11) do not exclude the possibility of stereospecificity for the 1-position, esterified with palmitic acid.

Significant hydrolysis did not occur in experiment 2, and it was decided to reincubate the radioactive lipid present, constituting experiment 3. The small amount of hydrolysis that did occur in experiment 2 resulted in increased proportions of radioactive diglyceride and monoglyceride, but no production of radioactive free fatty acid. This suggests that hydrolysis specific for the 1- or 3-positions had occurred. That little hydrolysis of diglyceride and monoglyceride took place is indicated by the presence of radioactive diglyceride and the absence of radioactive free fatty acid in contrast to the results of experiment 1. It is possible that only small numbers of lipolytic microorganisms were present, such that available lipase may have been overloaded by substrate. The cow from which the rumen fluid was obtained had been starved overnight and the rumen contents were quite liquid, suggesting that food material in the rumen would have been almost completely digested and that the microorganisms responsible for initial breakdown and hydrolysis of dietary material may not have been present in very great numbers.

Following reincubation in experiment 3 of the radioactive lipid from experiment 2, a marked increase in the proportions of radioactivity in free fatty acid occurred. This radioactivity was derived mainly from the diglyceride and monoglyceride fractions, as the proportions of radioactive diglyceride and monoglyceride were reduced to a greater extent than the proportions of triglyceride.

Isomerisation in the diglyceride and monoglyceride fractions recovered from experiment 2 would be extensive due to increased temperatures involved during extraction of radioactive lipid prior to reincubation (Mattson and

FIGURE 4



Pathways of hydrolysis in rumen fluid of glycerides recovered from experiment 2 (experiment 3)

Volpenhein, 1962). It is probable, therefore, that most of the 1-^{14}C-linolenic acid in the monoglycerides migrated to vacated 1- or 3-positions, together with some 1-^{14}C-linolenic acid in the diglycerides. Hydrolysis specific for the 2-position in experiment 3 seems unlikely, because in all probability insufficient 1-^{14}C-linolenic acid would have remained in the 2-positions of the diglycerides and monoglycerides to lead to an increase from 0% to 32% in radioactive free fatty acid (reincubation for 180 minutes).

A suggested scheme for hydrolysis occurring in experiment 3, beginning with the three glycerides likely to be recovered from experiment 2, is shown in Figure 4 (see Figure 3 for key to symbols).

No significant hydrolysis occurred in experiment 4 (Table 11), as radioactivity was confined to the triglyceride fraction. Hydrolysis specific for the 2-position did not take place, as no radioactive diglyceride was detected. Similarly, hydrolysis specific for the 1- or 3-positions would have produced radioactive free fatty acid, which also was not detected. The radioactive lipid was extracted and reincubated, constituting experiment 5.

Because the triglyceride used in experiment 5 contained 1-^{14}C-linolenic acid esterified at the 3-position, hydrolysis specific for the 2-position would have resulted in a high proportion of radioactivity in diglyceride and a slow increase in proportions of radioactive monoglyceride and free fatty acid. This latter effect would have been aggravated by the tendency for isomerisation of fatty acids in glyceride combination away from the 2-position. Hydrolysis specific for the 1- or 3-positions would result in a rapid increase in radioactive free fatty acid, with some radioactivity remaining in diglyceride and monoglyceride. This distribution of radioactivity was obtained in experiment 5. After reincubation for 240 minutes radioactivity in the triglyceride fraction decreased from 100% to 30%, and that present in free fatty acid increased from 0% to 68%.

FIGURE 5

Scan of fatty acid and glyceride fractions after reincubation of glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate for 240 minutes

KEY:

1	=	triglyceride	4	=	1,2-diglyceride
2	=	free fatty acid	5	=	monoglyceride
3	=	1,3-diglyceride	M	=	marker

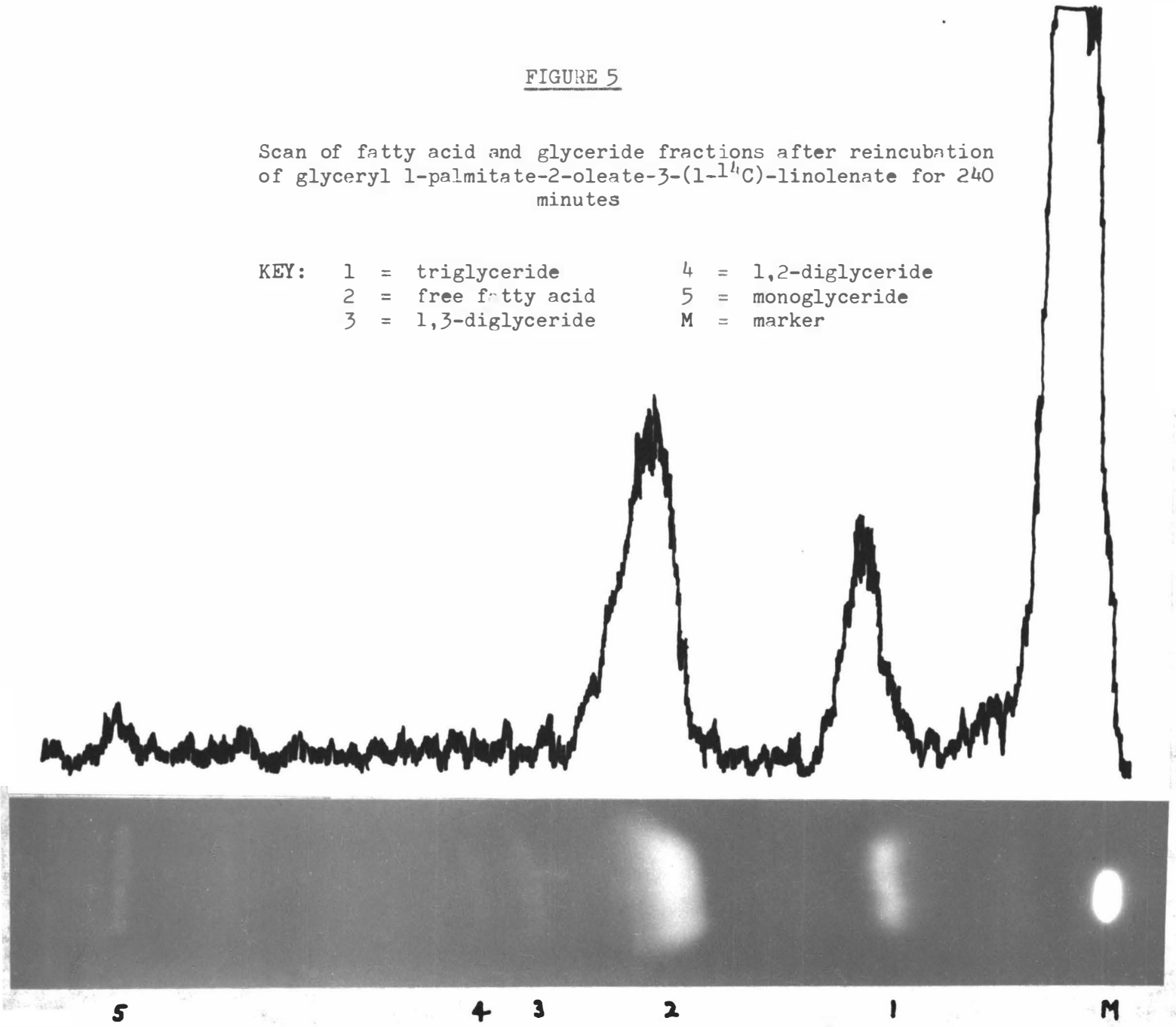


FIGURE 6

Scan of free fatty acid and glyceride fractions after reincubation of glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate for 240 minutes (see Figure 5 for key)

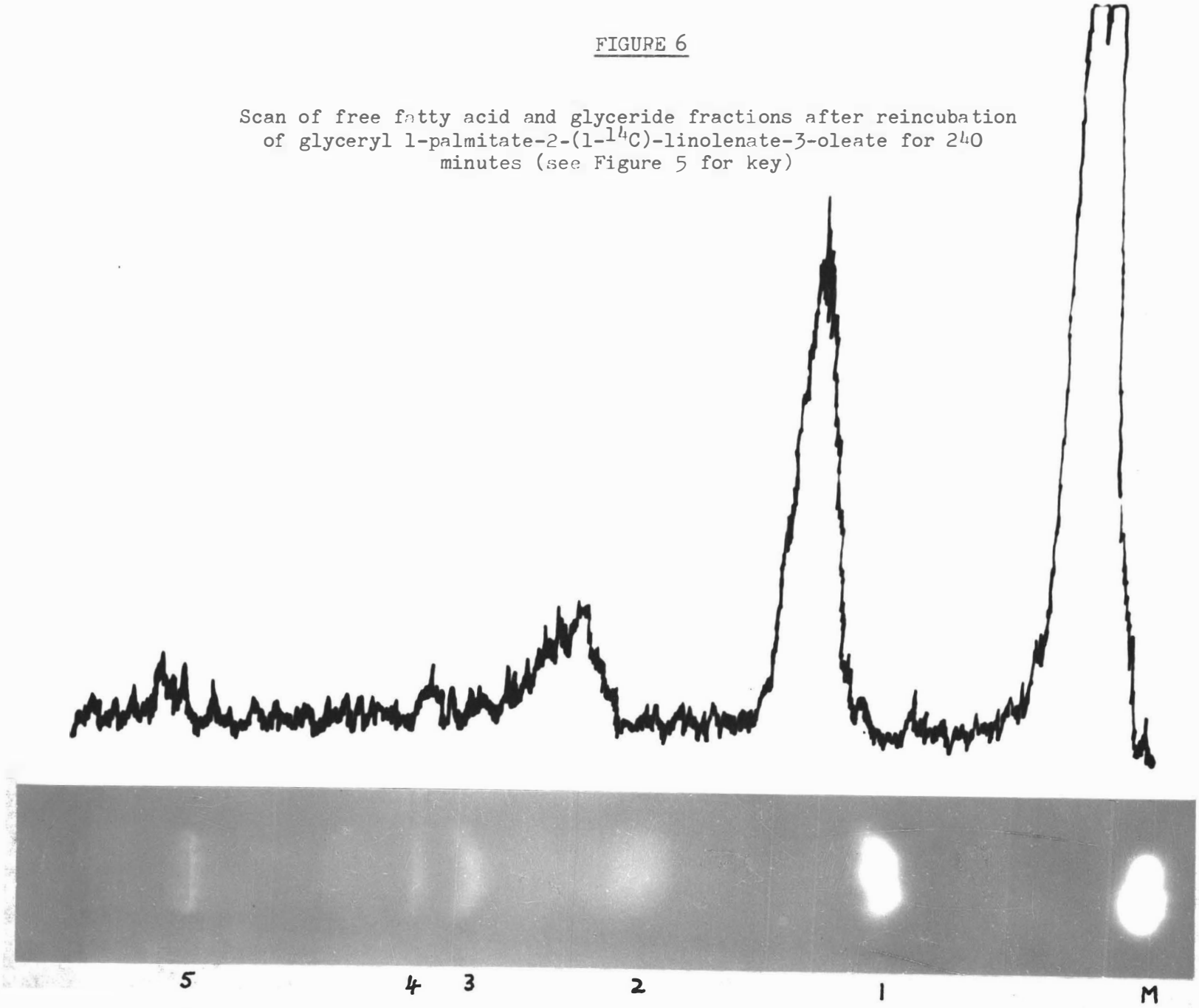
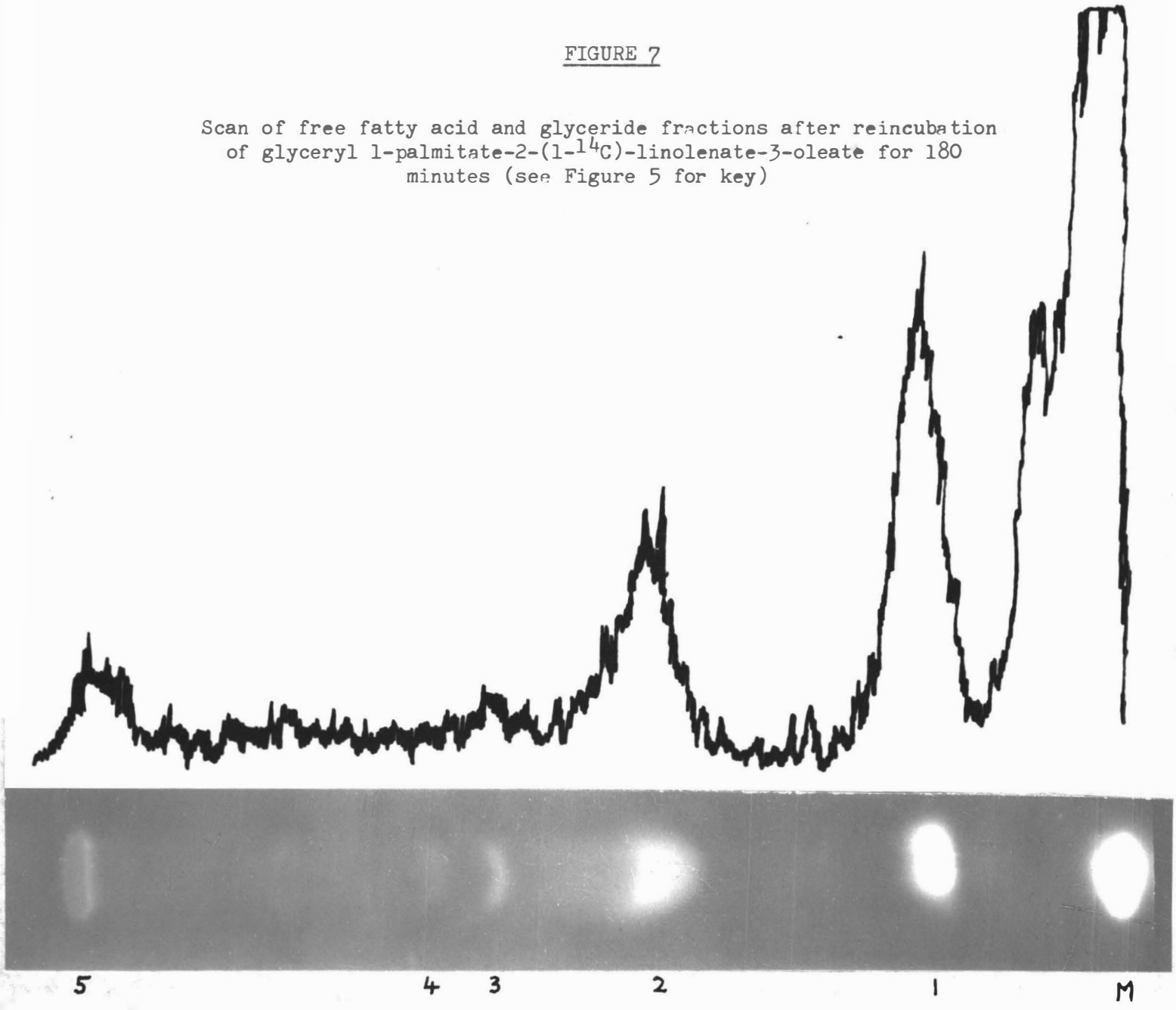


FIGURE 7

Scan of free fatty acid and glyceride fractions after reincubation of glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate for 180 minutes (see Figure 5 for key)



After reincubation of triglyceride containing 1-^{14}C-linolenic acid esterified at the 3-position for 240 minutes (experiment 5), only 30% of the radioactivity remained in the triglyceride fraction, whereas after reincubation for 240 minutes of triglyceride containing 1-^{14}C-linolenic acid esterified at the 2-position (experiment 3) the proportion of radioactivity in triglyceride was reduced from 90% to 72%. The greatest decrease in triglyceride radioactivity in experiment 3 was from 90% to 56% after reincubation for 180 minutes. These results are shown in Figures 5, 6, and 7, and suggest that fatty acids esterified at the 1- or 3-positions were hydrolysed more rapidly than those esterified at the 2-position.

The results of partial hydrolyses of linseed oil (Garton et al, 1961) shown in Table 3 may be explained on the basis of hydrolysis specific for the 1- or 3-positions. Hilditch (1956) concluded that in triglycerides of vegetable fats oleic and linoleic acids are preferentially esterified to the secondary hydroxyl group of the glycerol molecule. Linseed oil contains about 30% of oleic and linoleic acids, and, if the conclusion drawn by Hilditch (1956) applies to linseed oil, the primary hydroxyl groups of the glycerol molecule would be esterified exclusively with linolenic acid. Thus, removal of linolenic acid from the linseed oil triglycerides indicates specificity of hydrolysis for the 1- or 3-positions of the triglycerides. This possibly could explain the decreased proportion of linolenic acid in the "residual glycerides" isolated by Garton et al (1961).

5.6. Relationships Between Hydrogenation and Hydrolysis of Unsaturated Triglyceride in Rumen Fluid

The results in Table 12 indicate that significant hydrogenation of 1-^{14}C-linolenic acid originally present in synthetic triglyceride had not

occurred until after reincubation for 240 minutes in experiment 5. However, in Table 11 it is seen that after reincubation for 240 minutes in experiment 5 only 30% of radioactivity remained in the triglyceride fraction. After reincubation for shorter periods of time, at least 50% of the radioactivity was present in triglyceride, and from Table 12 it is seen that this was 1-^{14}C-linolenic acid. From these results it appears that significant hydrogenation of radioactive fatty acid did not occur until most of the 1-^{14}C-linolenic acid was hydrolysed from the triglyceride, suggesting that unsaturated fatty acids must be free in the rumen before hydrogenation can take place.

To study this relationship in more detail, the radioactive fatty acids of the glyceride and free fatty acid fractions of experiment 5 were identified. The results are shown in Table 13.

These results show that most hydrogenation occurred amongst unsaturated fatty acids free in the incubation medium. The observation that after reincubation for 60 minutes there was no hydrogenation of unsaturated fatty acids free in the incubation medium indicates that hydrolysis was a prerequisite for hydrogenation of unsaturated fatty acids. However, significant hydrogenation occurred in the diglyceride fatty acids, suggesting that, although slower, hydrogenation of unsaturated fatty acids in ester linkage may occur.

Hydrogenation and hydrolysis are probably enzymatically controlled reactions (Reiser, 1951; Garton et al, 1961) and it is possible that unsaturated triglycerides are competed for as substrate by lipase and hydrogenating enzyme. Resulting competitive inhibition would slow hydrogenation of esterified fatty acids by comparison with hydrogenation of free fatty acids, where lipase would not be in competition with hydrogenating enzyme. The results of the present study support this

hypothesis in that hydrogenation of esterified fatty acids does not proceed to the same extent as does hydrogenation of free fatty acids.

Possible pathways of hydrolysis suggested by Garton et al (1961), implying that triglyceride hydrolysis is carried out to completion before substrate is released from the enzyme, would preclude enzymatic hydrogenation of esterified fatty acids. These suggestions (Garton et al, 1961) were partly based on failure to isolate diglycerides or monoglycerides from rumen fluid during hydrolysis of triglycerides. Hawke and Robertson (1964) isolated diglycerides and monoglycerides from the rumen, and in the present study these intermediates were also isolated from rumen fluid during in vitro hydrolysis of triglyceride. Further, hydrogenation of esterified fatty acids has been demonstrated in the present study.

The tentative identification of radioactive octadecatrienoic acid with two conjugated double bonds (Table 13) is supported by the suggestion of Wilde and Dawson (1966) that this acid is the first intermediate in hydrogenation of linolenic acid in rumen fluid. Kepler and Tove (1967) isolated a linoleate 12-cis, 11-trans-isomerase from B. fibrisolvens which was shown to catalyse isomerisation of linolenic acid (octadeca-9-cis,12cis, 15-cis-trienoic acid) to an octadeca-9-cis,11-trans,15-cis-trienoic acid.

Results shown in Table 14 provide additional evidence for hydrogenation of unsaturated fatty acids esterified to glycerol. Significant proportions of radioactivity were present in conjugated octadecatrienoic acid in the triglyceride and diglyceride fractions. Hydrogenation proceeded further in the diglyceride fraction, where 23% of the radioactivity was identified as octadecadienoic acids. Shorland et al (1957) suggested that linoleic acid itself was not among the octadecadienoic acids formed during hydrogenation of linolenic acid. A similar result was obtained by Wilde

and Dawson (1966) where, after permanganate-periodate oxidation of the non-conjugated octadecadienoic acids resulting from a 20-minute hydrogenation of linolenic acid, only 1% of the monocarboxylic acids formed was hexanoic acid. Thus, a maximum of 1% of linoleic acid could have been present (Table 5). In the present study no attempt was made to identify any isomers of linoleic acid which may have been formed.

Hydrogenation proceeded to a greater extent in the triglyceride and diglyceride fractions as recorded in Table 14 than in those shown in Table 13. If 1- or 3-specific hydrolysis of triglycerides occurs in rumen fluid, 1-^{14}C-linolenic acid esterified at the 2-position in the triglyceride of experiment 3 (Table 14) would be in triglyceride combination longer than if esterified at the 3-position as in experiment 5 (Table 13). This would increase the possibility of hydrogenation being completed before hydrolysis.

The identity of the radioactive fatty acids of fraction 5A is in doubt. The retention times relative to methyl palmitate identify two of the methyl esters present as methyl oleate and methyl palmitate (Table 14). However, there is a possibility that oxidation of 1-^{14}C-linolenic acid at the double bonds took place during resolution of the total lipid extracted after reincubation for 240 minutes in experiment 3 into glyceride and free fatty acid fractions on acid-washed Florisil (Carroll, 1963). Complete oxidation could yield a mixture of three radioactive dicarboxylic acids. These are 1-^{14}C-azelaic acid, 1-^{14}C-tridec-9-enoic acid, or 1-^{14}C-pentadeca-9, 12-dienoic acid. However, none of the relative retention times of the methyl esters of these fatty acids correspond to those of the methyl esters appearing on the gas-liquid chromatogram (Ward *et al*, 1964). Partial oxidation of 1-^{14}C-linolenic acid at the double bonds would yield aldehydo-acids corresponding to the dicarboxylic acids listed above. The relative

retention times of the methyl esters of the C13 and C15 aldehydo-acids may correspond to those of the methyl esters appearing on the chromatogram. That saponification was necessary before isolation of the fatty acids of fraction 5A indicates that oxidation would have involved esterified 1-^{14}C-linolenic acid.

There is no evidence available indicating that oxidation of unsaturated fatty acids occurs on acid-washed Florisil. Anderson and Hollenbach (1965) separated unsaturated C18 acids on acid-washed Florisil impregnated with silver nitrate and obtained quantitative recoveries of the unsaturated acids involved. However, the columns used were cooled to 10° and covered with black cloth. These precautions may also have prevented oxidation occurring. The acid-washed Florisil columns used in the present study were exposed to the atmosphere and were not cooled.

If oxidation on acid-washed Florisil columns does not take place, the appearance of radioactivity in palmitic acid provides evidence for a conversion of C18 acids to palmitic acid, as suggested by Garton et al (1961).

5.7. Fatty Acid Composition of the Total Lipid Obtained after Incubation with Rumen Fluid of Synthetic Triglyceride

As shown in Table 15, hydrogenation of linolenic acid with a simultaneous increase in more saturated C18 acids took place in experiments 3 and 5. This trend continued in experiment 3 for 120 minutes, but after 240 minutes the proportion of linolenic acid had increased again. A similar result was obtained in trial G (Table 8). The amount of lipid extracted was not determined in experiments 3 and 5, and the explanation advanced in section 5.1. of this chapter may apply to these results.

After 240 minutes in experiment 3 the proportion of palmitic acid rose as in trial J (Table 8), accompanied by a marked decrease in the proportions of stearic acid and a marked increase in the proportions of palmitoleic acid.

A similar trend is noted in the results of experiment 5. These findings are supported by the suggestion of Garton et al (1961) and Hawke and Robertson (1964) that C18 acids may be converted to palmitic acid, and may indicate that palmitoleic acid is an intermediate in such a conversion.

5.8. Fatty Acid Composition of the Glyceride and Free Fatty Acid Fractions Obtained after Incubation of Synthetic Triglyceride in Rumen Fluid

The results in Table 16 show that while the proportion of linolenic acid in the triglyceride fractions remained relatively constant with increasing incubation time, oleic acid decreased and palmitic acid increased. Alteration of the proportions of fatty acids in triglyceride fractions implies either addition or removal of triglycerides, or interconversion of esterified fatty acids.

Addition of triglyceride may occur as a result of synthesis of new triglyceride by microorganisms in the incubation medium. That such a process has not been carried out to any extent by bacteria is indicated by the absence of any increase in triglycerides of fatty acids characteristic of the lipids of bacteria from the rumen, such as 13-methyltetradecanoic acid (Tweedie, Rumsby, and Hawke, 1966). However, protozoa from the rumen are known to accumulate palmitic acid but not branched chain fatty acids (Williams, Gutierrez, and Davis, 1963) and so synthesis by protozoa of triglycerides containing palmitic acid may have taken place.

Removal of triglyceride during incubation could occur only as a result of hydrolysis, and the fatty acid composition of recovered diglycerides is shown in Table 16. No removal in toto of triglyceride from the incubation flasks occurred, as sampling during incubation was not necessary.

Interconversion of esterified fatty acids could involve either hydrogenation of unsaturated fatty acids or alteration of chain length. Significant hydrogenation of unsaturated fatty acids in glyceride combination

did not occur, as no decrease of such fatty acids, especially linolenic acid, or corresponding increase of oleic or stearic acids was detected.

The increase in palmitic acid, seemingly at the expense of oleic acid, may indicate chain shortening of esterified oleic acid. Conversion of free oleic acid to palmitic acid prior to incorporation of the latter into new triglyceride by microorganisms implies hydrolysis of synthetic triglyceride to release oleic acid, and at the same time liberation of linolenic and palmitic acids also present in synthetic triglyceride. As microorganisms do not appear to incorporate linolenic acid into their body lipid (Tweedie et al, 1966), it would be lost from the triglyceride fraction. However, the proportion of linolenic acid in triglycerides remained steady, as shown in Table 16, indicating that esterified and not free oleic acid may be converted to palmitic acid. It follows that two carbon atoms must be lost from the methyl end of esterified oleic acid. Some evidence for such a conversion may have been obtained in the present study (Table 14). The possibility of chain-shortening occurring in rumen fluid is discussed in section 5.3. of this chapter.

Enzymic conversion of palmitic acid to oleic acid in glyceride combination could be in competition with hydrogenation of esterified fatty acids. Table 16 shows that in the diglyceride fraction hydrogenation of esterified 1-^{14}C-linolenic acid occurred when the proportion of oleic acid was at a low level (after reincubation for 240 minutes), suggesting that, after oleic acid had been converted to palmitic acid, hydrogenation of 1-^{14}C-linolenic acid was able to take place.

Linolenic acid was detected in significantly lower proportions in the diglyceride fractions than in the triglyceride fractions, suggesting either removal by hydrolysis or hydrogenation while in ester linkage. Some hydrogenation occurred, as shown in Table 16 (reincubation for 240 minutes).

However, although no hydrogenation of 1-^{14}C-linolenic acid in diglycerides had taken place after reincubation for 60 or 120 minutes, the proportions of linolenic acid were markedly reduced by comparison with the triglyceride fraction. Hydrolysis specific for the 1- or 3-positions could lead to such a decrease, although during such hydrolysis the proportions of palmitic acid would also be expected to decrease. Instead the proportions of palmitic acid in diglycerides increased from 31.1% to 43.6%, suggesting either stereospecific removal of linolenic acid, isomerisation of oleic acid to the vacated 1- or 3-positions and its subsequent hydrolysis, or conversion of esterified stearic (or oleic) acid to palmitic acid. As discussed elsewhere, stereospecific hydrolysis seems unlikely, although the possibility cannot be dismissed. It is likely that oleic acid has migrated to the 1- or 3-positions and been removed by 1- or 3-specific hydrolysis, although such hydrolysis would also remove palmitic acid, indicating that stereo-specific hydrolysis may have taken place. It is also possible that conversion of esterified stearic acid to palmitic acid has taken place, as discussed elsewhere. The increased proportions of palmitic acid may also have resulted from synthesis of glycerides by microorganisms, and subsequent hydrolysis of these glycerides as the microorganisms die. The glycerides of microorganisms would therefore become available for hydrolysis at a later time than do free glycerides such as the added synthetic triglyceride. There are high proportions of fatty acids characteristic of microorganism lipids in both the diglyceride and monoglyceride fractions (Table 16).

Both the fatty acid composition and the distribution of radioactivity in the free fatty acid fractions are indicative of a marked degree of hydrogenation of unsaturated fatty acids although liberation of radioactive unsaturated fatty acids partially hydrogenated while esterified must have contributed to the proportions of hydrogenated fatty acids free in the incubation medium.

That free fatty acid ultimately reaches a high degree of saturation is indicated by the high proportion of free stearic acid early in the incubation period, presumably before significant liberation and hydrogenation of linolenic acid had occurred.

Chapter 6

SUMMARY

1. A study has been made of hydrogenation of unsaturated fats in rumen fluid. Hydrogenation was shown to follow a similar course under both *in vivo* and *in vitro* conditions. The significance of this finding is commented upon in relation to the validity of further results obtained under *in vitro* conditions.

2. A comparison was made of the fatty acid composition of residues and fluid obtained after straining crude rumen contents through two layers of muslin cloth. The lipid of the residues contained higher proportions of oleic and stearic acids, with the proportion of the latter increasing during the sampling period due to hydrogenation of unsaturated fatty acids present. This finding is commented upon in relation to interpretation of results obtained in experiments utilising strained rumen fluid.

3. Some evidence for formation of palmitic acid in the rumen is presented, and pathways for its formation in the rumen are discussed.

4. The synthesis and purification of the triglyceride isomers glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate and glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate is described. These synthetic triglycerides were incubated under *in vitro* conditions and the fate of esterified 1-^{14}C-linolenic acid followed.

5. Data showing the distribution of radioactivity in free fatty acid and glyceride fractions after incubation of synthetic triglyceride isomers is discussed in relation to isomerisation of fatty acids esterified to glycerol, positional specificity and stereospecificity in hydrolysis of glycerides, and the relative rates of isomerisation and hydrolysis.

6. The release of 1-^{14}C-linolenic acid from glyceryl 1-palmitate-2-oleate-3-(1-^{14}C)-linolenate was more rapid than from glyceryl 1-palmitate-2-(1-^{14}C)-linolenate-3-oleate. This finding is discussed in relation to positional specificity of hydrolysis of glycerides in rumen fluid.

7. The data presented suggest specificity of hydrolysis for fatty acids esterified at the 1- or 3-positions of glycerol. The possibility of stereospecificity for either of these positions seems unlikely from the results obtained, but cannot be dismissed.

8. Radioactive fatty acids (derived from 1-^{14}C-linolenic acid) were found to be more saturated when free in rumen fluid than in esterified form. This finding suggests competitive inhibition of hydrogenating enzyme by lipase during hydrogenation of esterified fatty acids.

9. An octadecatrienoic acid with two conjugated double bonds has been tentatively identified from gas-liquid chromatographic data. It is concluded that this acid is an early intermediate in hydrogenation of linolenic acid in the rumen.

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