THE PROTECTION OF THE UNSATURATED FATTY ACIDS OF DRIED GRASS AND SUNFLOWER SEED AGAINST BIO-HYDROGENATION BY RUMEN MICRO-ORGANISMS.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Agricultural Science in Animal Science at Massey University.

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ABSTRACT

Ryegrass which had been dried and treated with HCHO was incubated with the rumen contents of a pasture-grazed cow. The protein in the grass was protected from degradation by the rumen microbes.

The degree of protection of the protein increased with the rate of HCHO application over the range 0.5-2.0 g HCHO per 100 g of dried grass. At the highest rate of HCHO application, the digestibility of the forage dry matter (measured *in vitro*) was a little less than that of the untreated forage.

In vitro incubations with rumen fluid also showed substantial protection of 18:3 in dried grass which had been treated with HCHO. Again, the degree of protection increased with the rate of application of HCHO. The upper level of HCHO treatment which was also the optimum was higher than the level recommended by other workers for the protection of protein in dried forage.

Dried grass obtained from a commercial source was treated with HCHO (2 g HCHO/100 g dried grass) and was fed to a cow from a monozygous twin pair. Intake was reduced and an underfeeding response was observed. The proportions of 18:2 and 18:3 in the milk fat of the cow were not elevated. This lack of response probably was due to a combination of the depressed intake by the cow and the low levels of endogenous lipid (compared with spring pasture) in the grass used.

A supplement of sunflower seed and casein which had been treated with HCHO was fed to a cow. Milk fat containing about 10 moles % 18:2 was produced. When a supplement of sunflower seed and casein which had not been treated with HCHO was fed, a much smaller increase in the content of 18:2 in the milk fat was observed.

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ABBREVIATIONS USED

Ammonia	NH_3
approximately	approx.
carbon dioxide	CO_2
centimetre(s)	cm
chloroform	$CHCl_3$
cubic centimetre(s)	cm^3
degrees Celcius	^oC
dry matter	D.M.
estimated	est.
ethanol	ETOH
Fatty Acids	
butyric acid	4:0
caproic acid	6:0
caprylic acid	8:0
capric acid	10:0
lauric acid	12:0
myristic acid	14:0
palmitic acid	16:0
stearic acid	18:0
oleic acid	18:1
linoleic acid	18:2
linolenic acid	18:3
formaldehyde	HCHO
gas liquid chromatography	G.L.C.
gram(s)	g
grams per 100 cubic centimetres solution	% (w/v)
hours	h
hydrochloric acid	HCl
internal diameter	i.d.
kilogram(s)	kg
methanol	MeOH
millilitre(s)	ml
millimetre(s)	mm
minute(s)	min
molar	M
nitrogen	N_2
oxygen	O_2

per cent	%
plus or minus	±
potassium carbonate	K_2CO_3
second(s)	s
standard error of mean	S.E.
thin layer chromatography	T.L.C.
ultra-violet	U.V.
volume(s)	vol(s)

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

It has been known for some time that the lipids of ruminants differ in several ways from those of non-ruminants. Of significance is the difference in fatty acid composition of ruminant lipid compared with that of other herbivorous animals. The fatty acid composition of ruminant lipids does not vary much even when there are wide variations in the dietary lipids. These characteristics are due to the activities of the micro-organisms in the rumen. These effects, their consequences and how they may be modified or avoided are discussed in this review.

1.2 The nature of pasture and animal lipids

1.2.1 Plant lipids

The composition of lipids in leaf tissue has been described in a review by Hawke (1973).

Hawke (1963) found that the lipid content of pasture decreases with maturity; short succulent ryegrass consisting entirely of leaf tissue contained approx. 8% lipid compared with mature grass which contained 5% lipid on a dry weight basis. The lipid content of leaves is also dependent on light intensity; leaves grown in bright light contain more lipid than shaded leaves (Hawke, 1973).

Ryegrass contains a high proportion of unsaturated fatty acids in the total leaf lipid (60-80% of the fatty acids are 18:3 and 7-13% are 18:2, Hawke, 1963). According to Weenink (1959), about 60% of the lipid fraction of clover and forage grasses consists of galactosyl glycerol esters of fatty acids, about 90% of which are linolenic acid (Shorland 1961) - mostly of the *cis* configuration (Katz and Keeney, 1966). The main galactolipids are mono- and di-galactolipid with monogalactolipid being the dominant ester in spinach leaves (Webster and Chang, 1969; Benson *et al.* 1959). Much of the lipid of the leaf appears to be concentrated in the chloroplasts. Kates (1970) found that 33-36%

of the dry weight of spinach chloroplasts consists of lipid. A large portion of the chloroplast lipids, mainly the galactolipids, are present in a continuous matrix in which protein particles containing pigment and lipids are embedded (Bamberger and Park, 1966).

1.2.2 Animal lipids

Dietary fats have little influence on the composition of ruminant depot fat in which 16:0, 18:0 and 18:1 are the major fatty acids (Shorland, 1952). The fatty acid composition of non-ruminant depot fat on the other hand tends to reflect the fatty acid composition of the diet as shown in the following table from Shorland (1953).

That the virtual absence of 18:3 in the ox and sheep (compared with the horse, rabbit and pasture) may be due to a difference in the nature of fat metabolism in these animals was proposed by Brooker and Shorland (1950). The influence of the rumen was noted by Reiser (1951) who incubated rumen fluid containing metabolising microbes with emulsions of linseed oil and found that the unsaturated fatty acids were substantially hydrogenated.

A further feature of ruminant depot and milk fat is the presence of small amounts of branched- and odd-numbered carbon fatty acids (Hansen, et al. 1955); Shorland, et al. 1955; Hansen, et al. 1956). These fatty acids are not characteristic of plant lipids and it became evident that the synthesis of these acids must occur in the rumen (Church, 1970).

Table 1.1 Comparison of fatty acid composition of pasture and depot fats of pasture-fed animals. Fatty acids (wt %). (from Shorland, 1953)

Species	Source of fat	Fatty acids (weight %)										
		Saturated				Unsaturated						
		12:0	14:0	16:0	18:0	C12	C14	16:1	18:1	18:2	18:3	C20
Ryegrass	Leaf	0.4	1.4	10.6	1.5	0.2	0.5	4.1	4.6	11.6	62.8	1.9
Ox	Caul & kidney	-	2.7	27.8	21.6	-	0.3	2.5	42.5	0.5	0.3	1.8
Sheep	Total fatty tissues	1.3	3.5	25.0	22.2	-	0.5	1.7	44.2	tr	tr	0.9
Horse	" " "	-	2.4	29.7	4.3	-	1.4	6.5	32.5	3.8	16.1	3.1

1.3. Digestion and absorption of lipids in the ruminant

There have been a number of comprehensive reviews written on aspects of this topic (Church, 1970; Dawson and Kemp, 1970; Garton, 1963, 1965, 1967; Hungate, 1966; Keeney, 1970; Lough, 1970; Van Soest, 1963). Consequently, only material relevant to this thesis will be discussed here.

1.3.1. Initial fate of ingested pasture

The breakdown of the cell and release of contents during chewing by the animal has been discussed by Reid et al. (1962). Cells of freshly cut clover which are still turgid may be expected to rupture more easily than those of flaccid leaves, with a higher proportion of chloroplasts released in the former case. They further stated that whereas in some cases the cell wall is apparently ruptured with consequent release of whole cell contents, in other cases the cell may be crushed and its contents disorganised without rupture of the cell wall. Furthermore, the differences in absolute and relative rates of release of cell constituents will depend on the duration and vigour of chewing by the animals.

1.3.2. Rumen metabolism of lipids

Much of the investigatory work in this field, some of which is discussed below, has been done using in vitro techniques of various types.

a) Biohydrogenation. Although biohydrogenation is not the first reaction undergone by dietary lipids it was discovered before the other processes.

Since the initial investigations of Reiser (1951), biohydrogenation of unsaturated fats in the rumen has been extensively studied. Despite this, however, the exact nature of the pathway(s) involved and the source of hydrogen have not been conclusively established. Hartman et al. (1954) found that biohydrogenation in the rumen was accompanied by

the partial formation of trans isomers. Shorland et al. (1957) supported this conclusion. In fact, Katz and Keeney (1966) found that whereas unsaturated fatty acids in herbage are almost entirely of the cis configuration, 75% of the 18:1 acids leaving the rumen (about 7% of the total) consisted of trans acids with trans 11:12 being the major component. Conjugated fatty acids are also formed during hydrogenation (Hoflund et al. 1955; Shorland et al. 1957). Shorland et al. (1957) stated that the conjugated systems appeared to be partially resistant to hydrogenation. This probably would be due to stabilisation of the conjugated double bonds as a result of resonance (Hart and Schuetz, 1966).

It has been established that both protozoa and bacteria are capable of carrying out biohydrogenation, (Wright 1959, 1960; Gutierrez et al. 1962; Polan et al. 1964; Katz and Keeney, 1966) although the bacteria appear to be largely responsible for the process (Viviani, 1970).

That two systems of biohydrogenation may operate was suggested by Polan et al. (1964). They found that in the in vitro hydrogenation of 18:2, the saturated acid did not appear until after the level of 18:1 was greater than the level of 18:2 in the incubating system. The second system (18:1 $\longrightarrow$ 18:0) appeared to be inhibited by 18:2 and was dependent on the formation of a high level of 18:1 to offset this inhibition.

Dawson and Kemp (1970) in summarising the results of workers to this date have postulated the possible pathways of hydrogenation of α- 18:3 by rumen micro-organisms, (Fig. 1.1).

Of interest is a recent report by Harfoot et al. (1973) who have suggested that food particles may act as a site for the biohydrogenation of dietary unsaturated fatty acids. Hawke and Silcock (1970) found that centrifugation of rumen fluid at 50 g for 5 min resulted in the prevention of biohydrogenation of 18:1 to 18:0 by the supernatant. This was attributed to the removal of hydrogenating bacteria adhering to the food particles by the centrifugation process. Harfoot et al. (1973), however, dispute this theory. They suggested that biohydrogenation is effected by extracellular hydrogenases

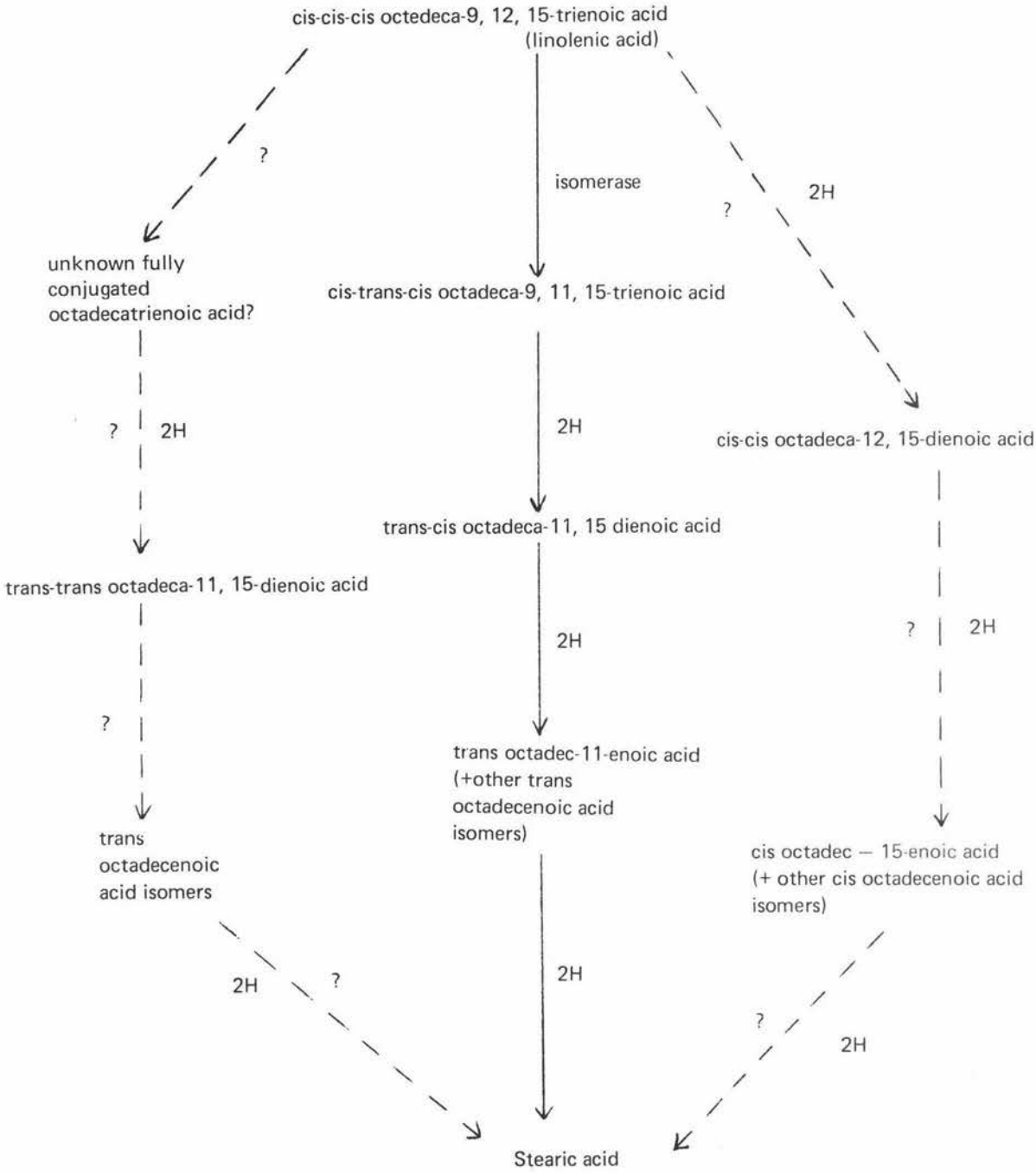

Fig. 1.1. Main and other possible pathways of hydrogenation of a linolenic acid by rumen micro-organisms. The diagram represents a simplification and many more positional isomers, of both conjugated and non-conjugated dienoic acids are found as intermediaries. (Dawson and Kemp, 1970)

produced by the bacteria in suspension. Clearly more work is required to resolve this issue.

b) The role of biohydrogenation. The precise role and implication of biohydrogenation is not known although Dawson and Kemp (1970) in their review have summarised the possibilities.

Polan et al. (1964) suggested that 18:2 is hydrogenated by an enzyme system which normally functions in the hydrogenation of an endogenously produced unsaturated compound.

There is also the possibility that hydrogenation plays a role in preventing toxicity due to the ingestion of unsaturated fatty acids which appear to inhibit microbial growth (Nieman, 1954). The toxicity of the unsaturated fatty acids may depend on their greater surface activity (compared with saturated fatty acids) and the consequent effect on changing the permeability of cell membranes (Kodicek and Worden, 1945). Czerkawski et al. (1966) and Blaxter and Czerkawski (1966) described experiments in which they found that unsaturated fatty acids depressed methane production by the methanogenic bacteria and that the reduction in methane production increased with increased unsaturation. Czerkawski et al. (1966) considered this depression in methane production might be energetically favourable to the ruminant as it would depress energy losses due to methane production. How substantial or significant this energy saving might be was not stated. In fact, MacLeod and Wood (1972) observed no improvement in energetic efficiency when soybean oil was added to a low fat basal diet fed to dairy cows.

Hydrogenation may also reduce the toxicity of other compounds, e.g., the hepatoxic alkaloid heliotropine which can cause chronic liver disease (Bull et al. 1956) unless hydrogenated (Culvenor et al. 1962). Plant phenolics or phenolic acids produced by ruminal amino acid metabolism may also be reduced in the rumen thus depressing their toxicity to the micro-organisms present (Booth and Williams, 1963; Scott et al. 1964).

Hydrogenation may protect ruminant tissues from the effects of high intakes of polyunsaturated fatty acids, Blaxter et al. (1952). That ingestion of these acids, coupled with a low intake of vitamin E, can lead to myopathic conditions in animals was suggested by Blaxter (1957), and is supported by evidence from Blaxter et al. (1952) and Boyd (1964) in work with pre-ruminant calves. However, Scott et al. (1970),and Faichney et al. (1972) made no mention of this problem when they fed protected polyunsaturated fats to ruminants although no more than 6 animals were used in their experiments.

c) Lipolysis. Garton et al. (1958) incubated triglycerides (linseed oil and tung oil) with sheep rumen contents. They found that both hydrogenation and lipolysis occurred. Of the total lipid at the end of incubation, 75% was in the form of free fatty acids. They found no lipolytic activity in saliva and presumed that the rumen microorganisms were responsible for lipase activity in the rumen. Their results were in agreement with later observations made by Garton et al. (1961) that the fatty acids of higher molecular weight in the digesta leaving the rumen were in the free state compared with those of non-ruminants in which ingested lipids are not subjected to hydrolysis until they reach the small intestine. They could detect no mono- or diglycerides during in vitro lipolysis of triglycerides suggesting that these intermediates, if formed at all, have a very transient existence. Hawke and Robertson (1964) in an in vivo experiment with dairy cattle were able to detect small amounts of the above intermediates. As with Garton et al. (1961) they noted that hydrogenation was probably preceded by lipolysis. Experiments utilising ^{14}C-labelled triglyceride in incubations with bovine rumen fluid revealed no detectable hydrogenation products in either the unhydrolysed triglycerides or in the radioactive mono- and diglycerides isolated from the incubation mixtures (Hawke and Silcock, 1970). Bacteria rather than other microorganisms appear to be responsible for most of the lipolysis occurring in the rumen (Wright, 1961). Faruque et al. (1974) showed that plant lipases released during chewing and rumination by the animal also play a role in lipolysis. It seems likely

that the rate of lipolysis has a maximum value. Bath and Hill (1967) noted the appearance of triglycerides in the lower gastrointestinal tract and said that this was a result of saturation of the lipolytic system by triglycerides.

d) Glycerol and galactose metabolism. Since the principal form of lipid in green leaves is galactosylglyceride, significant amounts of galactose (and glycerol) are released during lipolysis. These are readily fermented into volatile fatty acids. Propionic acid is the major product of glycerol fermentation (Garton et al. 1961; Hobson and Mann, 1961; Johns, 1953) although Kirk et al. (1971) found that more acetic than propionic acid might be formed. A mixture of acetic, propionic and butyric acids results from the fermentation of galactose by several species of rumen bacteria (Hobson and Mann, 1961).

e) Absorption. There appears to be little if any uptake of free fatty acids by the rumen epithelium although Hird et al. (1966) found that small amounts of free fatty acids were taken up by isolated sheets of sheep rumen epithelium and were possibly metabolised to ketone bodies. Bickerstaffe et al. (1972) using C^{14}-labelled 18:3 and 18:2 found little or no fatty acid uptake or absorption from the rumen which was also in agreement with other workers, e.g. Wood et al. (1963), Cook et al. (1969).

1.3.3 Metabolism and absorption of lipids in the lower gut

Very little if any degradation of long-chain fatty acids apparently takes place in the rumen. The lipids which are present in the digesta, passing more or less continuously from the rumen through the omasum and abomasum to the small intestine, consist largely of free fatty acids (of which 18:0 is normally the major component). The lipids in the digesta also contain the structural lipids of the micro-organisms (Garton, 1969).

There appears to be little change in the relative proportions, or fatty acid composition, of the various classes of lipid present in the digesta during its passage through the abomasum (Bath and Hill, 1967). Most of the

microorganisms are broken down which assists in the subsequent digestion of their structural lipids (Smiles and Dobson, 1956).

In the upper jejunum the lipid composition of the digesta changes because of the influx of bilary lipids which have a high content of phospholipids (Garton, 1969).

There appears to be little selectivity in the uptake of the fatty acids of the digesta. Bickerstaffe et al. (1972) using ^{14}C-labelled fatty acids found no selectivity in the metabolism of the geometric and positional isomers of 18:1. The rates of absorption of the isomers from the small intestine, transfer into lymph, uptake by the mammary gland and appearance in milk fat were similar. That some desaturation of stearic to oleic acid may occur in the wall of the small intestine was demonstrated by Bickerstaffe and Annison (1968, 1969) and Bickerstaffe et al. (1972). The physiological significance of this desaturation was not determined. Quantitatively it does not seem likely to be very important (Garton, 1969).

Fatty acids up to C_{10} are absorbed direct into the portal vein from the digestive tract whereas the long chain fatty acids are absorbed as lipoprotein complexes (chylomicra) which enter the jugular vein via the thoracic lymph duct (Senior, 1964).

1.4 The synthesis of milk fat in the ruminant

This topic has been extensively reviewed by Garton (1963); Peeters and Lauryssens (1964); Dimick et al. (1970); Storry (1970); Bickerstaffe (1971) and Storry (1972). It will be discussed only briefly here.

Milk lipids consist almost entirely of triglycerides (98% of total); the remaining 2% consisting of cholesterol esters, phospholipids, hydrocarbons and carotenoids. The cholesterol esters and phospholipids are synthesised in the mammary gland (Storry, 1972).

The milk fat triglycerides contain fatty acids of chain length C4-C20 (Hansen and Shorland, 1952). These fatty acids are arranged within the triglyceride in a non-random

manner (Blank and Privett, 1964) despite earlier indications from Boatman et al. (1961) and Garton (1963) that this was not so. The triglycerides of milk fat may be fractionated into a high and low molecular weight fraction (Blank and Privett, 1964) although the boundary between the two fractions is not precise as can be seen in the data of Kuksis et al. (1963). Some 38 types of triglycerides of which there may be up to 6 isomers were specifically identified by Breckenridge and Kuksis (1968a). These workers also confirmed an earlier claim by Kuksis et al. (1963) that 4:0 and 6:0 occur in the triglycerides almost exclusively in combination with medium and long chain fatty acids. These two fatty acids are confined to the 3 position of the triglyceride molecule (Pitas et al. 1967; Breckenridge and Kuksis, 1968b). A theory to account for the specific distribution of the fatty acids on the triglyceride molecule was advanced by Breckenridge and Kuksis (1969). They assumed a common pool of long chain 1,2-diglyceride precursors from which the bulk of both low and high molecular weight triglycerides are synthesised by a stereospecific introduction of C4-C18 fatty acids to position 3 of the glycerol moiety. This would explain the rough division into low and high molecular weight butterfat fractions obtained by Blank and Privett (1964).

The fatty acids of milk are basically derived from two sources (Bickerstaffe, 1971);

a) plasma lipids
b) intra-mammary gland synthesis.

About 50% of the milk lipids (in particular the long chain fatty acids, Tove 1965) are derived from blood lipids of which the major sources for milk fat are the chylomicra and low density lipoproteins. Bickerstaffe et al. (1971) cited in Bickerstaffe (1971) estimated that the low density lipoproteins appeared as approximately 36% of milk fatty acids with chylomicra and very high density lipoproteins contributing about 15% each of the total milk fat. This is surprising as Tove (1965) amongst others states that the high density lipoproteins contribute almost nothing to the fatty acids of milk.

Before the fatty acids in blood can be absorbed by the mammary gland they must be de-esterified by lipoprotein lipase in the capillary wall to free fatty acids (West et al. 1967a,b).

Briefly, the synthesis of milk fat within the mammary gland proceeds as follows (Storry, 1972):

i) incorporation of acetyl CoA into fatty acids of up to 16C's by the malonyl CoA pathway (Nandedkar et al. 1969).

ii) direct incorporation of β OH butyrate as a 4C molecule which may subsequently be elongated by further additions of acetyl CoA (Nandedkar et al. 1969). β hydroxy butyrate may also be incorporated after being degraded to two acetyl CoA units (Linzell et al. 1967). About 8% of the carbon in milk fat is derived from β OH butyrate (Palmquist et al. 1969).

iii) incorporation of acetic acid into short and intermediate chain acids by mitochondria (avidin-insensitive or non-malonyl CoA pathway, Nandedkar and Kumar, 1969; McCarthy and Smith, 1972).

Absorbed 16:0 and 18:0 may be desaturated in the mammary gland (Annison et al. 1967; Bickerstaffe and Annison, 1968; McCarthy et al. 1965). Desaturation proceeds only as far as 16:1 and 18:1. This desaturase enzyme accounts for much of the cis 18:1 present in ruminant milk fat (Bickerstaffe and Annison, 1968; Annison et al. 1967; Linzell et al. 1967). The desaturase enzyme is most active for 18:0 - the extent of desaturation of 16:0 being only about 20% of that of 18:0.

1.5 Variations in the composition of milk fat in the ruminant

Variations in the fatty acid composition of milk and body fat are small in cows receiving pasture. McDowall (1962) suggested that differences in butterfat characteristics could be due to the differences in the extent to which body fat reserves are drawn upon by the cow for the maintenance of lactation. Subjecting cows to under-feeding appears to result in an increase in the mobilisation and/or use of

fatty acids from depot fat for milk fat synthesis (Robertson et al. 1960; Munford et al. 1964). This, coupled with a lower availability of blood acetate, would account for the increase in butterfat % often encountered in underfed cows (Flux and Patchell, 1957; Robertson et al. 1960) and the increase in the degree of unsaturation and depression in the proportions of short chain fatty acids in the milk fat (Munford et al. 1964). Restricted feeding generally also results in a depression in milk, protein, lactose and fat yields with fat yield falling less than the other constituents (Flux and Patchell, 1957; Robertson et al. 1960). An increase in the supply of volatile fatty acids via infusion (Wilson et al. 1967) or presumably after a return to normal levels of feeding tends to alleviate the starvation effects.

Using identical twins, McDowall and McGillivray (1963a), investigated the possible influence of differences in pasture species (white clover and ryegrass). Their results indicated that butterfat characteristics were in fact influenced more by the stage of maturity than the botanical composition of the pasture. Stage of maturity appears to be an important factor in influencing the properties of New Zealand butterfat throughout the season (McDowall et al. 1961; McDowall and McGillivray 1963b). Hawke (1963) analysed fatty acid compositional changes. Using identical twins, he found that the milk fat of cows on short succulent ryegrass (ref. section 1.2.1) contained higher proportions of 18:1 and other C18 acids whilst the proportions of 14:0 and 16:0 were lower compared with the milk fat of cows grazed on stalky pasture. He concluded that the higher degree of unsaturation of the milk fatty acids of the cows on the succulent grass may have been related to (a), the higher levels of unsaturated fatty acids in the young ryegrass diet and (b), the extent to which these unsaturated fatty acids were hydrogenated in the rumen.

More marked changes in milk fat composition can be achieved if cows are fed fat or oil diets as discussed in the following section.

1.6 The effects of fats and oils on the composition of milk fat in the ruminant.

This has been reviewed by Davis and Brown (1970), Storry (1970, 1972) and Bickerstaffe (1971).

Interest in feeding fats and oils has arisen from the fact that small amounts can be beneficial in increasing palatability, aiding in the process of pelleting concentrate rations and in preventing bloat. Fats are also a rich source of energy (more than twice the energy content of carbohydrate, Storry, 1972) although their contribution in this regard depends on palatability and their effects on mechanisms involved in the control of the intake of energy, (MacLeod and Wood, 1972). A further limitation is that, in general, whereas the more saturated fats tend to maintain or increase milk fat and/or yield, unsaturated fats tend to decrease the fat content of milk. The type and quantity of roughage are significant factors as are also the amount and degree of unsaturation of fats in the basal diet (MacLeod and Wood, 1972).

Despite the extensive research on the effects of fats and oils, there are conflicting reports on all aspects of the subject (Bickerstaffe, 1971). That polyunsaturated fats in the diet can cause a depression in milk fat was established by Shaw and Ensor (1959). Whereas feeding cod liver oil resulted in milk fat depression, feeding hydrogenated cod liver oil did not. They also found large changes in the proportions of volatile fatty acids in the rumen - a finding not substantiated by anyone since.

The effects of supplements of lipids in the diet of ruminants has been summarised by Storry (1970) as follows:

a) Long-chain fatty acids (> C16 acids)

Feeding high levels of 18:3, 18:2, 18:1 and 18:0 to cows either as the free acid or in oils containing these acids have been associated with increased yields of 18:1 and 18:0 but not 18:2 or 18:3 in the milk. The increased yields of 18:1 and 18:0 may be due to:-

i) direct increase in the dietary intake of the fatty acid

ii) indirect increase from the diet due to hydrogenation of the less saturated acids in the rumen,

iii) increased 18:1 as a result of desaturation of 18:0 in the mammary gland.

Oils containing C20 or C22 acids do not appear to be secreted in more than trace amounts in milk even in cows given intravenous infusions (Storry et al. 1969).

b) Short and intermediate chain fatty acids (4-16 C's)

The effects of diet on the appearance of these fatty acids in milk are more variable because of intra-mammary gland synthesis of these acids from acetic acid and βOH butyric acid.

The synthesis of fatty acids of short and intermediate chain lengths within the mammary gland is decreased by the addition of oils and long chain acids to the diet. Brumby, et al. (1972) said that this decreased intra-mammary synthesis of fatty acids could be due to inhibition of acetyl CoA carboxylase by the increased supply of long chain fatty acids from blood plasma.

In summary, Storry et al. (1967) concluded that the fatty acid composition of the milk fat tended to change towards that of the dietary lipid except where the dietary fatty acids were hydrogenated in the rumen.

1.7 Philosophy for changing the nature of ruminant lipids

Recently, much interest has focused on the possibility of obtaining unsaturated ruminant milk and body fats. The contention is that saturated fats are associated with high levels of cholesterol in human blood serum and that this in turn predisposes towards heart disease. The issue is an unproven and controversial one as demonstrated by Reiser (1973) in his critical and comprehensive review. Despite the uncertain nature of the issue however, much research has been done with ruminants to either alleviate or bypass the process of biohydrogenation in the rumen. This research (discussed later) has been assisted by investigators attempting to

protect protein from rumen degradation. Black (1971) calculated the theoretical advantages of bypassing the rumen on the efficiency of use of dietary energy and protein in lambs. He considered that the rumen resulted in less efficient use of dietary energy on diets low in fibre and high in protein.

As a result of the above work, a number of ways have been devised in which components of the diet can be made to bypass the rumen or its effects.

1.8 Techniques for protection against biohydrogenation by the rumen micro-organisms.

1.8.1. General

Bypassing the rumen could be expected to have highly significant effects on the composition of ruminant milk and body fat. Changes that might be expected can be seen on inspection of the composition of horse fat. Fat from horses fed on pasture is highly unsaturated (Table 1 Section 1.2.2). Dietary fats are absorbed without being hydrogenated in horses as microbial fermentation of the diet does not occur in a rumen but rather, in the caecum - beyond the small intestine. As in monogastric animals, lipid material is absorbed from the small intestine. That C18 polyunsaturated fatty acids can be readily secreted in milk when the rumen is bypassed has been noted by Tove and Mochrie (1963) and Ogilvie and McClymont (1961) among others. These unsaturated acids can also be readily deposited in adipose tissue as such as demonstrated by Siren (1962), Cook *et al.* (1970), Faichney *et al.* (1972) and Connolly (1973).

1.8.2 Intravenous infusions

Tove and Mochrie (1963) infused 18:2 intravenously into a cow and obtained an increase in 18:2 in the milk fat. Storry *et al.* (1969) infused continuously for 2 days triglycerides of the fatty acids 3:0, 4:0, 6:0, 8:0, 9:0, 10:0, 12:0, 14:0 and 18:1 intravenously into cows and found that all acids except 3:0 and 4:0 gave increased yields of the infused acid in the milk fat. Infused triglycerides of fatty acids greater than C10 were transferred to milk in

large enough quantities to increase the yield of milk fat. Short chain acids were elongated by the addition of two C units up to C15 and C16 fatty acids. Intravenous infusions of soya bean oil (about 50% 18:2 - Hilditch and Williams, 1964), (Storry et al. 1969) and sunflower oil (55-60% 18:2 - Hilditch and Williams, 1964) (Stewart and Irvine, 1970) resulted in increased levels of the C18 unsaturated acids in milk fat. Infused cod liver oil (C20 and C22 unsaturated fatty acids, 25 and 20% respectively, Hilditch and Williams, 1964) resulted in increased levels of C20 and C22 unsaturated fatty acids in plasma triglycerides. However, they did not appear in the milk fat (Storry et al. 1969). They suggested that the C20-C22 unsaturated acids inhibited lipoprotein lipase in the mammary gland which would inhibit the uptake of all long chain fatty acids from the plasma by the mammary gland.

1.8.3. Duodenal and abomasal infusions

Infusion of a 40 ml emulsion (8:1, v/v, fat/bile) of linseed oil (rich in 18:2 and 18:3, Hilditch and Williams, 1964) per day into a sheep was carried out by Ogilvie and McClymont (1961) who then observed the effects on the composition of depot fat. They found that depot fat levels of 18:2 and 18:3 rose from the normal value of 1-2% of total fatty acids to approx. 8 and 10% for 18:2 and 18:3 respectively. Moore et al. (1969) infused emulsions of linseed oil, maize oil or 18:2 into the abomasums of 3 sheep and found that the polyunsaturated fats were preferentially used by the liver for the synthesis of phospholipids and cholesterol esters but not for the re-synthesis of triglycerides (their interpretation). In fact plasma concentrations of 18:2 and 18:3 in the triglycerides and free fatty acids remained unchanged during the infusion period.

The effects obtained by Ogilvie and McClymont (1961) are in agreement with work by Bickerstaffe and Annison (1971) who infused up to a maximum of 650 g of sunflower oil per day into a cow. The 18:2 level in the milk fat rose from 3.0 to 27.0 moles %. The yield of milk fat rose from 750 g to a maximum of 1050 g/day.

1.8.4. Use of young ruminants

Sirén (1962) and Hoflund et al. (1956) found that in young ruminants, in which a functional rumen had not developed, the composition of the depot fat was dependent on the nature of the dietary fat (as in non-ruminants, Sirén, 1962). The metabolism of polyunsaturated fatty acids in young calves is very dependent on the anatomical and functional development of the rumen (Sirén, 1962). They found that depot fat of calves receiving a linseed oil milk replacer (no roughage) diet contained approximately 9 and 10.6% 18:2 and 18:3 respectively. However, the depot fat of calves fed hay contained less than 3.5% 18:2 and 18:3. Presumably the roughage promoted earlier development of a fully functioning rumen.

1.8.5 Oesophageal groove reflex

The use of this reflex with respect to ruminant nutrition has been reviewed by Ørskov (1972). By closure of the oesophageal groove, milk and other suckled fluids are passed directly into the abomasum thus bypassing the rumen. Factors affecting closure of the groove are discussed by Ørskov (1972) and Ørskov et al. (1970). The application of the reflex in the feeding of "high quality" protein has been investigated by Ørskov and Benzie (1969a, 1969b).

The use of the groove reflex in the production of polyunsaturated body fats in ruminants was investigated by Connolly et al. (1973). Lambs were suckled with ewe milk-replacer diets, enriched with polyunsaturated lipid (18:2). The diets bypassed the rumen and when the lambs were slaughtered at 36 kg liveweight, their depot fats showed a 2-10 fold increase in 18:2 content over control animals. In fact, 18:2 in some cases rose to approximately 20% of the total fatty acids of depot fat.

The use of this reflex would appear to be the most practicable technique discussed so far. However, it is unlikely to result in unsaturated milk or body fats in mature ruminants due to the loss of the reflex action in these

animals. Maintenance of the groove function appears to be dependent on conditioning by the feeding procedure (Ørskov et al. 1970).

1.8.6 Antibiotics

These have been used in association with protection of protein against ruminal degradation. Hogan and Weston (1969) found that different antibiotics had different effects but that chloramphenical appeared to give the best results in preventing protein degradation. However, while the digestion of protein in the rumen was depressed, feed intake was also seriously reduced. In fact, there was no increase in the amount of protein passing out of the rumen into the lower gastrointestinal tract. No work of this nature appears to have been done with respect to attempts to prevent hydrogenation of polyunsaturated fatty acids.

1.8.7 Autoclaving or heating

This technique has also only been used in attempts to protect proteins against rumen microbial attack. Danke et al. (1966) found that heating proteins (cottonseed meal) could denature them and render them less soluble and thus less liable to degradation in the rumen.

1.8.8 HCHO treatment

In one respect this is similar to the technique discussed in section 1.8.7. in that it results in a depression in the solubility of protein in the rumen. The use of HCHO in preventing degradation of protein by rumen microbes has been investigated by a number of workers and has been reviewed (Ferguson 1970, 1972). Ferguson et al. (1967) found that HCHO treatment of casein (10 vol. 4% HCHO) rendered the casein virtually insoluble at pH 6.0 but soluble at pH 3.0 (c.f. rumen pH which normally is greater than 6.0 but abomasal pH which is less than 3.0). Microbial degradation of the HCHO-treated casein was found to be almost completely prevented with little overall loss in digestibility of the casein (90% digestible compared with 96% for untreated material - Reis and Tunks, 1969). Other

studies with protected protein have since been done with similar results, e.g. Barry (1969, 1970), MacRae (1970), Carrico et al. (1970) and MacRae et al. (1972).

Walker (1964) states that HCHO has been used for some time in the tanning industry to harden proteins, decrease their water sensitivity and increase their resistance to the action of chemical reagents and enzymes. During the tanning process, water appeared to be split off and it seemed likely that simple methyl derivatives were the primary products formed in all protein - HCHO reactions. Protection is probably due to the formation of methylene bridges and other cross linkages between the protein chains which are acid reversible (Ferguson et al. 1967). The HCHO-protein reaction occurs most rapidly with proteins in the dissolved state (Walker, 1964).

Use has recently been made of the protein-HCHO reaction in the protection of polyunsaturated fatty acids against biohydrogenation by the rumen contents. The subject has been discussed by Johnson (1974). Protection of unsaturated fat was first achieved by Scott et al. (1971). They prepared an oil-casein (1:1 w/w) emulsion which was then spray-dried resulting in particles consisting of oil droplets coated with protein. These particles were then HCHO-treated at 4-5% (w/w, HCHO/casein). When HCHO-treated particles of linseed oil/protein were fed at 500 g/day to goats, milk fat containing 20-25% of total fatty acids as 18:3 resulted, compared with control levels of 0.5-0.7%. Feeding 1500 g of treated supplement to cows resulted in 12-22% 18:3 in the milk fat compared with approximately 1% in the controls. Safflower oil - (75% 18:2 Cook et al. 1970) protein-treated particles resulted in 35% 18:2 in the milk fat compared with normal values of approximately 2% (Scott et al. 1970).

When safflower oil-casein supplements were fed to 8-10 week old lambs there were significant increases in the proportions of 18:2 in perinephric, mesentric and subcutaneous fat. After 3 weeks of feeding (170-180 g supplement per day),

the unsaturated fatty acid content of perinephric fat had risen from 4-5% to approximately 15-20% (Cook et al. 1970). The depot fats of sheep were altered in a similar manner after feeding the HCHO-treated particles for 6 weeks (Scott et al. 1971). Faichney et al. (1972) found that incorporation of the protected unsaturated fat appeared to be more extensive in the deeper body tissues than in tissues nearer the skin. Incorporation followed a curve of diminishing increments over a period of 8 weeks of feeding 18:2 supplement to young steers. Faichney et al. (1972) obtained up to 25-35% 18:2 in the depot fat of these young animals. These results indicated that patterns of incorporation of 18:2 into plasma and tissue lipids would not be unlike that in non-ruminants as confirmed in a further study by Cook et al. (1972a). The protected fat supplement was also found to provide the animal with a greater intake of metabolisable energy, nett energy and amino acids than a basal diet of lucerne hay (Hogan et al. 1972). This would be expected because of the "high quality" of protected casein as a source of amino acids and the high energy content of oils (Ref. section 1.6).

The effects of protected oil supplements on the composition of milk fat obtained by the Australians were also obtained by Plowman et al. (1972). They produced milk fat containing up to 35% 18:2 after feeding cows a HCHO-treated supplement of casein and safflower oil (5.8:4.0, w/w protein/oil) at 1500 g per day. The effects on milk composition were studied in further detail by Pan et al. (1972) and Cook et al. (1972b). Pan et al. (1972) found that feeding 1 kg of HCHO protected supplement (1:1, w/w, safflower oil/casein) per day resulted in an average increase of 15 and 6% in the contents of fat and protein respectively. The usual effect of a depression in milk fat associated with the feeding of unsaturated lipids to ruminants (ref. section 1.6) was eliminated. Cook et al. (1972b) found that the supplement yielded increased proportion of 18:2 both in plasma triglycerides and the milk fat. This compares with abomasal infusions by Moore et al. (1969) of 18:3 and 18:2 into sheep where they obtained no increase in plasma

triglycerides. However tissue uptake of triglycerides may have been greater resulting in triglyceride levels of 18:3 and 18:2 in plasma remaining constant despite increased uptakes into the triglycerides. Cook et al. (1972b) found that the increased proportions of 18:2 in the milk fat were associated with decreased proportions of 16:0 and 14:0. Conversely, oil supplements which had not been treated with HCHO were hydrogenated in the rumen resulting in an increase in 18:1 in the milk fat again at the expense of 16:0 and 14:0.

The metabolism of HCHO ingested with these supplements was investigated by Mills et al. (1972). Using ^{14}C-HCHO they were able to conclude that ruminants can effectively metabolise HCHO with no accumulation of the compound in either the carcass or milk. Of the ingested HCHO, 60-80% was metabolised to CO_2 and CH_4, 11-27% was excreted in the faeces and 5-6% appeared in the urine.

The efficiency of transfer of protected unsaturated fat into milk fat was measured by Bitman et al. (1973). Formaldehyde treated material (safflower oil:casein:HCHO, 58:40:2, w/w) was fed at rates varying from 200-3200 g/day. Efficiency of transfer of dietary 18:2 ranged from 17-42% for various prepared lots. However, preparations of HCHO-treated soybean particles were incorporated into milk fat with only 2-8% efficiency. This low and variable rate of incorporation probably explains why Hutjens and Schultz (1971) were unable to see any advantage in treating soybeans with HCHO. They found that incorporation of 18:2 into milk fat from HCHO-treated material was no better than for untreated material, although protection against ruminal biohydrogenation with the HCHO-treated material did appear to be occurring to a small extent. Analysis of the results of Cook et al. (1972b) shows that efficiency of transfer of dietary 18:2 (in 2 Jersey cows) was of the order of a maximum of 40% which compares well with the maximum value obtained by Bitman et al. (1973).

Of further interest is work by Barry (1971), Hemsley et al. (1970) and more recently Sharkey et al. (1972) and Barry (1973), in which dried forage was sprayed with HCHO.

Barry (1971) and Hemsley *et al.* (1970) achieved protein protection with this method which resulted in a variable increase in wool growth, when the material was fed to sheep. Sharkey *et al.* (1972) did not confirm this result. They explained their lack of response on the basis that the level of HCHO retained in the hay (0.3% of crude protein) was too low and that excessive loss of formalin after treatment reduced its effectiveness. Barry (1972 *pers.com.*) recommended an application rate of 5 g/100 g crude protein for forages. This is higher than the 1.0-1.5% level recommended by Ferguson (1970). Presumably also, the effectiveness of the HCHO treatment as measured by changed wool growth would depend on the protein content and quality of the dried grass. There would appear to be little point in protecting "poor protein" - as in mature forage - from degradation by the rumen.

If forage protein can be protected then it would also seem possible that forage lipid could be protected from hydrogenation by rumen microbes. As discussed in section 1.2.1 the bulk of pasture leaf lipid occurs in the chloroplasts - cell organelles enclosed by lipoprotein membrane complexes (Gurr and James, 1971). Furthermore, as mentioned, chloroplastic lipid is present in a continuous matrix in which protein particles containing pigment and lipids are embedded. It may be that this protein-lipid association would be close enough to provide protection against rumen biohydrogenation of the leaf lipids if the material was HCHO-treated before feeding to ruminants. If this were so, it would undoubtedly be of great practical significance.

1.9 Aim of the present study

The aim of the present study was to determine whether HCHO could be used to protect the unsaturated fatty acids in leaf tissue from rumen biohydrogenation. The protection of grass lipid was investigated using both *in vitro* and *in vivo* techniques. The proportion of polyunsaturated fatty acids transferred from the diet into the milk was determined for

both the HCHO-treated dried grass and a protected sunflower seed supplement. The two diets were compared.