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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
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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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LIST OF CONTENTS

	Page
CHAPTER 1 LITERATURE REVIEW	1
1.1 Introduction.	1
1.2 The nature of pasture and animal lipids.	1
1.2.1 Plant lipids.	1
1.2.2 Animal lipids.	2
1.3 Digestion and absorption of lipids in the ruminant.	4
1.3.1 Initial fate of pasture lipids.	4
1.3.2 Rumen metabolism of lipids.	4
a) Biohydrogenation.	4
b) The role of biohydrogenation.	7
c) Lipolysis.	8
d) Glycerol and galactose metabolism.	9
e) Absorption.	9
1.3.3 Metabolism and absorption of lipids in the lower gut.	9
1.4 The synthesis of milk fat in the ruminant.	10
1.5 Variations in the composition of milk fat in the ruminant.	12
1.6 The effects of fats and oils on the composition of milk fat in the ruminant.	14
1.7 Philosophy for changing the nature of ruminant lipids.	15
1.8 Techniques for protection against biohydrogenation by the rumen micro-organisms.	16
1.8.1 General.	16
1.8.2 Intravenous infusions.	16
1.8.3 Duodenal and abomasal infusions.	17
1.8.4 Use of young ruminants.	18
1.8.5 Oesophageal groove reflex.	18
1.8.6 Antibiotics.	19
1.8.7 Autoclaving or heating.	19
1.8.8 HCHO treatment.	19
1.9 Aim of the present study.	23

CHAPTER 2	USE OF FORMALDEHYDE IN THE PROTECTION OF LINOLENIC ACID IN DRIED GRASS AGAINST RUMEN MICROBIAL BIOHYDROGENATION <u>IN VITRO</u> .	Page
		25
2.1	Materials.	25
2.1.1	Reagents and solvents.	25
2.1.2	Source and preparation of dried grass.	25
2.1.3	Rumen fluid.	26
2.2.	Methods.	26
2.2.1	Incubation of rumen fluid.	26
2.2.2	Analytical techniques.	27
	a) Chemical analysis of the dried grass.	27
	b) pH	27
	c) Determination of NH_3 .	27
	d) Digestibility.	27
	e) Extraction of lipids.	28
	f) Preparation of methyl esters.	28
	g) Analysis of methyl esters.	29
2.3	Experimental and results.	29
2.3.1	Chemical analysis of dried grass.	29
2.3.2	The effect of spraying dry grass with 1 g HCHO/ 100 g dry grass on NH_3 production and digestion <u>in vitro</u> .	30
2.3.3	The relationship between the level of HCHO treatment of dry grass and NH_3 production, pH and digestibility following incubation with rumen fluid for 24 h.	32
2.3.4	The relationship between biohydrogenation of linolenic acid, NH_3 production, pH and duration of incubations of HCHO-treated dried grass with rumen fluid.	37
2.3.5	The effect of level of HCHO-treatment of dried grass on the pH, NH_3 level and fatty acid composition of the incubation mixture after 24 h of fermentation.	40
2.3.6	The effects of addition of free HCHO to incubations of rumen fluid and untreated dry grass.	42

CHAPTER 3.	THE USE OF FORMALDEHYDE TO PROTECT UNSATURATED FATTY ACID CONSTITUENTS OF THE DIET AGAINST RUMINAL BIOHYDROGENATION <u>IN VIVO</u>	Page
		45
3.1	Materials.	45
3.1.1	Reagents and solvents.	45
3.1.2	Source and preparation of feedstuffs.	45
	a) Dried grass.	45
	b) Oil-seed supplement.	48
	c) Barley meal.	48
3.1.3	Animals and their treatment.	48
3.2	Methods.	48
3.2.1	Chemical analysis of feedstuffs used.	48
3.2.2	Analysis of milk.	49
	a) Analysis by the infra-red milk analyser (IRMA).	49
	b) Fatty acid analysis of milk fat.	49
	i) Extraction of milk fat.	
	ii) Preparation of methyl esters.	
	iii) Analysis of methyl esters.	
3.3	Experimental design.	50
3.4	Results.	52
3.4.1	Analysis of feedstuffs.	52
3.4.2	Intake and milk yield.	55
3.4.3	Milk lactose.	57
3.4.4	Milk protein.	57
3.4.5	Milk fat.	60
	a) The fat content of milk and total milk fat yield.	60
	b) Composition of milk fat.	60
CHAPTER 4.	DISCUSSION AND CONCLUSIONS.	69
4.1	Introduction.	69
4.2	The protection of protein and 18:3 in dried grass against digestion by rumen micro- organisms in rumen liquor.	69
4.2.1	Protection of forage protein.	69

	Page	
4.2.2	The optimal rate of HCHO-application for protection of protein in dried grass.	69
4.2.3	The extent of biohydrogenation of 18:3 in dried grass by rumen micro-organisms.	70
4.2.4	The effects of free HCHO on the activity of micro-organisms in rumen liquor.	71
4.3	The protection of unsaturated fatty acids in the diet against biohydrogenation in the rumen of the cow.	72
4.3.1	Feedstuffs used.	72
4.3.2	The influence of dried grass which was HCHO-treated and the effects of underfeeding on milk yield and composition.	73
4.3.3	The influence of supplements of oil-seed and barley meal on milk yield and composition.	75
4.4	Summary and conclusions.	78
4.4.1	The protection of protein and 18:3 in dried grass against digestion by the micro-organisms in rumen liquor.	78.
4.4.2	The protection of unsaturated fatty acids in the diet against biohydrogenation in the rumen of the cow.	79
	REFERENCES.	80

LIST OF FIGURES

	Page
Fig.1.1 Principal and other possible pathways of hydrogenation of α -linolenic acid by rumen micro-organisms.	6
Fig.2.1 The effects of HCHO-treatment of dried grass (1 g HCHO/100 g dried grass) on NH_3 production when incubated with rumen fluid.	31
Fig.2.2 The relationship between the level of HCHO-treatment of dried grass and NH_3 production when incubated with rumen fluid for 24 h.	33
Fig.2.3 The relationship between the level of HCHO-treatment of dried grass and (a) the final pH (b) the digestion of dry matter after incubation with rumen fluid for 24 h.	34
Fig.2.4 The relationship between pH and digestibility values when dried grass was treated with different levels of HCHO.	36
Fig.2.5 The relationship between fatty acid composition and time of fermentation of HCHO-treated (2.0% w/w) and untreated dried grass.	38
Fig.2.6 The effect of HCHO treatment of dried grass (2.0% w/w) on (a) pH, (b) NH_3 conc. during incubation with rumen fluid.	38
Fig.2.7 The effect of level of HCHO-treatment of dried grass on the fatty acid composition of the incubation mixture after fermentation with rumen fluid for 24 h.	41
Fig.3.1 Daily grass D.M. and concentrate intake.	52
Fig.3.2 Daily milk yield.	56
Fig.3.3 Daily milk lactose content and milk lactose yield.	58
Fig.3.4 Daily milk protein content and milk protein yield.	59
Fig.3.5 Daily milk fat content and milk fat yield.	61

	Page
Fig.3.6 Daily milk fat composition; short chain fatty acids - 4:0, 6:0, 8:0, 10:0.	63
Fig.3.7 Daily milk fat composition; medium chain fatty acids - 12:0, 14:0, 16:0.	65
Fig.3.8 Daily milk fat composition; long chain fatty acids - 18:0 and 18:1.	66
Fig.3.9 Daily milk fat composition; long chain fatty acids - 18:2 and 18:3.	67
Fig.A.1 Sample milk fat G.L.C. trace.	

LIST OF TABLES

		Page
Table 1.1	Comparison of fatty acid composition of pasture and depot fats of pasture fed animals.	3
Table 2.1	Rates of application of 5% HCHO solution to dried grass (Batch 2).	26
Table 2.2	Composition of dried grass used in <u>in vitro</u> incubations with rumen fluid.	30
Table 2.3	The effect of HCHO-treatment of dried grass (1 g HCHO/100 g dry grass) on NH ₃ production and digestion of dry matter when incubated with rumen fluid.	32
Table 2.4	The effects of HCHO-treatment of dried grass on pH and NH ₃ concn. after incubation for 24 h with rumen fluid.	42
Table 2.5	A comparison of the effects of free and protein-bound HCHO on the activity of rumen micro-organisms.	44
Table 3.1	The effects of HCHO-treatment on the formation of NH ₃ , biohydrogenation of 18:3 and final pH after incubating dried grass for 24 h with rumen fluid.	46
Table 3.2	Analysis of all feed stuffs used.	54
Table 3.3	The variation obtained in the analysis of one sample of milk fat on the gas liquid chromatographs.	62
Table A.1	Gas liquid chromatograph correction factors.	
Table A.2	Example calculation on milk fat sample from cow 110, date, 10/4.	

per cent	%
plus or minus	±
potassium carbonate	K ₂ CO ₃
second(s)	s
standard error of mean	S.E.
thin layer chromatography	T.L.C.
ultra-violet	U.V.
volume(s)	vol(s)

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; Carton, 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 → 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

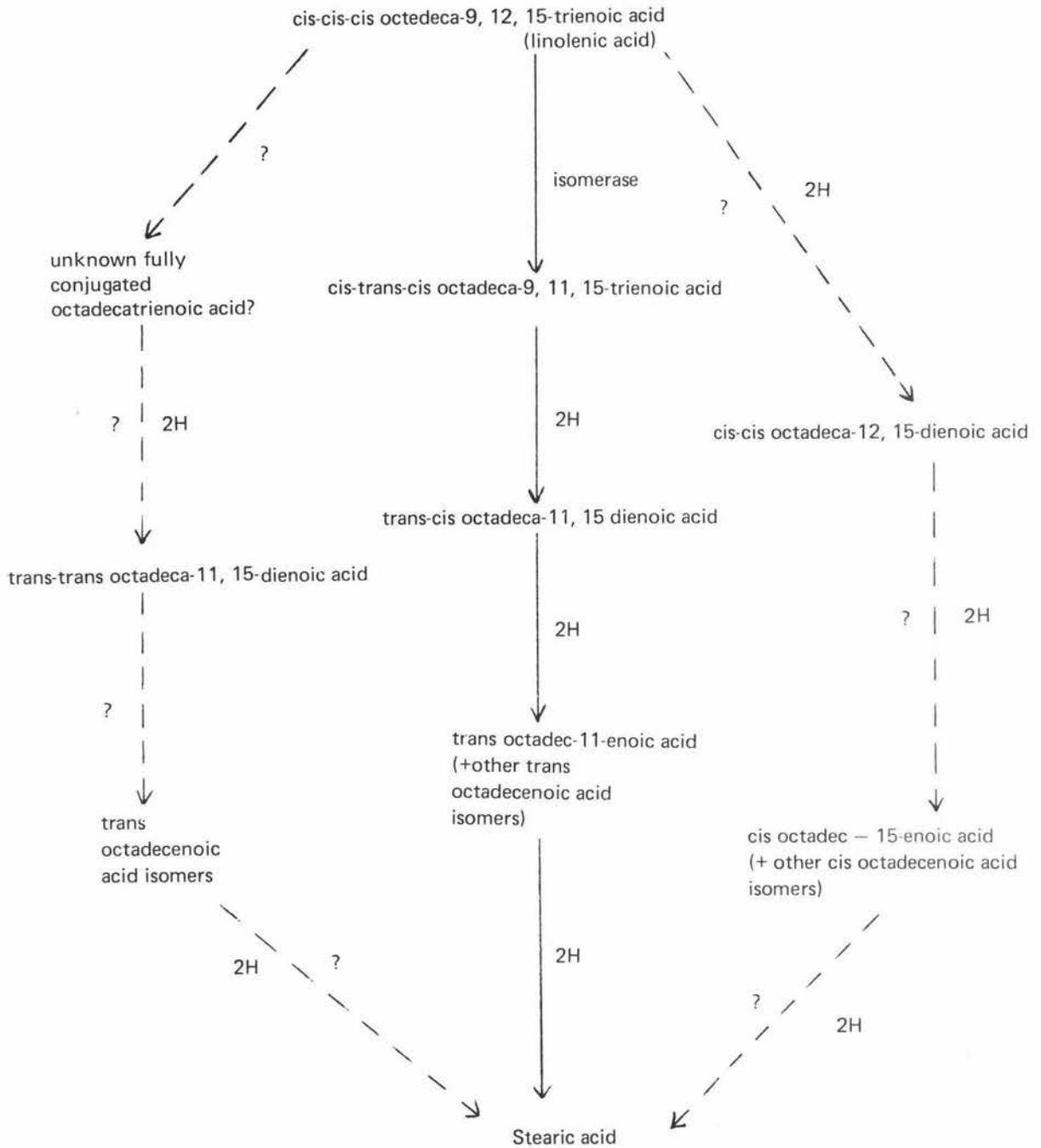


Fig. 1.1. Main and other possible pathways of hydrogenation of a linolenic acid by rumen micro-organisms. The diagram represents a simplification and many more positional isomers, of both conjugated and non-conjugated dienoic acids are found as intermediaries. (Dawson and Kemp, 1970)

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 hepatotoxic 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 C¹⁴-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 biliary 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 Laurysens (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 C_4 - C_{20} (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 β OH 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 chloramphenicol 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.

CHAPTER 2 USE OF FORMALDEHYDE IN PROTECTION OF DRIED
GRASS LINOLENIC ACID AGAINST RUMEN MICROBIAL
BIOHYDROGENATION IN VITRO.

2.1 Materials

2.1.1 Reagents and solvents

All chemicals were of either analytical or laboratory grade. Solvents were distilled before use.

2.1.2 Source and preparation of dried grass

The dried grass was prepared on the basis of the methods used by Hemsley et al. (1970) and Barry (1971).

Two batches of "Grasslands Ariki" ryegrass (Lolium x hybridium Hausskin) were collected from the Massey University No.2 Dairy Farm. The grass (approx. 20 cm long) was cut about 5 cm above the ground. The cut grass (17% dry matter content) was dried in a forced air-draught oven at 28 °C, or slightly lower, for 48 h. One half of the first batch (Batch 1) of dried grass was sprayed with 5% (w/v) HCHO at a rate of 1 g HCHO per 100 g of dried grass (Hemsley et al. 1970). Five percent HCHO was the maximum concentration that could be used while still maintaining even coverage of the grass. Both the sprayed and unsprayed samples were then stored in plastic bags for 5 days after which they were further oven dried at 40 °C for 24 h. The grass was then ground in a Wiley Mill (2 mm sieve) and stored in glass flasks in the dark.

The second batch of grass (Batch 2) was treated as described above except that three rates of HCHO application were used. These are summarised in Table 2.1.

Table 2.1 Rates of application of 5% HCHO solution to dried grass, (Batch 2).

Basis for calculation of rate of application	HCHO (% w/w)		
per 100 g dried grass	0.5	1.0	2.0
per 100 g dry weight of dried grass	0.55	1.11	2.22
per 100 g crude protein (est.)*	2.90	5.80	11.60
per 100 g crude protein (determined) ^t	2.06	4.12	8.24

* est. on 19% D.M. basis

^t by Kjeldahl, D.M. determined at 26.7%

2.1.3 Rumen fluid

A non-lactating Jersey cow fitted with a rubber rumen cannula and grazed on fresh pasture was fasted over night (15-16 h) to provide a source of rumen fluid. Rumen fluid was removed from the mid-region of the rumen (Bryant, 1964; Davey, 1965) and strained through two layers of cheese-cloth into a previously warmed thermos flask. The rumen fluid was then transferred to a conical flask which was filled to the top, lightly stoppered and placed in a water-bath at 38 °C until required.

2.2 Methods

2.2.1 Incubation of rumen fluid

Incubations were carried out in 100 cm³ glass bottles containing the following:

- 1) 0.3 g dried grass sample
- 2) 20 cm³ of phosphate buffer (Clarke and Hawke, 1970) adjusted to pH 6.8
- 3) 10 cm³ of well mixed rumen fluid.

The bottles were gassed with 95% N₂ and 5% CO₂ for about 20 s before sealing with rubber stoppers fitted with bunsen valves (Tilley and Terry, 1963). Incubation was carried out at 38 ± 0.5 °C. In later experiments, 1.0 g dried grass, 25 cm³ phosphate buffer and 5 cm³ rumen fluid were used in incubations.

This was found necessary in order to allow hydrogenation of dried grass 18:3 to be assessed accurately.

Since the object of the experiments was to determine the effects of HCHO treatment of grass on rumen digestion only, the second stage incubation, as recommended by Tilley and Terry (1963) for digestibility measurements, was omitted.

When fatty acid analyses of the incubation mixtures were to be determined, incubations were stopped by heating with an equal volume of MeOH at 80 °C for one min.

2.2.2. Analytical techniques

a) Chemical analysis of the dried grass. Moisture, crude protein, crude fibre and lipid were estimated by the methods recommended by the A.O.A.C. (1965).

b) pH. The pH of the incubation mixtures was measured before and after fermentation using a Radiometer (Copenhagen) 25 pH meter. This provided a measure of the extent of volatile fatty acid formation in the incubation mixtures (Robertson, 1968; Bryant, 1961).

c) Determination of NH₃. The concentration of NH₃ in the incubation mixtures was determined by the method of Conway and O'Malley (1942). One cm³ of 0.5% boric acid (with bromocresol green/phenol red as indicator) in 20% EtOH was placed in the centre compartment of a Conway dish. One cm³ of rumen fluid and one cm³ of saturated K₂CO₃ was placed in the outer chamber. The dish was then quickly sealed and swirled to mix the rumen fluid and K₂CO₃. Ammonia was then allowed to diffuse over by heating the dish at 38 °C for 1 h. The concentration of NH₃ (mg NH₃/100 cm³ rumen fluid) was then calculated from the titration of the contents of the centre compartment with approximately 0.02 M standard HCl.

d) Digestibility. The method used generally followed that of Tilley and Terry (1963) as modified by Alexander and McGowan (1961) where centrifugation was replaced by filtration.

At the end of incubation (24 h), approx. 1 g of filter aid (Hyflo Super-cell) was accurately weighed into all

incubation bottles. The contents of the bottles were then filtered under vacuum on to weighed glass fibre filter papers using Hartley 3-piece funnels. The residue was washed with distilled water and dried on the filter paper at 100 °C for 24 h before weighing (Wr).

$$\begin{aligned} & \text{Then; \% digestibility of the dry sample} \\ & = \text{Dry wt of sample} - \\ & \quad \frac{(\text{residual dry wt} - \text{av. blank residue dry wt})}{\text{sample dry wt.}} \times 100\% \end{aligned}$$

where: sample dry wt. = wt. of grass sample x DM %

residual dry wt. = Wr - (filter paper + filter aid)

av. blank residue dry wt. = Wr - (filter paper + filter aid)

Av. blank residue dry wt. was meaned over all the blank incubations.

e) Extraction of lipids. At the end of the incubation period and after the addition of an equal volume of MeOH, the solids, which were retained by filtration through miracloth (Cal biochem California) were refluxed for 3 h with CHCl₃: MeOH (2:1, v/v). The filtrate was extracted twice in a separating funnel by adding 3 vols. of CHCl₃ and draining off the CHCl₃:MeOH layer after each extraction. These extracts were concentrated under vacuum using a Büchi rotary evaporator and combined with the refluxed portion. Water was added to the combined extract to give two phases and the CHCl₃ phase was washed with 0.9% (w/v) aqueous NaCl and then twice with water. The lipid solution, after evaporating to approx. 2cm³ under vacuum, was transferred to a test-tube and the remaining solvent removed by a stream of O₂-free N₂.

f) Preparation of methyl esters. Methyl esters of the constituent fatty acids of the lipids were prepared by the method of Van Wijngaarden (1967) as follows: The lipid sample (< 150 mg) was refluxed with 0.5 cm³ 0.5 M NaOH in MeOH for about 2 min on a sandbath. Boron trifluoride reagent (BDH - 14% or Fluka - 10% in MeOH) (0.7 cm³) was then added through the condenser and refluxing continued for a further 3 min. Approximately 2 cm³ of hexane was added and 15 s

later the reflux tube was removed from the sandbath. The methyl esters, dissolved in hexane were floated to the top of the tube by adding distilled water. The hexane layer was then removed with a Pasteur pipette. If the methyl esters were highly pigmented they were purified by TLC on silica gel G using hexane:diethyl ether (80:20 v/v) as the developing solvent. The areas on the developed chromatograms containing the methyl esters were identified by lightly spraying the plates with 0.2%, 2, '2'-dichlorofluorescein in ETOH and viewing under u.v. light. The appropriate zones were scraped off the plates into centrifuge tubes, extracted with diethyl ether by means of a vortex shaker and the ether layer decanted following centrifugation.

g) Analysis of methyl esters. The methyl ester solution was concentrated under N_2 and injected into the gas-liquid chromatographic column. Two gas-liquid chromatographs were used for analysis of the fatty acid methyl esters:

i) a Packard gas chromatograph fitted with a 1.8 m x 4 mm (i.d.) glass column packed with 12% diethylene glycol adipate (DEGA) on a solid support of Anakrom Q (60/70 mesh).

ii) An aerograph gas chromatograph model 1520 fitted with a 1.8 m x 4 mm (i.d.) glass column packed with 12% diethylene glycol succinate (DEGS) on a solid support of Anakrom P (60/70 mesh).

Both chromatographs were fitted with flame ionisation detectors. Nitrogen was used as carrier gas and columns were run at 165 °C.

Peaks were identified by means of standards and their relative areas calculated using a planimeter. The % fatty acid composition of each sample was calculated directly from the relative peak areas.

2.3. Experimental and Results

2.3.1. Chemical analysis of dried grass

Table 2.2. shows the results of the analysis.

Table 2.2. Composition of dried grass used in in vitro incubations with rumen fluid

	Dried grass	
	Batch 1	Batch 2
% D.M. of freshly harvested grass	18.5 \pm 0.3	17.0 \pm 0.4
% D.M. after final drying and grinding	91.0 \pm 1.0	90.4 \pm 0.2
Crude protein % (on D.M. basis)	21.3 \pm 0.6	26.7 \pm 0.6
Lipid extract % (" " ")	N.A.*	5.7 \pm 0.3
Crude fibre % (" " ")	N.A.	26.6 \pm 0.4

Values given are means of 4 replicates \pm SE.

* N.A. Not available.

Batch 2 came from a stand of Ariki which appeared younger and more succulent than that of Batch 1. This observation was confirmed by the values obtained for crude protein measurements, i.e., 26.7% of D.M. for Batch 2 and 21.3% for Batch 1. The difference in fresh D.M.% between batches is however fairly small.

2.3.2. The effect of spraying dry grass with 1 g HCHO/100 g dry grass on NH₃ production and digestion in vitro

Two experiments were carried out using the first batch of dry grass to establish the extent of reaction of HCHO with grass protein and whether this HCHO-protein reaction affected the digestion of the grass by rumen microorganisms.

The results of the first experiment (Fig.2.1) show that after 8 h incubations of HCHO-treated grass with rumen fluid, the NH₃ concentration was 36.7 mg/100 ml NH₃ whereas with untreated grass the concentration was 33 mg/100 ml. The difference between these values was highly significant ($p < 0.001$) as was the difference after the 24 h incubation ($p < 0.01$).

The second experiment (Table 2.3) again showed that during 24 h-incubations, HCHO-treatment of dry grass significantly depressed NH₃ production ($p < 0.01$) compared with the control. Ammonia production by the HCHO-treated grass

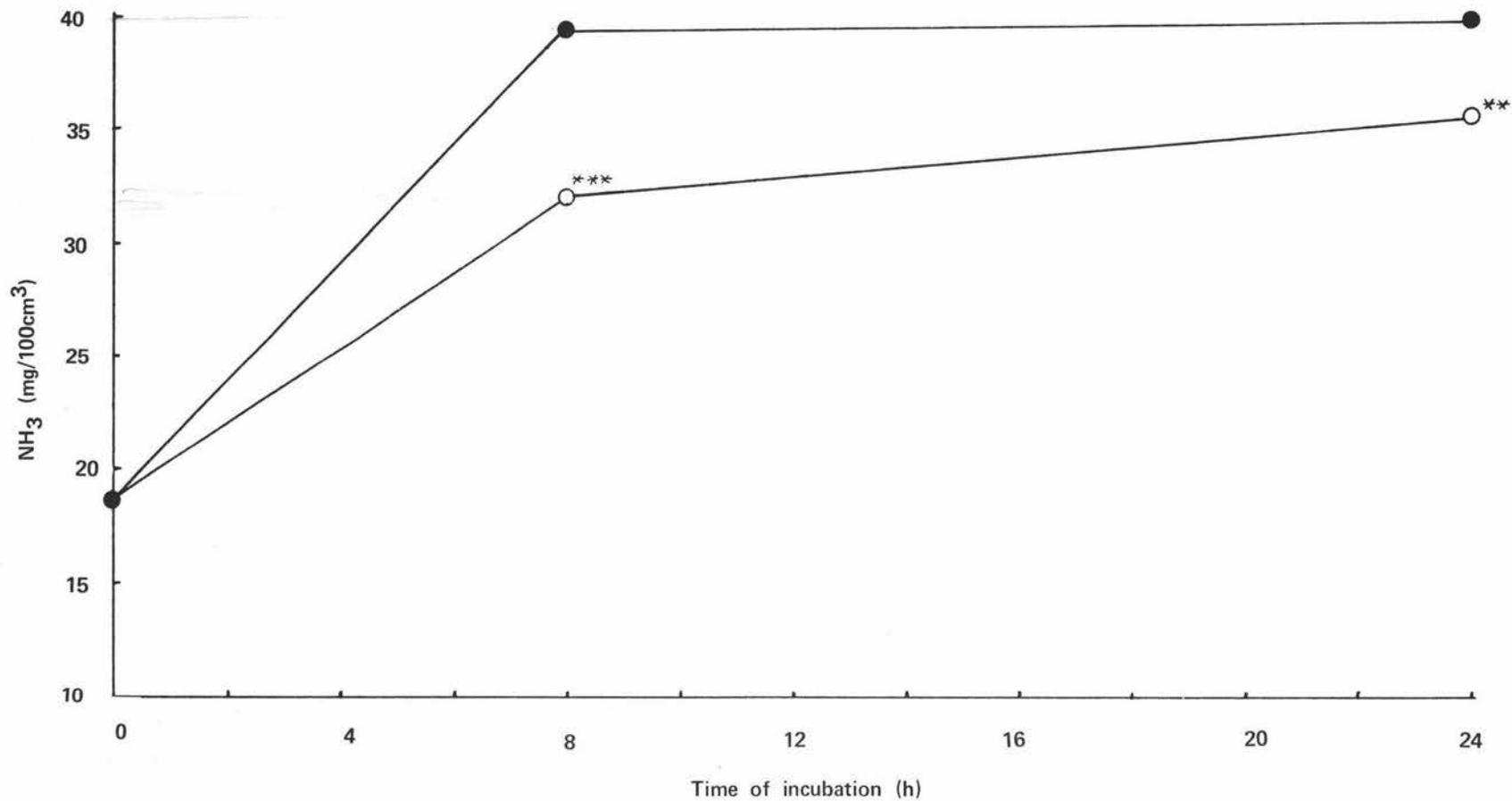


FIG. 2.1. The effects of HCHO-treatment of dried grass (1g HCHO/100 dried grass) on NH_3 production when incubated with rumen fluid. Conditions of incubation: 0.3g dried grass, 10 cm³ rumen fluid, 20 cm³ buffer; gassed with 5% CO_2 in N_2 and incubated at 38°C with occasional shaking.

Points plotted are means of 3 replicates at 0 and 8h and 6 replicates at 24h. S.E.'s are <0.9.

Significantly different from corresponding control mean, $p < 0.001$ (***) , $p < 0.01$ (**)

- , incubations with untreated dried grass
- , incubations with HCHO-treated dried grass

incubations was about three-quarters that of the untreated controls.

D.M. digestibility was not significantly affected by the HCHO treatment.

Table 2.3. The effect of HCHO-treatment of dried grass (1 g HCHO/100 g dry grass) on NH_3 production and digestion of dry matter when incubated with rumen fluid.

Time of incubation (h)	Grass Treatment	
	Control (untreated)	HCHO treated (1 g HCHO/100 g dried grass)
	(mg NH_3 per 100 cm ³) ¹	
0	12.2 ± 0.5	
24	46.8 ± 0.7	38.7 ± 1.0 **
	D.M. digestibility (%)	
24	65.3 ± 4.6	66.4 ± 2.2

Conditions of incubation are given in Fig.2.1.

Values in the table are means of 4 replicates ± SE.

** Difference between treatment and control means significant at the $p < 0.01$ level.

2.3.3. The relationship between the level of HCHO-treatment of dry grass and NH_3 production, pH and digestibility following incubation with rumen fluid for 24 h.

The second batch of dry grass was used for this experiment. The results in Fig.2.2 show that increasing rates of HCHO application resulted in an almost linear decrease in NH_3 production after 24 h of incubation. Analysis of variance followed by least significant difference tests revealed that:

a) NH_3 production by all the HCHO treatment incubations was significantly lower than the control incubations ($p < 0.001$).

b) the difference between 2.0, and 1.0 and 2.0 and 0.5 g HCHO/100 g dry grass in depressing NH_3 production was significant at the 1% level.

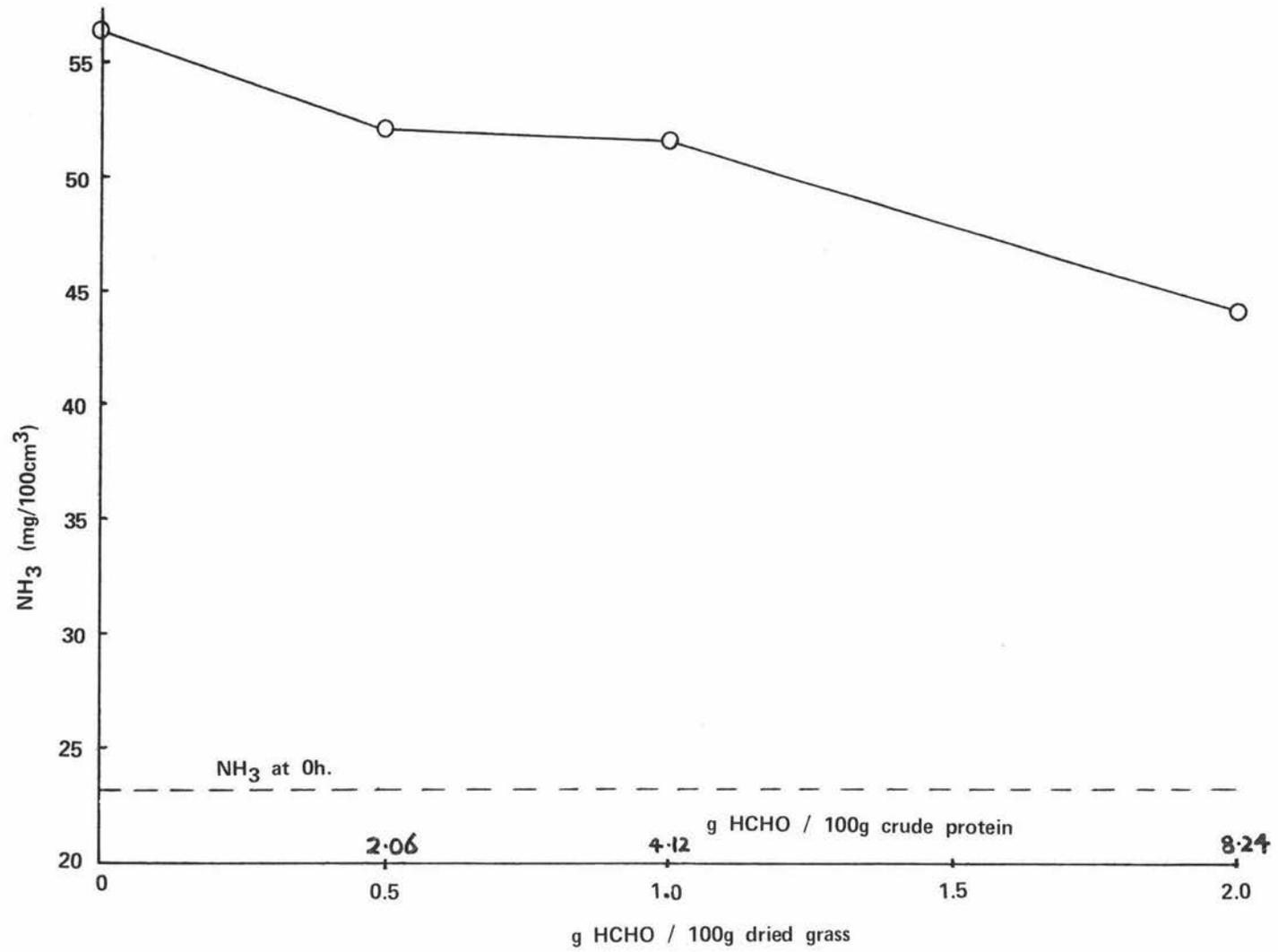


FIG. 2.2. The relationship between the level of HCHO-treatment of dried grass and NH₃ production when incubated with rumen fluid for 24h.

Conditions of incubation given in Fig. 2.1.
 Points plotted are means of 4 replicates. S.E.'s are <0.85

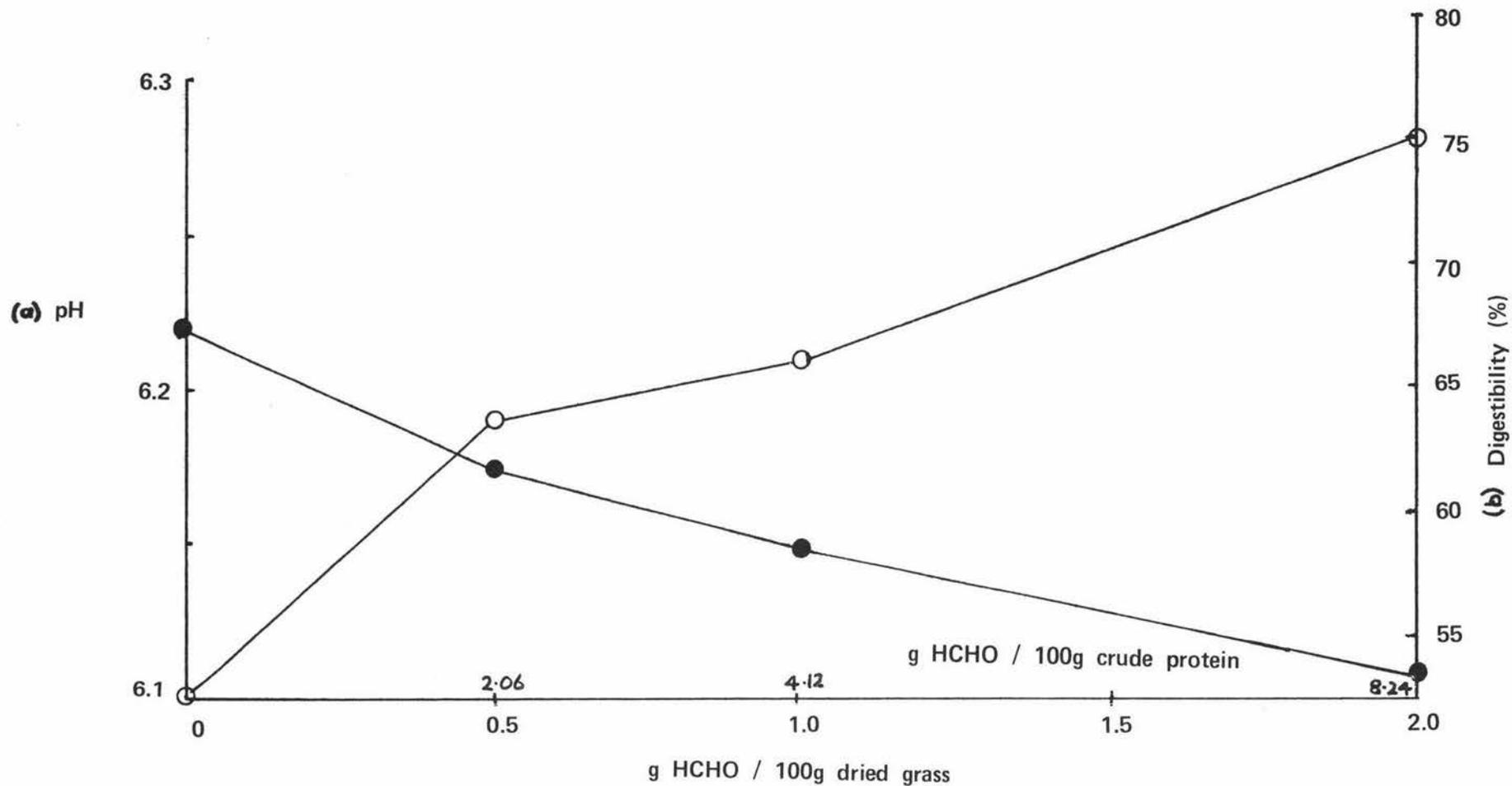


FIG. 2.3. The relationship between the level of HCHO-treatment of dried grass and (a) the final pH (b) the digestion of dry matter after incubation with rumen fluid for 24h.

Conditions of incubation given in Fig. 2.1.

Points plotted are means of 4 replicates. S.E.'s are <1.50.

○—○, pH. ●—●, dry matter digestibility (%).

c) the difference between 1.0 and 0.5 g HCHO/100 g dry grass was significant ($p < 0.05$) but was smaller than the differences in (a) and (b).

The effects of different rates of HCHO application on the final pH are shown in Fig.2.3. The lowest pH (6.10), indicating the greatest volatile fatty acid production, was obtained when untreated grass was incubated with rumen fluid. Increasing the level of HCHO treatment increased the pH and at 2.0% (by wt) HCHO the final pH was 6.28. Analysis of variance followed by least significant difference tests showed that 1.0 and 2.0 g HCHO/100 g dried grass gave pH's which were significantly higher than the control pH ($p < 0.05$).

Measurement of digestibility (Fig.2.3) showed that increasing rates of HCHO application resulted in a gradual decline in digestibility, (67.5% in the control and 54% in the 2% HCHO-treatment). All the digestibility means were significantly different from each other ($p < 0.001$) except for:

- a) 2.0 and 1.0 g HCHO/100 g dried grass treatments which were significantly different at the 1% level
- b) 1.0 and 5.0 g HCHO/100 g dried grass treatments which were different at the 5% level.

The relationship between the pH attained after 24 h fermentations and digestibility is demonstrated in Fig.2.4. There was a high negative correlation between the pH and the digestibility of the dry matter ($r = -0.993$).

The conclusions drawn from this experiment which were relevant to the aims of succeeding experiments were:

a) the highest level of HCHO-treatment (2.0 g HCHO/100 g dry grass) depressed NH_3 -production over 24 h incubations more than the other treatments and would therefore be most likely to give protection against hydrogenation. Depression of digestibility of the dry grass was also greatest for the high HCHO treatment.

b) from correlation analysis, the final pH was found to be a good guide to the extent of digestion.

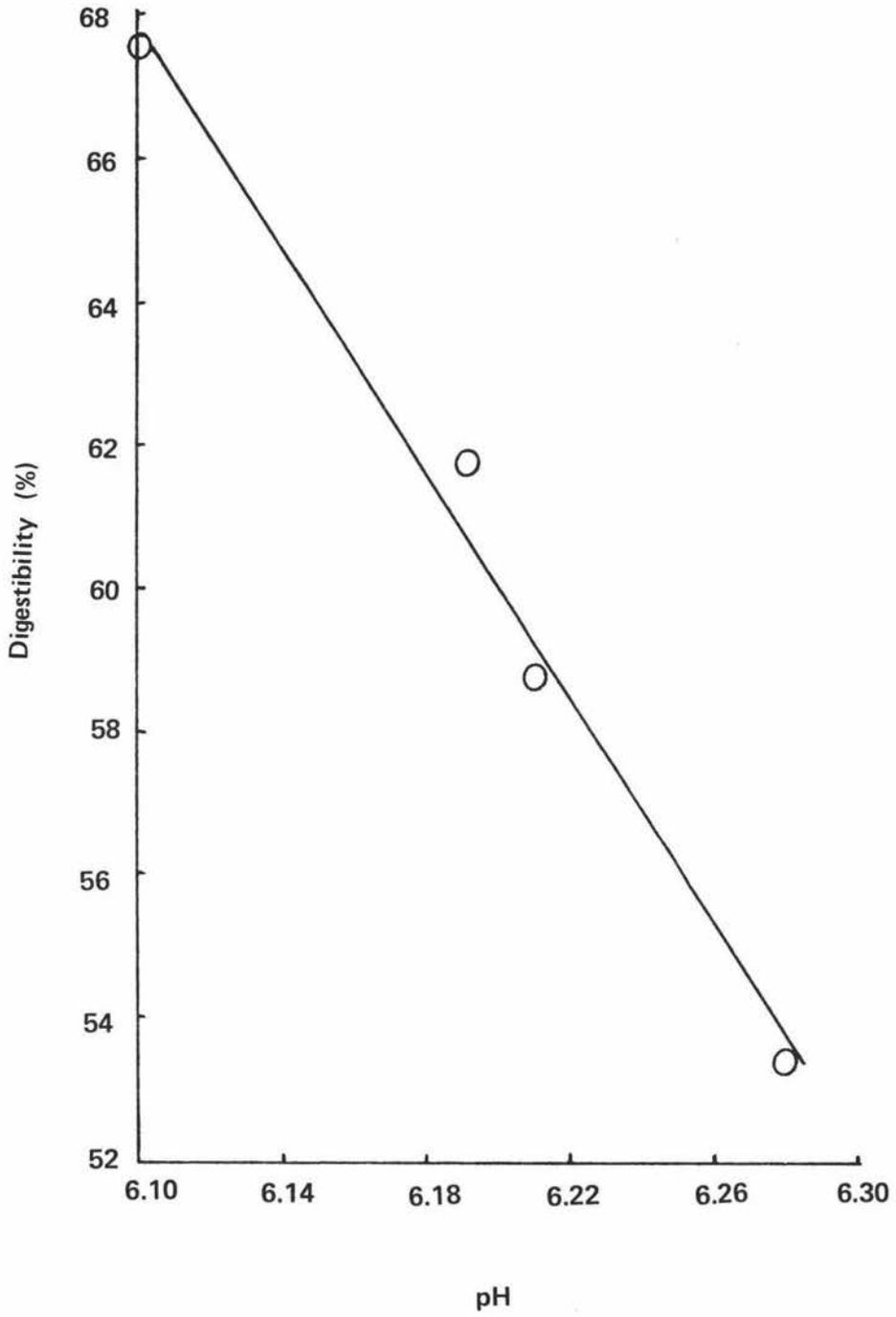


FIG. 2.4.

The relationship between pH and digestibility values when dried grass was treated with different levels of HCHO. (see Fig. 2.3).

2.3.4. The relationship between biohydrogenation of linolenic acid, NH_3 production, pH and duration of incubations of HCHO-treated dried grass with rumen fluid

On the basis of NH_3 formation, only the treatment of dried grass at 2% HCHO rate seemed likely to prevent appreciable biohydrogenation of linolenic acid (18:3). A preliminary experiment showed that because of interference from endogenous lipids in rumen fluid, the grass to rumen fluid ratio used in previous experiments (3:100, w/v) was too low to give readily detectable changes in 18:3 with time of incubation. One g dry grass and 5 cm³ rumen fluid (20:100, w/v) was used in all in vitro experiments in which changes in fatty acids were measured. HCHO-treatment of the dry grass almost completely prevented hydrogenation of 18:3 ($p < 0.01$) in that 18:3 diminished by only 5% from 39 to 34% (Fig.2.5). However, with untreated grass, 18:3 was rapidly hydrogenated and after 24 h comprised only 3.5% of the total fatty acids. Changes in 18:1 complemented the changes in 18:3. Oleic acid remained almost unchanged when HCHO-treated dry grass was used while in the untreated control, 18:1 increased rapidly from 5 to 32% of the total fatty acids. Changes in 18:2 with time are also of interest in that HCHO-treatment led to a slight and nearly linear increase from 8% to 11% while in the control, 18:2 increased to 15% between 8 and 12 h of incubation and then decreased to 9.5% after 24 h. In both treatment and control, the percentage of 16:0 remained nearly constant at about 19% throughout the incubations.

The results in Fig.2.6 indicate that HCHO-treatment of the dried grass diminished the rate of digestion of the grass by the rumen microorganisms. The pH of incubations containing untreated dry grass decreased from 6.8 to 5.5 after 8 h and remained at this level indicating that digestion was nearly complete after 8 h. However, the pH declined more slowly in a linear manner and reached 5.8 only after 24 h when HCHO-treated grass was incubated with rumen fluid.

Fig.2.6 also shows that production of NH_3 was very significantly depressed by HCHO-treatment compared with the

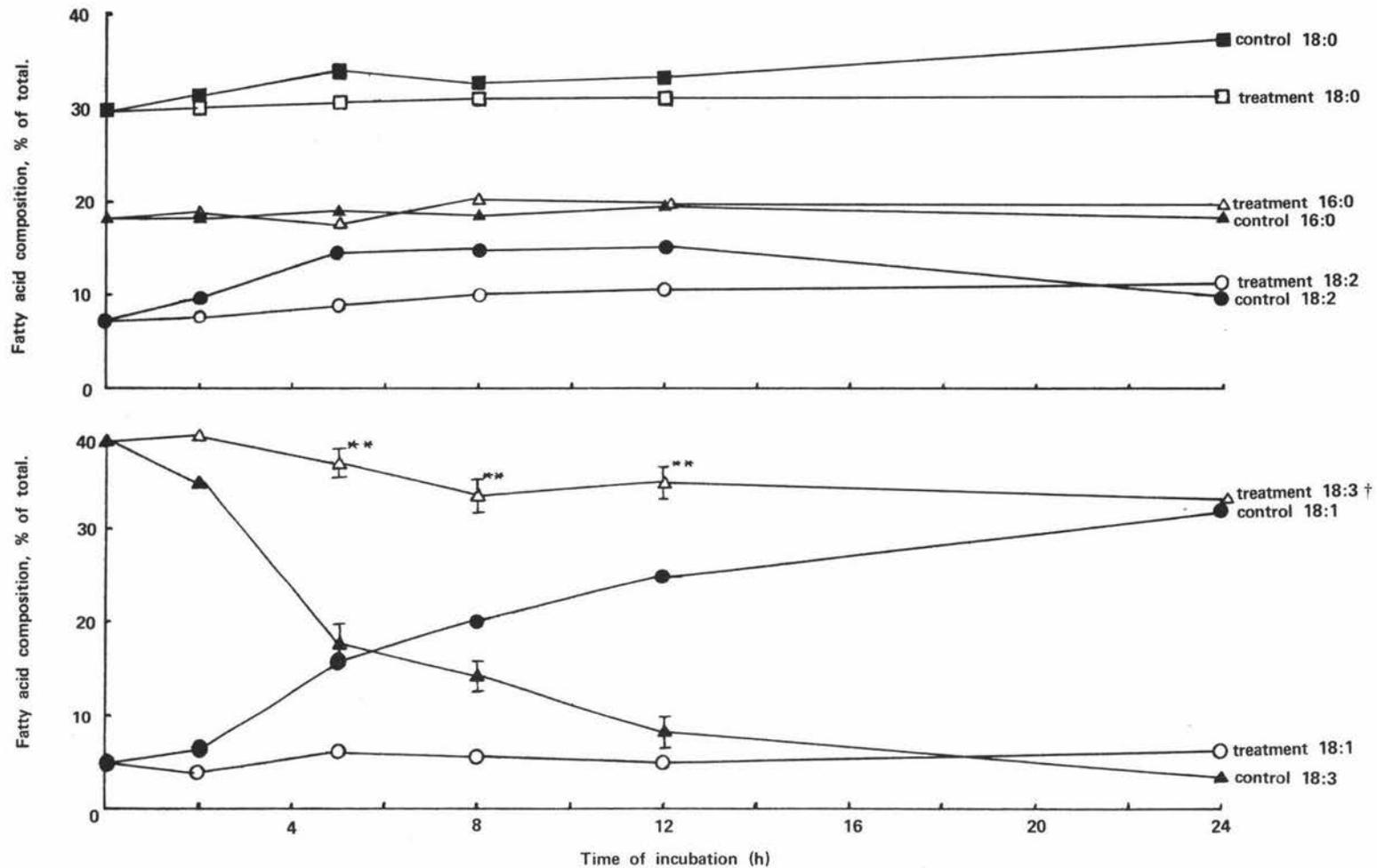


FIG. 2.5. The relationship between fatty acid composition and time of fermentation of HCHO-treated (2.0%, w/w) and untreated dried grass. Conditions of incubation: 1.0g dried grass, 5cm³ rumen fluid, 25cm³ buffer; gassed with 5% CO₂ in N₂ and incubated at 38°C with occasional shaking. Points plotted are means of 2 replicates \pm S.E. (for 18:3 only). †, replicate value not obtained. Significantly different from corresponding control mean $p < 0.01$. (**).

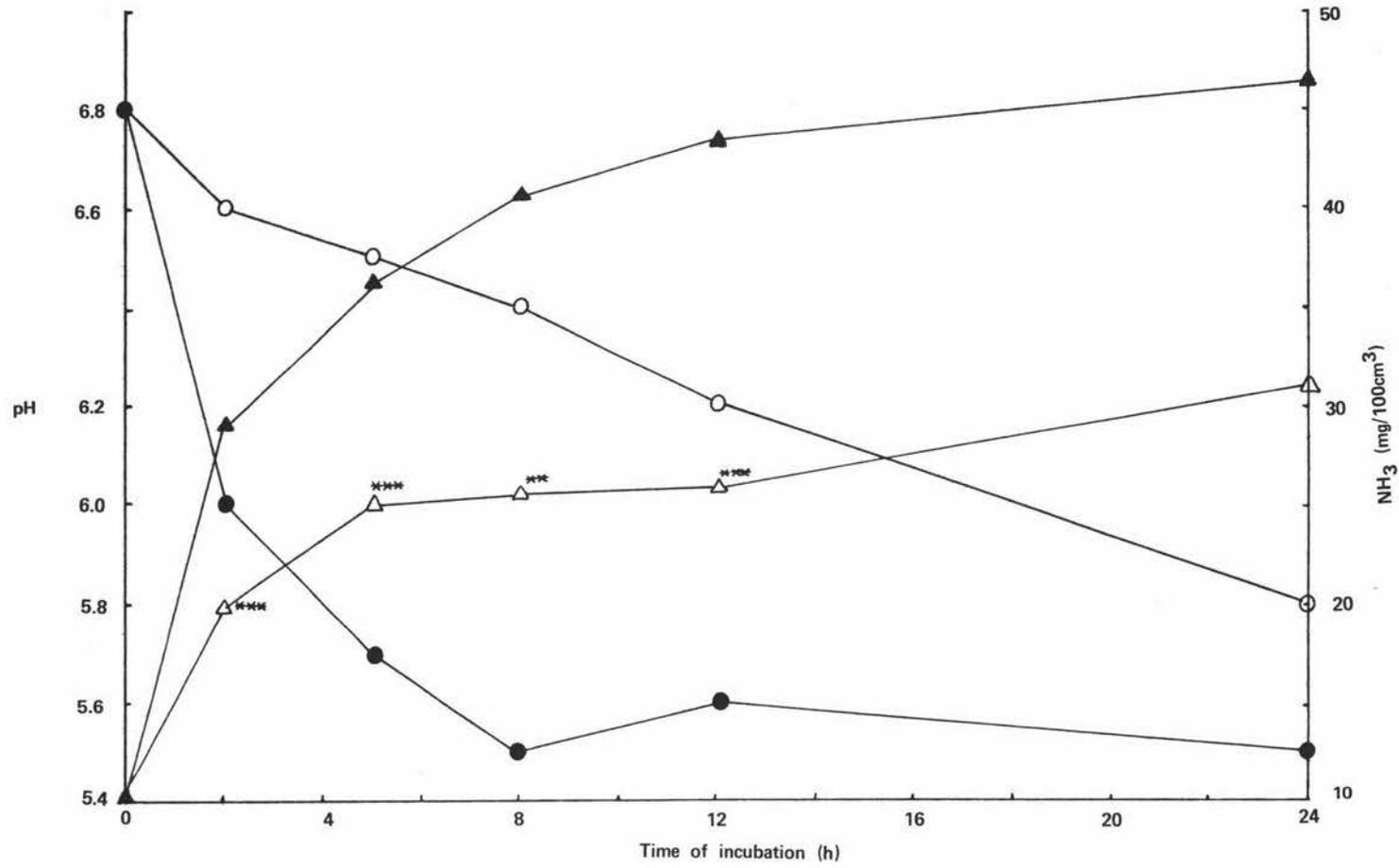


FIG. 2.6. The effect of HCHO-treatment of dried grass (2.0%, w/w) on (a) pH, (b) NH₃ concn. during incubation with rumen fluid. Conditions of incubation given in Fig. 2.5. Points plotted are means of 2 replicates. S.E.'s for NH₃ <0.5 except for 24h, (S.E. = ± 4.0). Significantly different from corresponding control mean, p<0.001 (***), p<0.01 (**).

○—○ pH, HCHO-treatment; ●—● pH, control;
 △—△ NH₃, HCHO-treatment; ▲—▲ NH₃, control.

untreated controls ($p < 0.001$). Ammonia concentration rose in a curvilinear fashion for both the treatment and untreated control but the concentration of NH_3 in the incubations of HCHO-treated grass was only a little over one-half that of the control after 24 h.

2.3.5. The effect of level of HCHO treatment of dried grass on the pH, NH_3 level and fatty acid composition of the incubation mixture after 24 h of fermentation.

The results depicted in Fig.2.7 and Table 2.4 indicate that 1 g HCHO/100 g dry grass was not as effective as 2 g% HCHO in preventing hydrogenation of 18:3 during incubation of the grass with rumen fluid.

The data presented in Fig.2.7 showed that 18:3 values, after 24 h-incubations, increased linearly with the level of application of HCHO to dried grass. With equivalent HCHO treatments in this experiment biohydrogenation of 18:3 did not appear to have been prevented as effectively as in experiment 2.3.3.

Even for the highest HCHO-treatment (2 g%) 18:3 had decreased from 40% of the total fatty acids before incubation to 16% after 24 h of fermentation. With the untreated grass, 18:3 decreased to 4.5% after 24 h. Percent 18:1 after 24 h incubation varied inversely with 18:3. This also indicated that increased HCHO/dry grass ratios in the range studied limited the degree of biohydrogenation more effectively.

The results in Table 2.4 were in agreement with the changes obtained in experiment 2.3.3 in that increasing rates of HCHO were associated with a linear decrease in NH_3 concentration and a linear increase in pH after a 24 h incubation.

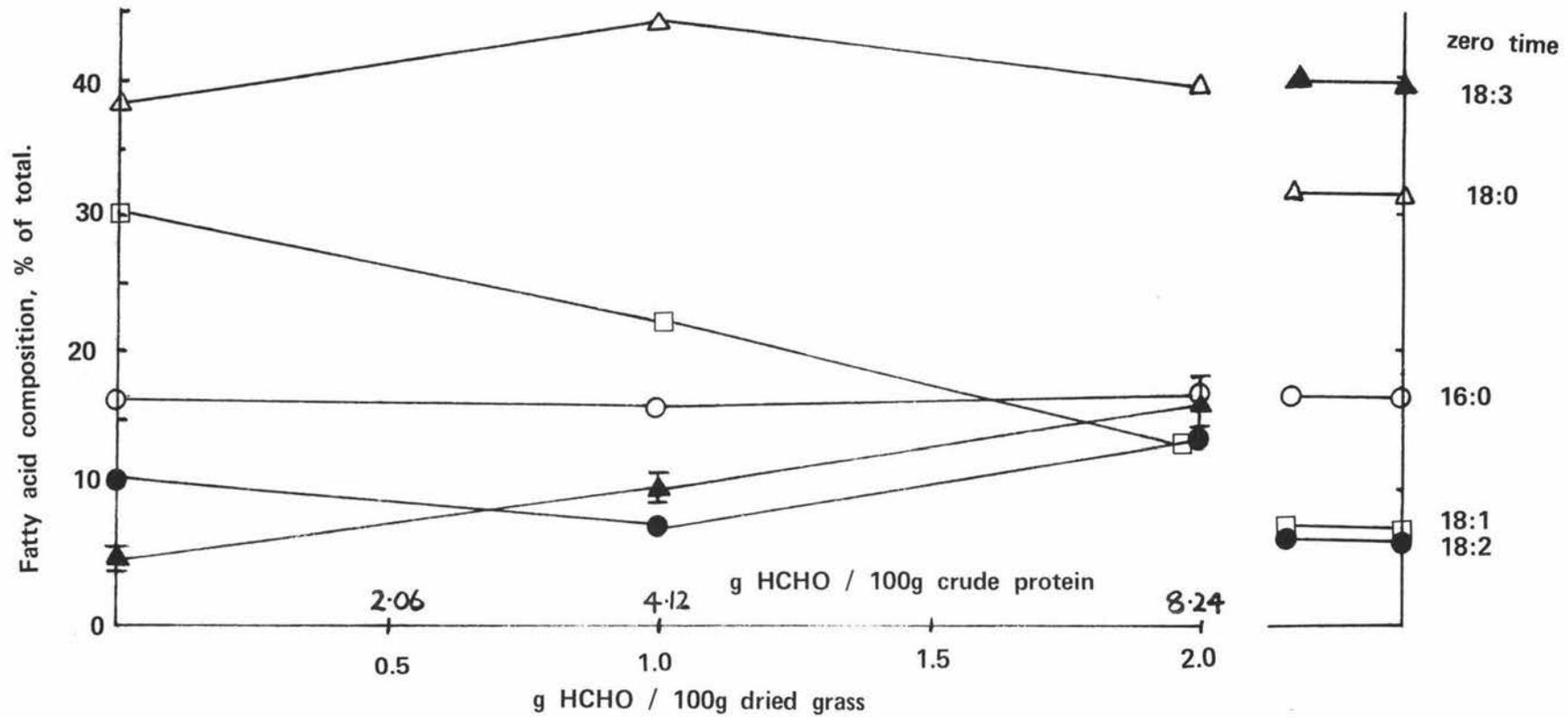


FIG. 2.7. The effect of level of HCHO-treatment of dried grass on the fatty acid composition of the incubation mixture after fermentation with rumen fluid for 24h.

Conditions of incubation given in Fig 2.5.

Points plotted are means (\pm S.E. for 18:3 only).

○—○ 16:0, △—△ 18:0, □—□ 18:1, ●—● 18:2, ▲—▲ 18:3

Table 2.4. The effects of level of HCHO-treatment of dried grass on pH and NH_3 concentration after incubation for 24 h with rumen fluid.

h of incubation	Control	Treatment	
		1 g HCHO/100 g dry grass	2 g HCHO/100 g dry grass
NH_3 -(mg/100 cm ³)			
0	8.38 (1)	8.08 (1)	8.67 (1)
24	51.1 \pm 1.5 (3)	41.1 \pm 3.1 (3)	32.7 \pm 1.6 (3)
pH			
24	5.44 \pm 0.02 (5)	5.50 \pm 0.02 (5)	5.70 \pm 0.02 (5)

Conditions of incubation as in Fig.2.5.

Values in the table are given as means \pm SE with replicates in parentheses.

2.3.6. The effects of addition of free HCHO to incubations of rumen fluid and untreated dry grass.

An experiment was carried out aimed at distinguishing between the effects of free and protein bound HCHO in incubations of dry grass with rumen liquor.

The results (Table 2.5) show that free HCHO at all levels resulted in NH_3 concentrations which were well below NH_3 concentrations in control and HCHO spray treated dried grass incubations. In fact, the highest free HCHO level (6.0 g free HCHO/100 g dried grass) resulted in an NH_3 concentration which was 3.6 mg % below the initial value of 14.6 mg %.

The effects of free versus protein-bound HCHO on rumen liquor activity were less distinct in the pH and fatty acid results. Free HCHO from 1.5 to 6.0% apparently prevented any digestion from occurring under these conditions; pH after 10 h was the same or slightly higher than the initial mean value of 7.8. However, 0.6% free HCHO resulted in a final pH of 7.3 which was similar to the final pH of incubations containing dried grass previously sprayed with 1.0 and 2.0 g % HCHO.

Free HCHO at all levels used, as well as pre-treatment of grass with 2.0% HCHO, resulted in almost complete prevention of biohydrogenation of 18:3 by the rumen microorganisms. Biohydrogenation of 18:3 in grass sprayed with 1.0 g % HCHO was about one third that in untreated grass incubations.

Visual and olfactory observations indicated that little or no fermentation had occurred in the incubations containing free HCHO at all levels whereas it appeared to be occurring in the control and HCHO sprayed dry grass incubations.

The results of this experiment could probably have been more successfully interpreted if digestibility and gas production measurements had been made.

Table 2.5 A comparison of the effects of free and protein bound HCHO on the activity of rumen microorganisms

Treatment	NH_3 (mg/100 cm ³)		pH		% fatty acid composition									
					16:0 18:0 18:1 18:2 18:3					16:0 18:0 18:1 18:2 18:3				
					Time of incubation (h)									
	0	10	0	10	0					10				
Control	14.6 \pm 0.1	47.3 \pm 0.4	7.80	6.7 \pm 0.0	16.0	25.4	4.7	6.8	47.1	15.9	47.7	19.3	6.4	10.7
Control + 0.6 g free HCHO/ 100 g dried grass		20.8 \pm 1.3*		7.3 \pm 0.2						16.2	24.2	6.3	10.7	42.7
Control + 1.5 g "		19.2 \pm 0.1 "		8.0 \pm 0.1						14.9	26.1	5.5	9.3	44.3
Control + 3.0 g "		15.7 \pm 0.1		7.9 \pm 0.0						18.2	24.1	4.5	9.2	46.0
Control + 6.0 g "		11.0 \pm 0.1		7.9 \pm 0.0						15.3	28.1	5.3	8.8	42.7
HCHO sprayed dried grass (1.0 g %)		28.3 \pm 0.1		7.4 \pm 0.0						14.9	34.9	12.5	6.7	31.2
HCHO sprayed dried grass (2.0 g %)		35.5 \pm 0.6		7.3 \pm 0.1						15.9	27.8	7.0	6.8	42.5
Number of replicates in each mean	2	3	2	3	1					1				

Conditions of incubation as in Fig.2.5

Values in the table given as means \pm S.E.

* All NH_3 levels were significantly different from the control value and from each other ($p < 0.05$) except for t and *.

CHAPTER 3 THE USE OF FORMALDEHYDE TO PROTECT UNSATURATED
FATTY ACID CONSTITUENTS OF THE DIET AGAINST
RUMINAL BIOHYDROGENATION IN VIVO.

3.1 Materials

3.1.1. Reagents and solvents.

As in section 2,1.1.

3.1.2. Source and preparation of feedstuffs.

a) Dried grass. This was prepared as in section 2.1.2 with the following modifications. Grass, dried and chopped, was obtained from N.Z. Grassmeal Ltd. Tahuna in 2 batches. Of the first batch, 135 kg was treated with 5% HCHO at the rate of 2 g HCHO/100 g dried grass. This was equivalent to 2.18 g HCHO/100 g of D.M. or 14.4 g HCHO/100 g of crude protein. The grass was sprayed with the 5% HCHO solution using a bucket sprayer and then stored in large plastic bags for 5 days. It was then dried in direct sunlight (21-27 °C) for 2 days. At this point in vitro assays were carried out using rumen fluid and the techniques as described in sections 2.1.3, 2.2.1 and 2.2.2.

The results of the first assay (Table 3.1 Part I) indicated that 18:3 in the HCHO-treated material has a resistance of about 50%⁺ against biohydrogenation. This compared with incubations of untreated material in which 18:3 after 24 h fermentation was only 12% of the initial proportion of 18:3. The HCHO-treatment of dried grass also depressed NH₃ production in comparison with control incubations, the difference between the 2 means being significant at the 0.1% level.

⁺ resistance against biohydrogenation
= % efficiency in prevention of biohydrogenation
= $\frac{\% \text{ 18:3 after 24 h incubation (in vitro)}}{\% \text{ 18:3 at 0 h incubation}} \times 100\%$

Table 3.1 The effects of HCHO-treatment on the formation of NH_3 , biohydrogenation of 18:3 and final pH after incubating dried grass for 24 h with rumen fluid.

Time of incubation (h)	Untreated dried grass	HCHO-treated dried grass Air dried only	
Part I (first assay)			
		pH	
0	7.80 \pm 0.05		
24	5.75 \pm 0.29	7.00 \pm 0.09***	
		<u>NH_3 production (mg/100 cm³)</u>	
0	9.1		
24	21.0 \pm 0.5	17.3 \pm 0.2***	
		<u>% 18:3 (% of total FA's)</u>	
0	35.4 \pm 2		
24	4.0 \pm 0.1	17.2 \pm 0.4***	
Part II (second assay)			
Time (h)	Untreated dried grass	Treatment (a) [†]	Treatment (b)
		pH	
0	7.85 \pm 0.05		
24	5.82 \pm 0.06	6.92 \pm 0.07***	7.07 \pm 0.04***
		<u>NH_3 production (mg/100 cm³)</u>	
0	10.37 \pm 0.22		
24	18.53 \pm 0.61	12.16 \pm 0.10*** [‡]	11.91 \pm 0.37***
		<u>% 18:3 (% of total FA's)</u>	
0	42 \pm 3		
24	4 \pm 0	9 \pm 1***	‡ 28 \pm 2***
<p>Conditions of incubation: 1 g dried grass, 5 cm³ rumen fluid, 25 cm³ clarified rumen fluid (prepared by centrifuging rumen fluid at 15 000 g for 30 min).</p> <p>Values in the table are means \pm SE of 3 determinations except for the estimate of % 18:3 (means \pm SE of 2 determinations).</p> <p>[†] Treatment (a) - HCHO-treated grass dried in air for 2 days and then dried in the oven at 35 °C for 24 h.</p> <p>Treatment (b) - as for treatment (a) but oven dried for 36 h.</p> <p>***Significantly different from corresponding control mean (p < 0.001)</p> <p>[‡] Treatment (a) mean significantly different from treatment (b) mean (p < 0.001).</p>			

The mean pH values indicated that digestibility was depressed significantly by HCHO-treatment.

The results in Table 3.1 Part II indicate that although HCHO treatment of the dried grass resulted in substantial protection of 18:3 against biohydrogenation there was considerable loss in digestibility. Furthermore, the treated material smelt strongly of HCHO despite 2 days of drying in the sun and on this basis alone would probably have been refused by the cow. A further period of drying in the oven at 35 °C for 24 and 36 h was carried out in an attempt to remove this surplus HCHO. Olfactory observations indicated that this had been reasonably successful. An *in vitro* assay of this oven dried material was also carried out. The results are shown in Table 3.1.II. The 2 drying regimes are referred to as:

i) treatment (a) dried grass - HCHO treated dried grass dried in air for 2 days followed by 24 h in an oven at 35 °C.

ii) treatment (b) dried grass - HCHO treated dried grass dried in air for 2 days followed by 36 h in an oven at 35 °C. The results show that treatment (a) dried grass was significantly ($p < 0.001$) more digestible (as measured by pH) than treatment (b) dried grass. Both these treatments were less digestible ($p < 0.001$) than dried grass which had not been treated with HCHO.

Mean NH_3 production during incubation of both treatment (a) and (b) dried grasses was not significantly different. The NH_3 concentration in both these treatment incubations rose to about 12 mg/100 cm³ rumen fluid; significantly lower than the control incubation mean of 18.5 mg/100 cm³.

Treatment (b) drying regimen resulted in protection of 18:3 against biohydrogenation which was 60% efficient. This compared with 21% for treatment (a).

The results in Table 3.1.II indicate that although treatment (b) dried grass was less digestible than treatment (a) dried grass this difference was small. Furthermore, the small loss in digestibility as a result of extra drying in the oven appears to have been compensated for by a large improvement in the efficiency of protection of 18:3 against biohydrogenation. On this basis drying treatment (b) was adopted.

The following is a summary of the steps used in the preparation of the HCHO-treated dried grass:

- 1) dried grass was sprayed with HCHO and stored in plastic bags for 5 days.
- 2) sprayed material was air dried for 2 days.
- 3) air dried grass was dried in the oven at 35 °C for 36 h.
- 4) HCHO-treated dried grass was then ready for feeding and was stored in jute bags until required.

b) Oil-seed supplement. A HCHO-treated (3%, w/w, HCHO/ Crude protein) sunflower oil supplement was donated by Alta Lipids N.Z. Ltd., Upper Hutt. The supplement had been prepared by the method as described in "Rural Research" (1973). The oil-seed was supplemented with casein (to aid in protection of 18:2 presumably). A similar supplement which had not been treated with HCHO was also made available. Analysis of the material appears in Table 3.2 p. 54.

c) Barley meal. This was obtained from the Poultry Research Unit, Massey University.

3.1.3. Animals and their treatment.

A pair of identical twin Jersey cows, No.109 and 110 (liveweights 336 and 332 kg respectively) was made available from the Massey University Identical Twin Herd. After feeding on concrete for 12 h/day for a week to harden their feet, the twins were transferred to an enclosed feeding barn. Water was continuously available to each animal. Food intakes and refusals were estimated twice daily during the milking periods at approx. 0800 and 1630 h.

Prior to the start of the trial, the animals were allowed to become accustomed to the barn and changed milking conditions. Dried grass, available from the Massey University No.3 dairy farm, was fed ad lib. After a week, the twins appeared to have become adapted to their new environment.

3.2 Methods

3.2.1. Chemical analysis of feedstuffs used.

This was carried out as in section 2.2.2 (g). In vitro digestibility % on a dry matter basis was measured as in

section 2.2.2.(c) except that 40 cm³ artificial saliva (McDougall, 1948) was used with 10 cm³ rumen fluid and 0.5 g dried grass. Second stage digestion as recommended by Tilley and Terry (1963) was also carried out.

3.2.2. Analysis of milk

a) Analysis by the infra-red milk analyser (IRMA).

On each day milk collected from a.m. and p.m. milkings was weighed and a combined sample taken for analysis. Analysis by IRMA gave the proportions and yields of fat, protein and lactose per day per cow.

b) Fatty acid analysis of milk fat

i) Extraction of milk fat. After mixing the sample taken for IRMA analysis, a sub-sample was taken for fatty acid analysis by G.L.C. The milk fat was extracted by the Rose-Gottlieb method (British Standards Institution, 1951) as follows:

1) 5 cm³ milk, 0.75 cm³ conc. NH₃, 5 cm³ EtOH, 11 cm³ hexane and 11 cm³ diethyl ether were shaken in a glass stoppered centrifuge tube.

2) the tube was centrifuged,

3) following (2), most of the top layer was removed using a Pasteur pipette and was then evaporated to dryness in a rotary evaporator.

ii) Preparation of methyl esters. Methyl esters of the milk fatty acids were prepared by the method of Shaketon et al. (1970). A 3 mg sample of fat was hydrolysed and methylated using 25 μ l transesterification reagent. This reagent was made up from 0.5 M sodium methoxalate in MeOH, petroleum ether and diethyl ether (3/12/5, v/v). The fat sample was placed with the transesterification reagent in a Kontes vial which was capped, gently shaken to dissolve the fat and left for 2 min. Petroleum ether (25 μ l) was then added, the vial re-sealed, gently shaken and then left to stand for a further 2 min. To avoid loss of volatile esters, GLC analysis of the sample was then carried out immediately.

iii) Analysis of methyl esters. The procedure for GLC was as described in section 2.2.2 (f) except that the column temperature was programmed to rise, from 60 °C at sample injection time, at 8 °C per min on the Packard chromatograph and 3 °C per min. on the Aerograph chromatograph until the final column temperature of 165 °C was reached. The column was held at this temperature until the analysis was complete. Only the saturated even chain (C4-C18) and the C18 unsaturated fatty acids were included in the calculations of fatty acid composition. A series of analyses on one milk fat sample using both chromatographs was carried out in order to determine variability in measurement and between instruments.

Fatty acid composition (moles %) was calculated from the triangulated areas of each peak using the following formula.

$$\text{moles \% of F.A. } x = \frac{\text{Ht}_x \times W @ \frac{1}{2} \text{Ht}_x \times \text{C.F.}_x}{\text{M.W.}_x} \div \sum_{i=1}^n \frac{\text{Ht}_i \times W @ \frac{1}{2} \text{Ht}_i \times \text{C.F.}_i}{\text{M.W.}_i}$$

where; Ht_x = triangulated peak height of fatty acid x.,
 $W @ \frac{1}{2} \text{Ht}_x$ = width at half peak height of fatty acid x.,
 M.W._x = molecular weight of fatty acid x.,
 F.A._x = fatty acid x.

$\sum_{i=1}^n$ = sum of all the fatty acids in the analysis.

A sample trace and calculation along with column correction factors is given in appendix 1.

3.3. Experimental Design

The limited availability of dried grass precluded the use of more than 2 animals in this trial. It was decided to use identical twins since the members of a set of such twins are of the same genotype (Patchell, 1956) and hereditary differences between animals are eliminated with a consequent reduction in the error variance. Consequently, treatment differences can be identified with greater confidence. The

relative efficiency of twins as compared with ordinary unrelated animals is variable as Patchell (1956) found. On the basis of the twins he used, one set of monozygous twins may replace without loss of statistical efficiency 2 groups of the following numbers of unrelated randomly selected animals.

Milk yield	4.6
Butterfat yield	5.5
Protein yield	3.5
Protein %	12.6
Butterfat %	23.3

(from Patchell (1956)).

The efficiency of twins obviously varies depending on the milk character being measured. The efficiency of twins with respect to milk fatty acid composition was not measured. However, it would seem likely to be greater than 1 and thus advantageous to use identical twins rather than 2 unrelated animals.

In the statistical analyses, the treatment period (minus the first 2 days to avoid a carry-over effect) was compared either with the results from the preliminary period or with the results from the other cow which was acting as a control. The student's t test was used. The type of comparison made will be described in the results.

The period where the supplement of untreated sunflower seed was fed was not analysed statistically because the supply of dried grass ran out before sufficient data had been collected.

The description of the trial and its duration are shown in Fig.3.1 (which also includes intake data). The trial was conducted in 2 sections.

Section 1. HCHO-treated dried grass diet. February 22 - March 27. Standardisation of the twins was achieved by the ad lib. feeding of untreated dried grass (Batch 1) for 14 days up to March 6. Cow 109 was then given the HCHO-treated material for 13 days until March 20. The intake of cow 109 fell ($p < 0.001$) and averaged 7.66 kg D.M./day over

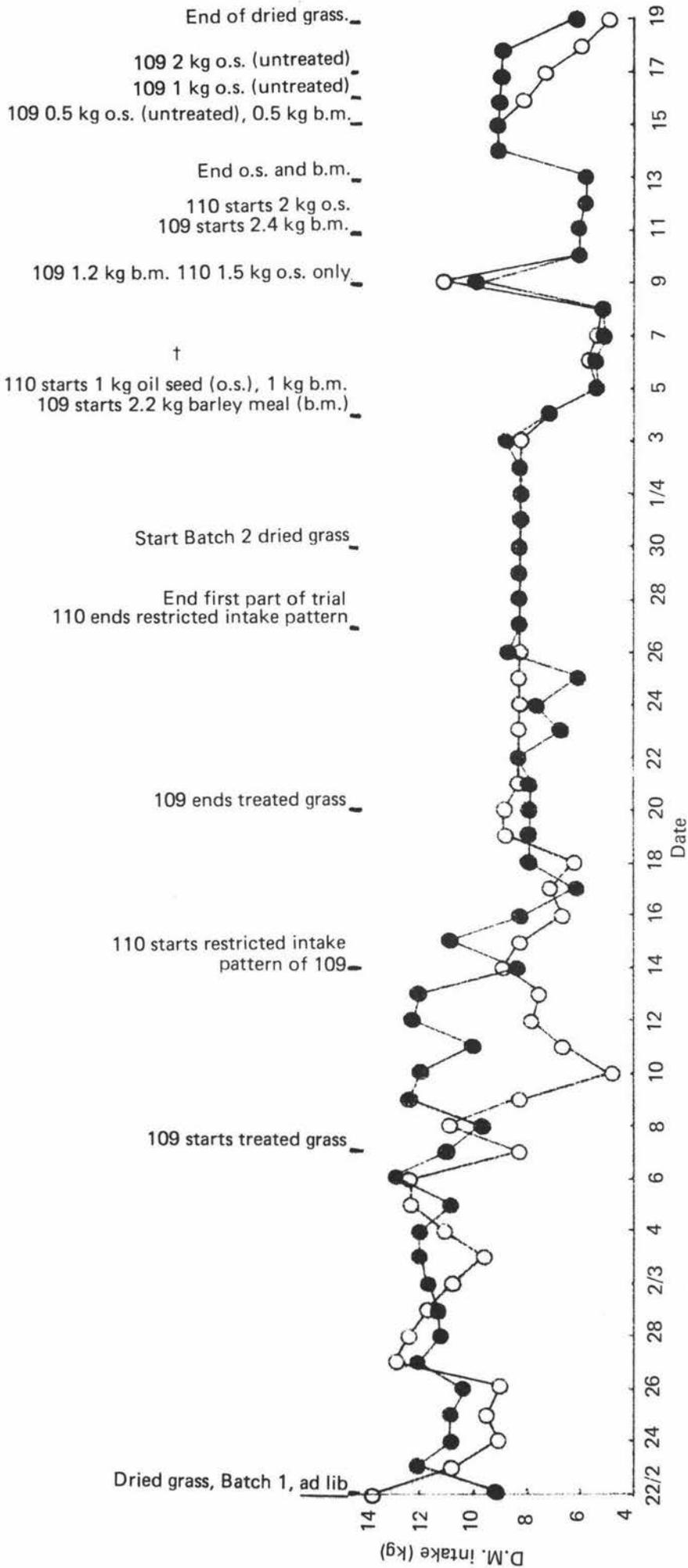


Fig. 3.1. Daily grass D.M. and concentrate intake.

○—○ COW 109, ●—● COW 110.

† HCHO-treated sunflower seed supplement.

this period compared with about 11.5 kg average in the preliminary period. Consequently the intake of cow 110 was restricted from March 14 to March 27, to follow approx. the intake pattern of cow 109 i.e. for the period that cow 109 had been on the HCHO-treated dried grass. The intake of cow 110 averaged 7.9 kg D.M./day during the restriction period.

Section 2. Oil-seed supplementation. March 27 - April 19. Following section 1 the twins were re-standardised by feeding 9 kg untreated dried grass per cow per day (Fig.3.1.). By April 3, IRMA and GLC analysis of milk components indicated that standardisation had again been achieved. Cow 110 was then given a diet of dried grass (untreated) supplemented with a mixture of HCHO-treated sunflower seed and barley meal (w/w, variable - Fig.3.1.) for 9 days from April 4-13. Over the same period cow 109 was fed a diet of untreated dried grass and barley meal (Fig.3.1.), equivalent in roughage and energy to the diet of the other cow. Following this regimen, each cow was fed 9 kg untreated dried grass per day. A small sample of sunflower oil-seed which had not been HCHO-treated became available on April 16. This was fed as a supplementary component to the diet of cow 109 until the supply of dried grass had been exhausted.

3.4 Results

3.4.1. Analysis of feedstuffs.

The analysis of all the feedstuffs used appears in Table 3.2.

Table 3.2 Analysis of all feedstuffs

Description of feedstuff	D.M.%	Cr.protein (% of D.M.)	Ether extract (% of D.M.)	Fibre (% of D.M.)	D.M.digestibility (% of D.M.)
Batch 1 dried grass	92.25 \pm 0.26 (7) [†]	15.14 \pm 0.56 (7)	3.25 \pm 0.32 (4)	33.24 \pm 0.44 (6)	65.6 \pm 0.8 (3) ^{***}
(HCHO-treated)	91.92 \pm 0.60 (6)	-	-	-	57.6 \pm 0.9 (4)
Batch 2 dried grass	91.14 \pm 0.21 (5)	14.12 \pm 0.23 (4)	4.40 \pm 0.32 (3)	36.71 \pm 0.20 (3)	64.99 \pm 2.28 (3)
HCHO-treated oil-seed suppl.	93.05 \pm 0.65 (2)	25.6 \pm 0.3 (4)	42.05 \pm 0.25 (2)	15.53 \pm 0.15 (2)	56.86 \pm 0.71 (4) [‡]
Barley meal supplement "	97.30	16.38	4.7	9.7	N.A. [∅]

[†] Values given are means \pm SE. Values in parentheses denote number of replicates for each mean. Each replicate a mean of 2 determinations except for digestibility % where each replicate is a mean of 3 determinations.

[‡] D.M. digestibility % estimated by adding oilseed/grass, (1:3, w/w) to make up 0.5 g substrate per incubation bottle.

[∅] N.A. Not available.

" From analyses, Dairy Husbandry Dept. Massey University.

^{***} Significantly more digestible than HCHO-treated dried grass. $p < 0.001$.

Important features of Table 3.2 are:

a) Cr.protein %'s of both Batch 1 and Batch 2

grasses are similar but considerably lower than the levels of 21-27% encountered in the grass used in chapter 2 - refer Table 2.2. This would be expected to influence the amount of HCHO which would remain fixed in the pasture. The rate at which the HCHO was sprayed (section 3.1.2(a)) should perhaps have been altered in accordance with the change in protein level. Ether extract was also lower - especially in the grass which was HCHO-treated.

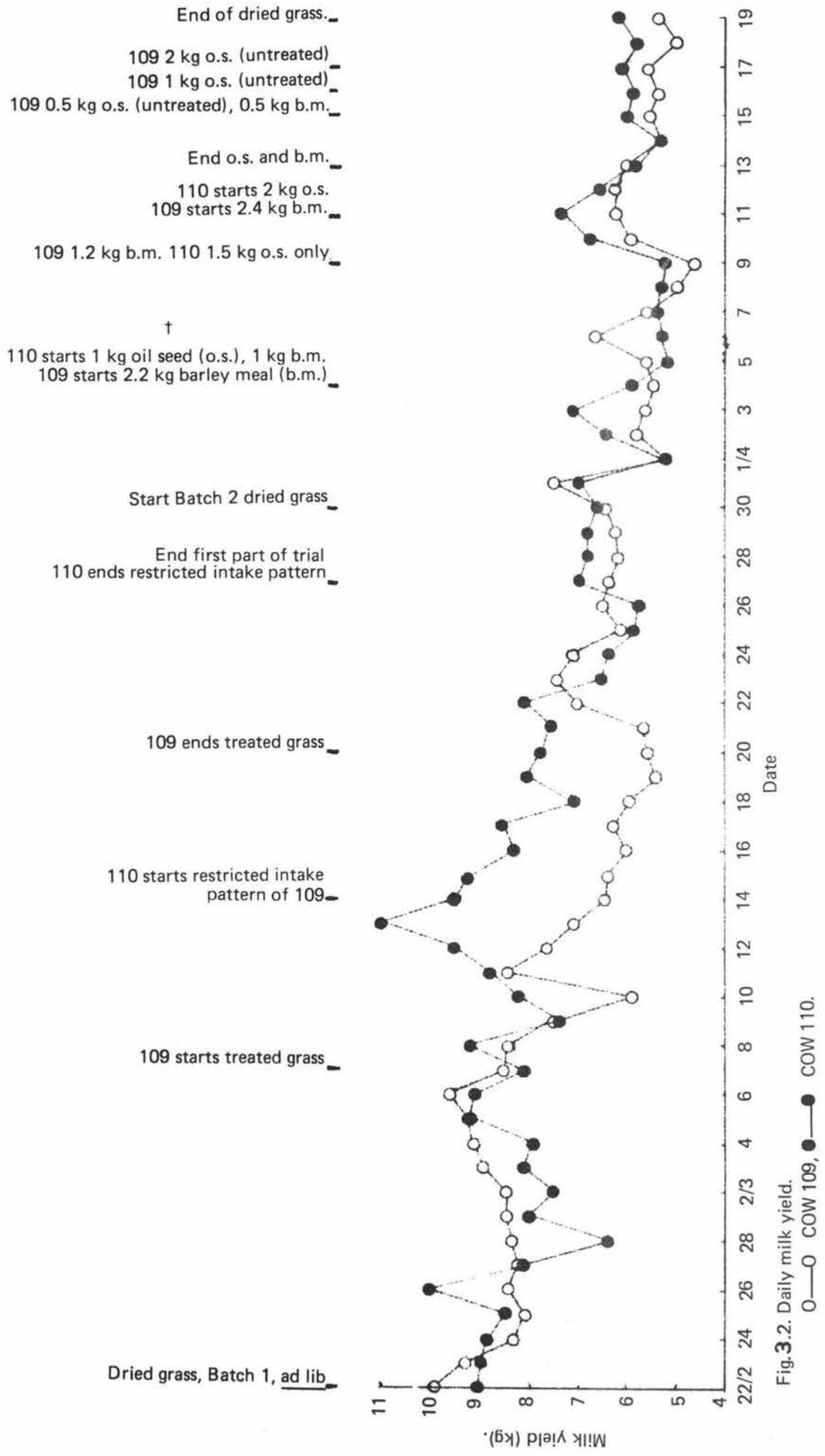
b) Digestibility % was lowered by HCHO-treatment of the dried grass as expected but this effect was not great. The diet of grass and sunflower seed which had been HCHO-treated was also less digestible than untreated dried grass fed on its own. Both batches of dried grass were of similar digestibility as in fact they were in all other measured characteristics.

c) The supplement of sunflower seed was, as anticipated, high in lipid, % ether extract being 42%.

3.4.2. Intake and milk yield

Fig.3.1 indicates that despite the drying regimen adopted as a result of the in vitro assays (section 3.1.2(a)), the dried grass which had been HCHO-treated was still not very acceptable to cow 109. Daily intake fell ($p < 0.001$) as described in section 3.3 when HCHO-treated grass was fed. As Fig.3.2 shows, milk yield fell accordingly compared with the preliminary period ($p < 0.001$) and cow 109 did not become accustomed to the HCHO-treated grass. When the intake of cow 110 was restricted to approx. follow that observed by cow 109 on the treated grass, milk yield fell ($p < 0.05$) from the average of 8.8 kg/day during the pre-restriction period to about 6.9 kg/day during underfeeding (Fig.3.2). However the effect was less than had been observed in cow 109.

During the second part of the trial (March 27-April 19) the cows were fed below appetite and all feed offered was consumed. Despite this, milk yield, on average, fell very slowly.



Cow 110 readily ate the mixture of barley meal and sunflower oil-seed which had been HCHO-treated when mixed with the untreated dried grass. It was anticipated that the barley meal would be required in the mixture to make the oil-seed palatable enough for its consumption. In the event this was found to be unnecessary and from April 10 onwards cow 110 was fed the HCHO-treated oil-seed on its own with the roughage component of the diet.

During the second part of the trial, when the concentrates were fed, the milk yield of cow 109 increased temporarily (April 10-13 $p < 0.05$ compared with previous 4 days) - possibly purely because of the extra dried grass given on April 9. This increase in basal diet was given to lessen the possibility of termination of lactation due to persistent underfeeding.

3.4.3. Milk lactose

The lactose content of the milk of both cows was fairly constant except over March 1-9 as shown in Fig.3.3. Lactose yield (Fig.3.3) closely followed milk yield in both animals.

3.4.4. Milk protein

Milk protein yield per day (Fig.3.4) was variable even during the preliminary period (February 22-March 6). When fed the HCHO-treated dried grass, cow 109 suffered a marked drop ($p < 0.001$) in milk protein per day from an average of 0.31 during the preliminary period to 0.23 kg/day during the treatment period. The milk protein yield of cow 109 remained low until the untreated dried grass was fed again (March 20) and then rose with rising intake. The yield of milk protein from cow 110 fell ($p < 0.05$), when intake was restricted, from an average of 0.30 in the preliminary period (March 1-13) to an average of 0.27 kg/day. This fall was somewhat less than that observed when cow 109 was fed the HCHO-treated material.

Yield of milk protein did not rise significantly during the feeding of the barley meal and barley meal/HCHO-treated oil-seed supplements. However, it did rise during the latter four days of the period when cow 110 was fed treated

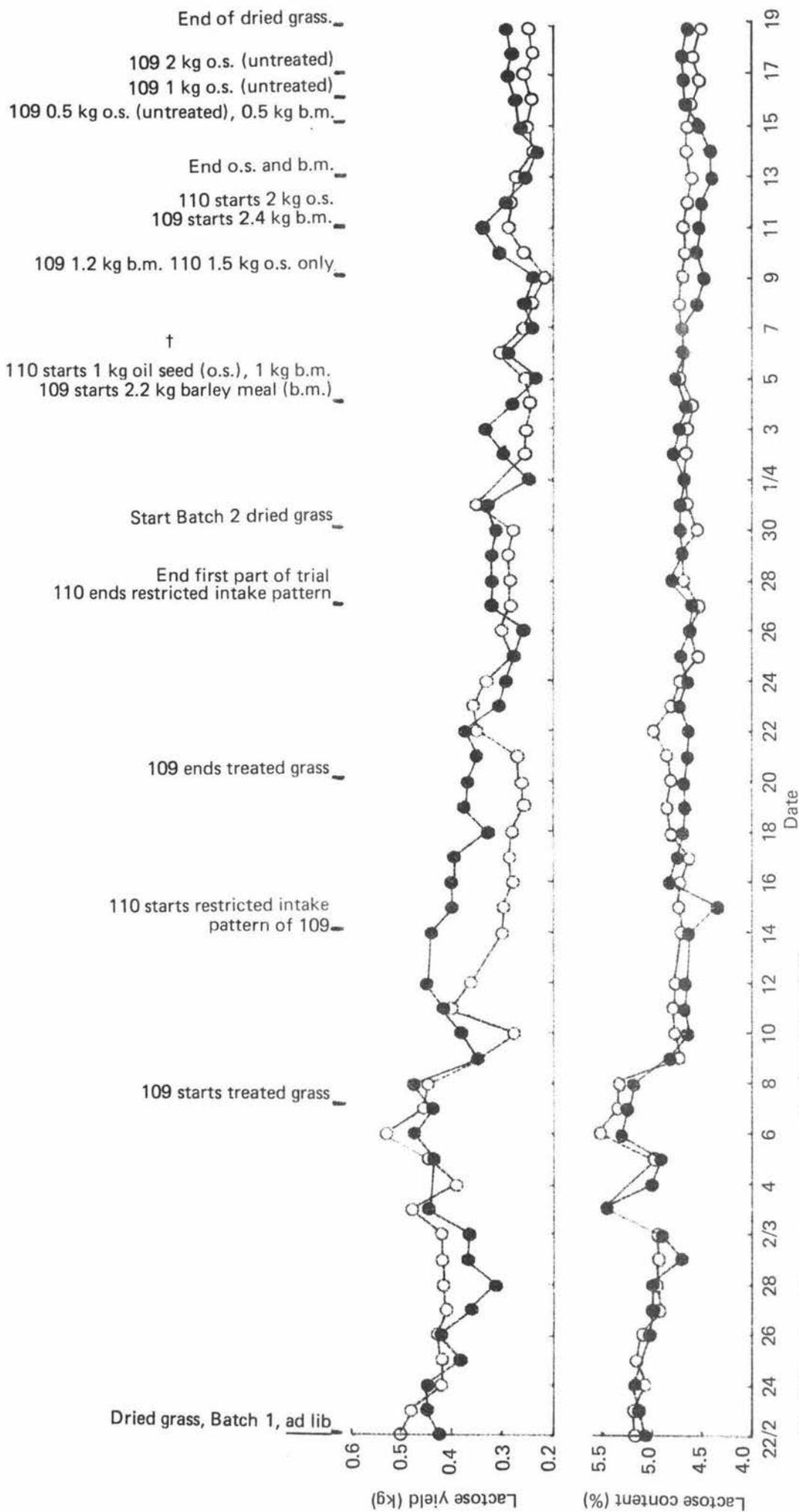


Fig. 3.3. Daily milk lactose content and milk lactose yield.

O—O COW 109, ●—● COW 110.

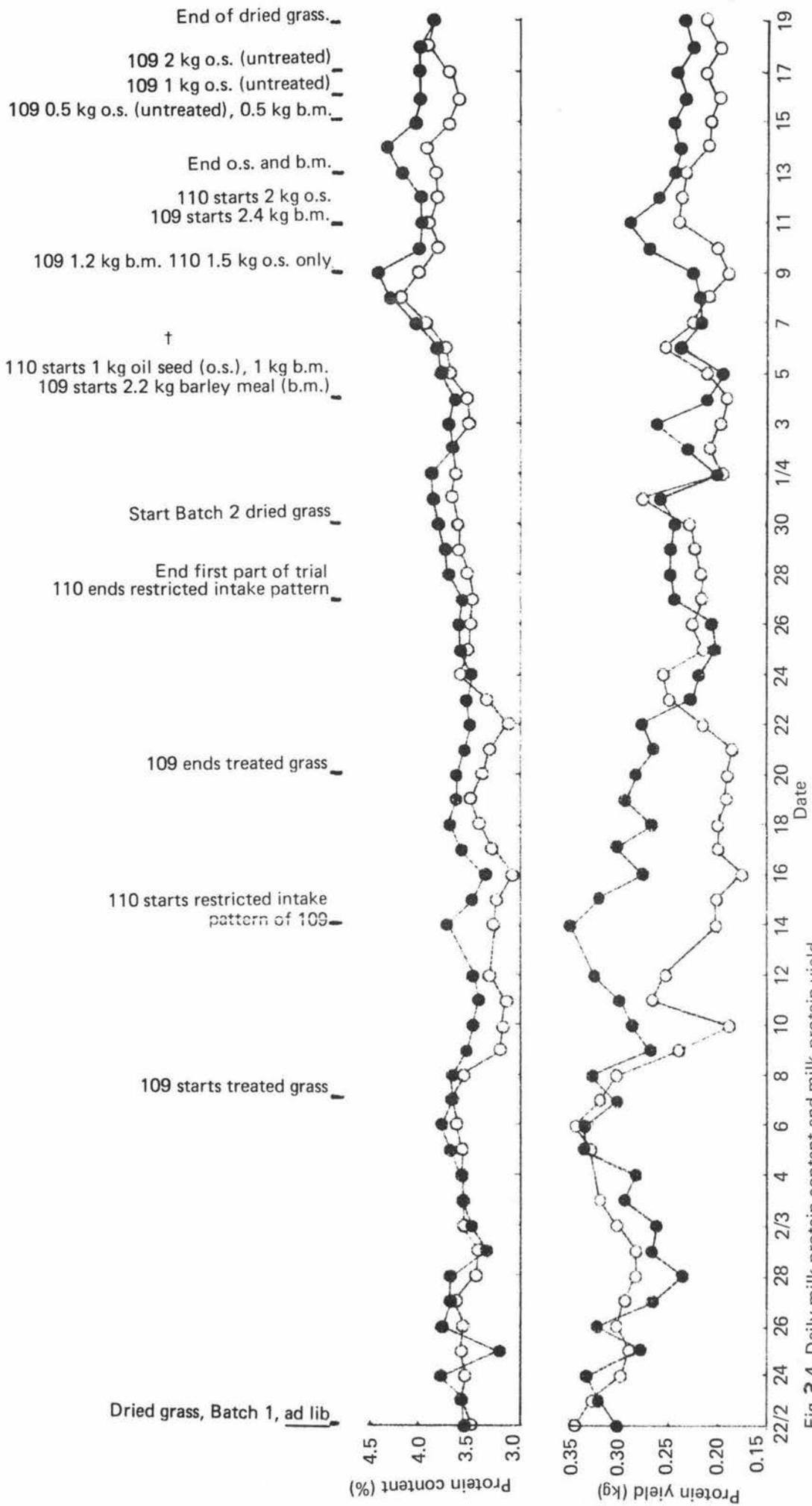


Fig. 3.4. Daily milk protein content and milk protein yield.

oil-seed, ($p < 0.05$) compared with the previous four days.

3.4.5. Milk fat

a) The fat content of milk and total yield of milk fat. The fat content in the milk of both cows was very variable (Fig.3.5). There was no significant depression in the content of milk fat in milk from cow 109 when fed the dried grass which had been HCHO-treated. The same was observed in cow 110 when underfed.

Fat content in the milk from cow 110 rose ($p < 0.05$) compared with cow 109 on addition of the supplement of HCHO-treated oil-seed to the diet. Comparison with the preliminary period (March 28-April 3) revealed that % milk fat rose in both cows 109 and 110 ($p < 0.05$) when fed barley meal and HCHO-treated oil-seed respectively.

The daily yield of fat was more variable than was protein in the milk of both cows. The yield of milk fat from cow 109 (Fig.3.5) declined ($p < 0.001$) from an average of 0.48 kg/day in the preliminary period (February 22-March 6) to 0.32 kg/day average when fed the dried grass which had been treated with HCHO. Underfeeding cow 110 resulted in a non-significant depression in milk fat yield ($p < 0.10$).

Milk fat yield increased when cow 110 was fed the sunflower seed supplement which had been HCHO-treated compared both with the preliminary period (March 28-April 3, $p < 0.05$) and with cow 109 fed the barley meal ($p < 0.05$). Barley meal supplementation had no significant effect on the yield of milk fat in cow 109 compared with the preliminary period of March 28-April 3. The yield of milk fat from cow 109 appeared to remain unchanged over the short period that the untreated oil-seed supplement was fed.

b) Composition of milk fat

Table 3.3 gives data on the variation obtained in the analysis of a single milk fat sample. The 2 gas liquid chromatographs are compared.

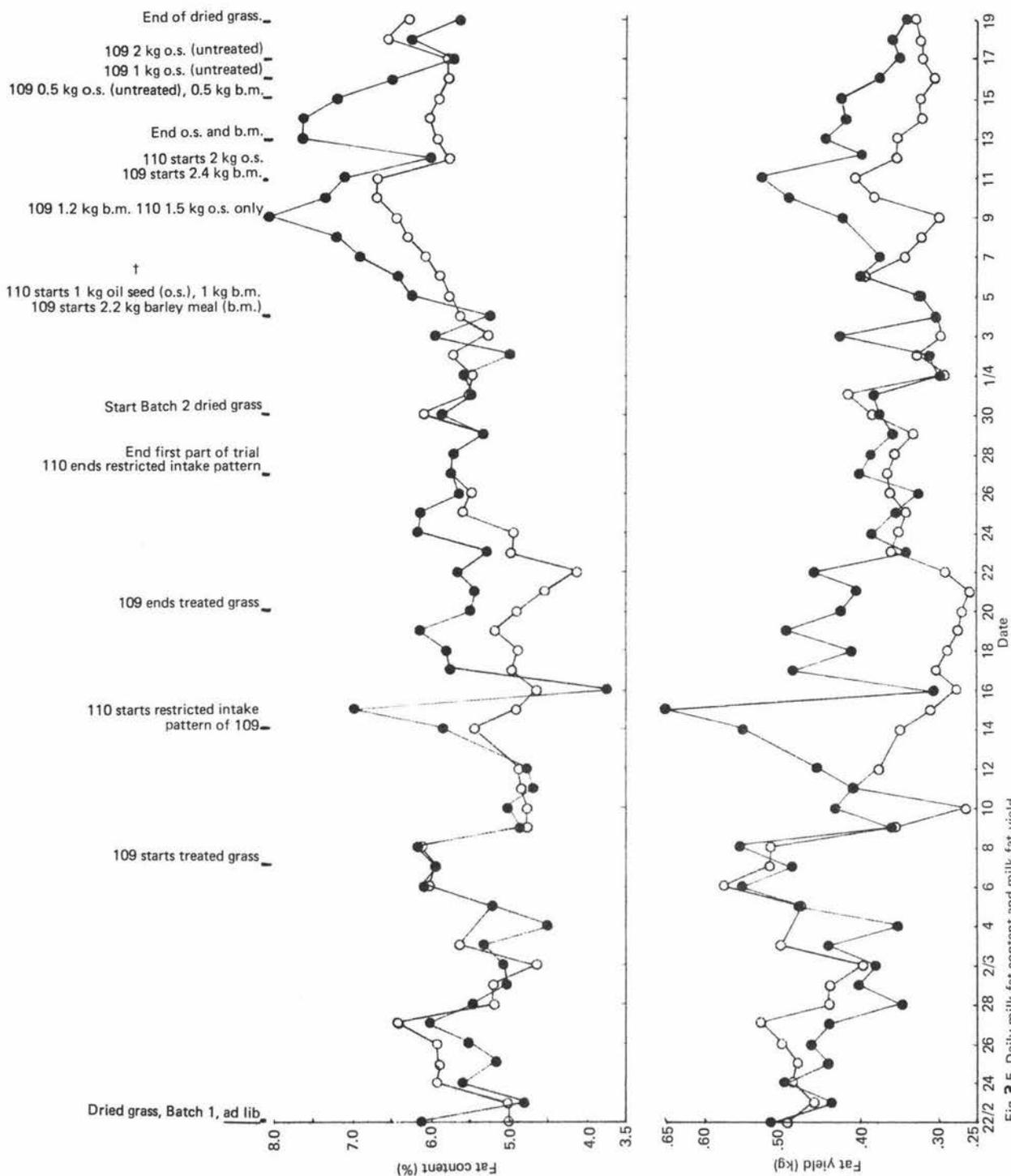


Fig. 3.5. Daily milk fat content and milk fat yield.

Table 3.3. The variation obtained in the analysis of one sample of milk fat on the gas liquid chromatographs.

Fatty acid [†]	Gas liquid chromatograph			
	Aerograph		Packard	
	Including 4:0	Excluding 4:0	Including 4:0	Excluding 4:0
4:0	14.1 \pm 0.37	-	13.9 \pm 0.57	-
6:0	5.1 \pm 0.19	6.0 \pm 0.26	5.1 \pm 0.05	6.0 \pm 0.09
8:0	1.8 \pm 0.05	2.1 \pm 0.08	1.8 \pm 0.06	2.1 \pm 0.06
10:0	2.6 \pm 0.09	3.1 \pm 0.13	2.6 \pm 0.05	3.0 \pm 0.05
12:0	2.2 \pm 0.06	2.5 \pm 0.07	2.2 \pm 0.08	2.5 \pm 0.09
14:0	7.2 \pm 0.11	8.3 \pm 0.13	7.6 \pm 0.21	8.8 \pm 0.25
16:0	16.2 \pm 0.22	18.9 \pm 0.19	16.2 \pm 0.39	18.9 \pm 0.49
18:0	15.3 \pm 0.55	17.9 \pm 0.57	15.3 \pm 0.22	17.7 \pm 0.23
18:1	26.4 \pm 0.29	30.7 \pm 0.28	26.3 \pm 0.53	30.6 \pm 0.45
18:2	7.5 \pm 0.09	8.8 \pm 0.13	7.5 \pm 0.26	8.7 \pm 0.30
18:3	1.5 \pm 0.12	1.7 \pm 0.14	1.6 \pm 0.27	1.6 \pm 0.08
	Means of 5 replicates		Means of 6 replicates	

[†] milk fat sample from cow 110, April 11.

Calculations have been made both including and excluding butyric acid. Mean values rounded to \pm 0.1; SE's rounded to \pm 0.01.

The results in Table 3.3 show that the molar content of 4:0 was more variable than the other fatty acids when analysed on the Packard. However, the exclusion of 4:0 from the calculations did not decrease the variability of the other fatty acids in most cases. Accordingly, 4:0 was included in all computations of the fatty acid composition of milk fat.

Fig.3.6 shows that feeding dried grass which had been treated with HCHO to cow 109 caused a depression in the short chain fatty acids 8:0 and 10:0 ($p < 0.01$ and < 0.001 respectively) compared with cow 110 on the untreated diet of dried grass. The other fatty acids of short chain length, 4:0 and 6:0, were not influenced significantly. Underfeeding cow 110 had no effect on the fatty acids of short chain length compared with the preliminary period where dried grass had been fed ad lib.

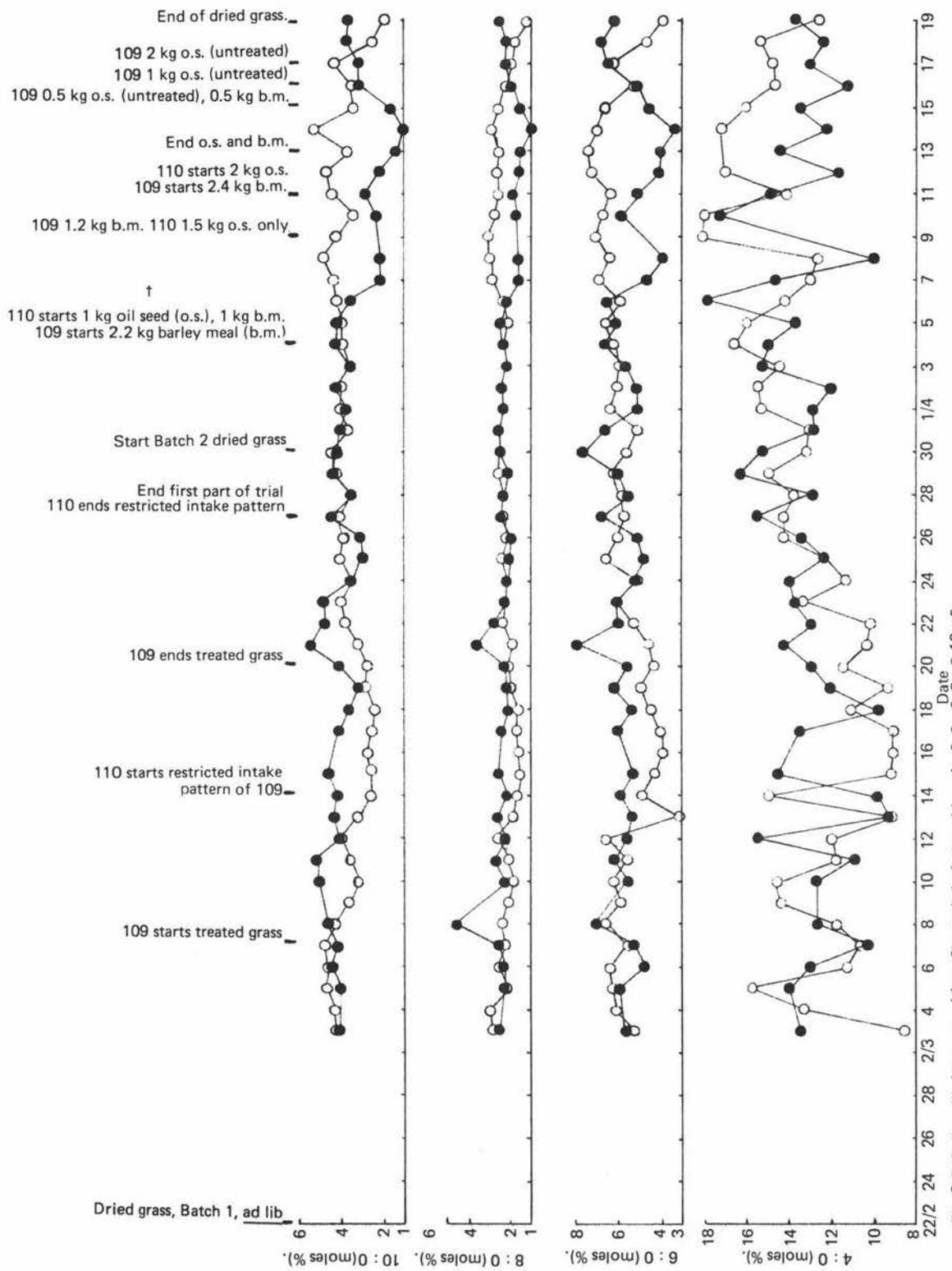


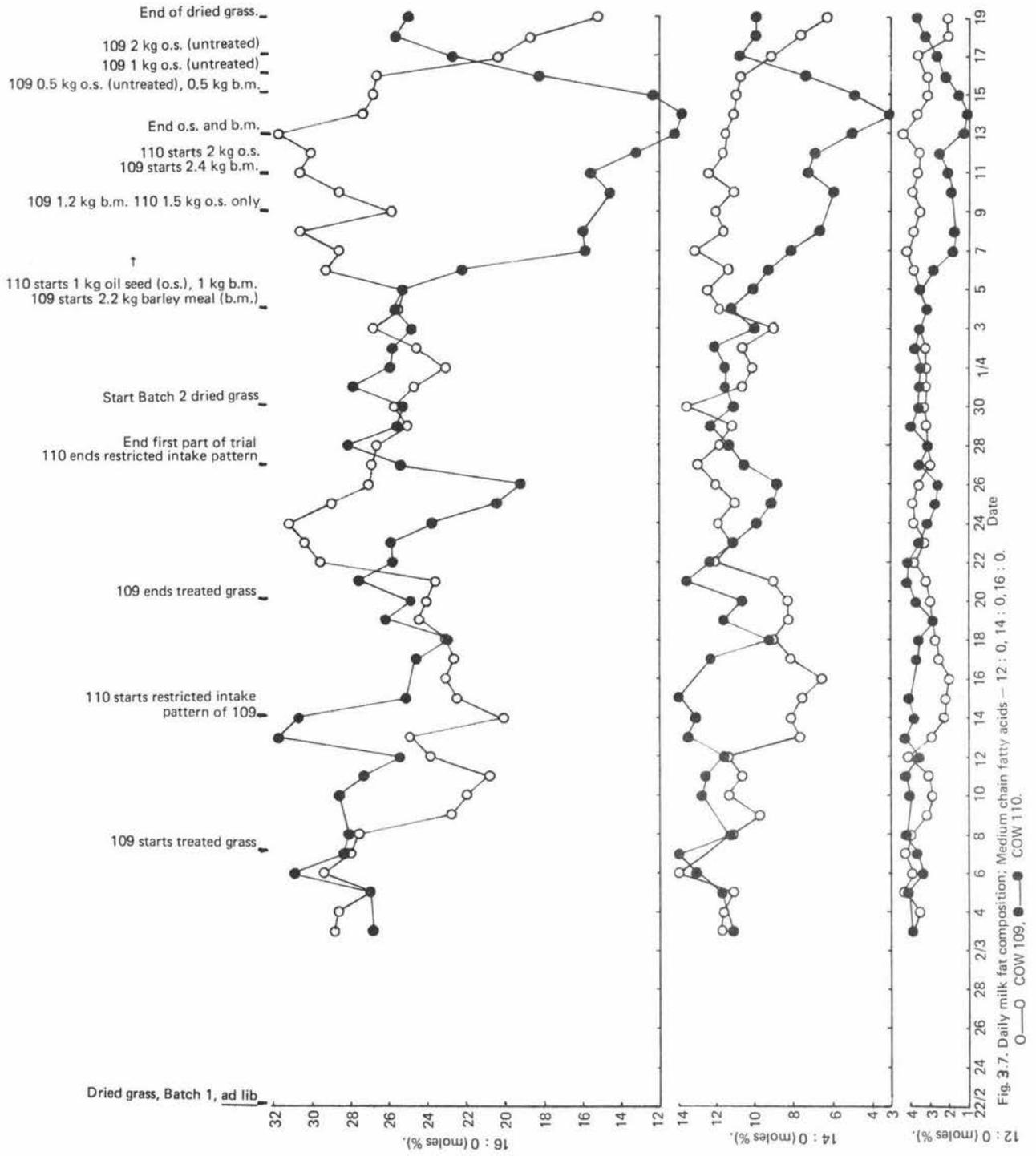
Fig. 3.6. Daily milk fat composition; Short chain fatty acids — 4 : 0, 6 : 0, 8 : 0, 10 : 0.
 O—O COW 109, ●—● COW 110.

During the second part of the trial (March 27-April 19), feeding the supplement of HCHO-treated oil-seed to cow 110 resulted in a significant decrease in the proportions of all the short chain fatty acids (except 4:0) compared with cow 109 fed the supplement of barley meal. A similar response was obtained when untreated oil-seed was fed to cow 109 although the results for the untreated supplement were not statistically analysed.

The fatty acids of medium chain length (12:0-16:0, Fig.3.7) varied in a manner similar to the fatty acids of shorter chain length. When the dried grass which had been HCHO-treated was fed to cow 109, the proportions of all the medium chain acids compared with the proportions in the milk of cow 110 fell ($p < 0.001$; 12:0, 14:0, 16:0). This effect disappeared about two days after the treatment period ended (March 19). Underfeeding cow 110 resulted in similar but less marked depressions in the proportions of the fatty acids of medium chain length compared with the preliminary period ($p < 0.05$; 12:0, 14:0 - $p < 0.001$ 16:0).

Supplementing the diet of cow 110 with a supplement of sunflower seed which had been HCHO-treated depressed the proportions of all the fatty acids of medium chain length ($p < 0.001$) in the milk compared with cow 109 fed a supplement of barley meal over the same period. The fatty acids 14:0 and 16:0 decreased from about 12 to a minimum of 3% and 29 to a minimum of 11% respectively. These effects were reversed a few days after cessation of feeding the supplement of HCHO-treated oil-seed. The supplement which had not been treated with HCHO appeared to have similar but less marked effects in cow 109.

Changes in the proportions of fatty acids of long chain length (C18 fatty acids, Figs. 3.8 and 3.9) mirrored the changes in the proportions of fatty acids of short and medium chain length. Feeding dried grass which had been HCHO-treated to cow 109 resulted in significant increases in 18:0 and 18:1 ($p < 0.001$ Fig.3.8) in milk fat compared with cow 110 fed untreated dried grass. In particular, moles %



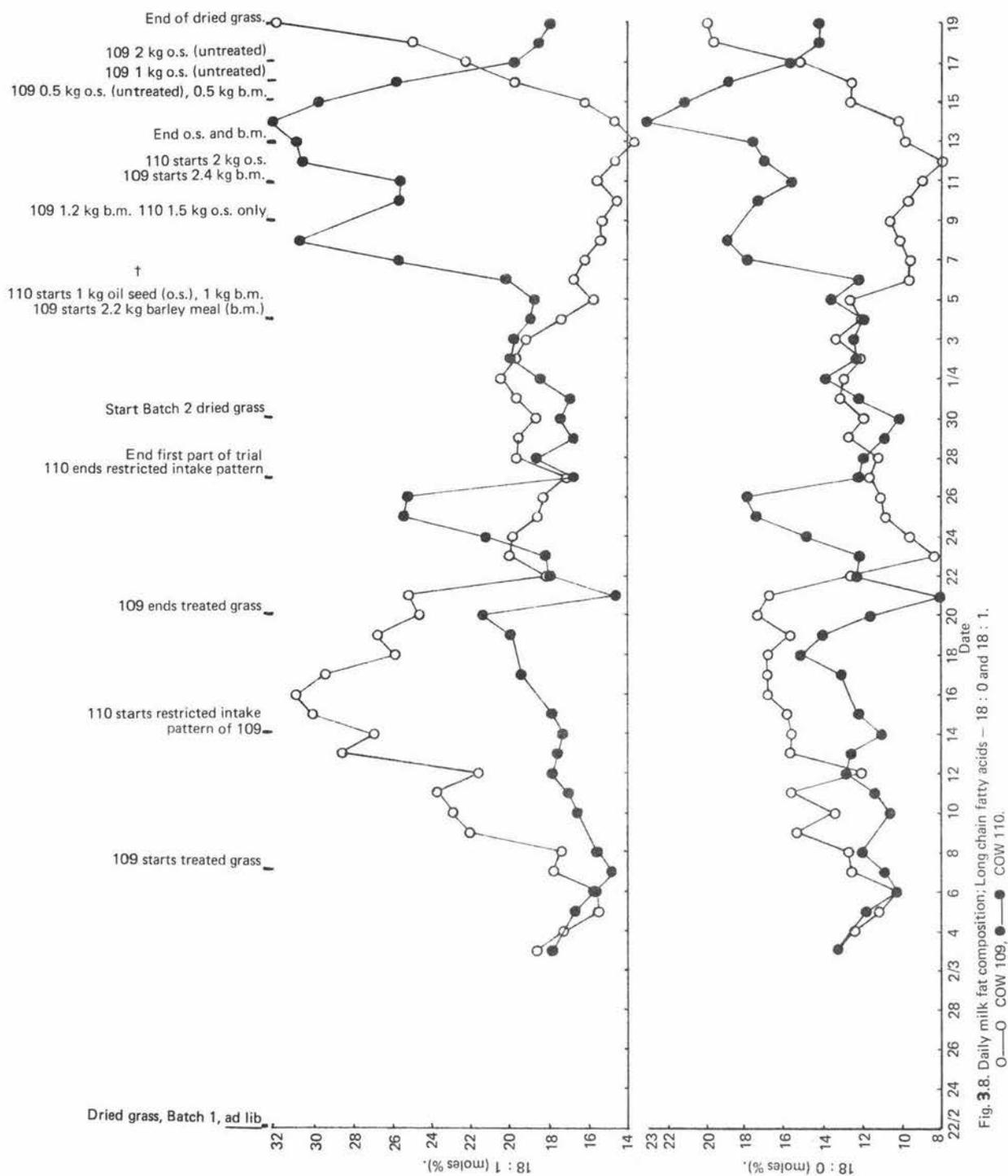


Fig. 3.8. Daily milk fat composition; Long chain fatty acids — 18 : 0 and 18 : 1.

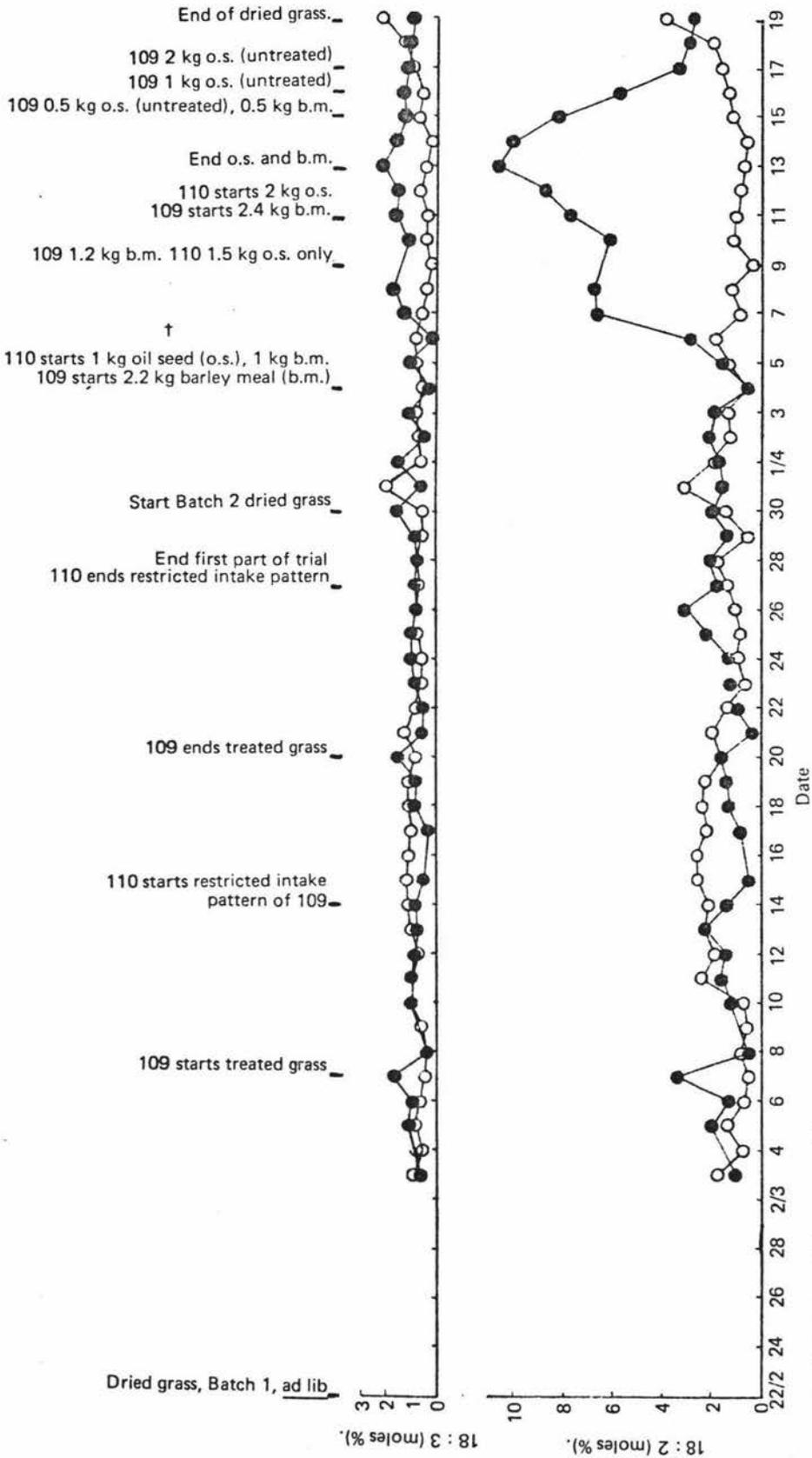


Fig. 3.9. Daily milk fat composition; Long chain fatty acids - 18 : 2 and 18 : 3.
 O—O COW 109, ●—● COW 110.

of 18:1 rose from an average of 17 to a maximum of 31% on March 16 after 9 days of feeding the treated grass.

Although 18:0 rose in cow 110 when underfed compared with her preliminary period, the rise was significant only at the $p < 0.1$ level. However, the level of 18:1 rose ($p < 0.01$) from a mean of 17% in the preliminary period to a maximum of 26% when underfed.

Feeding the supplement of sunflower seed which had been HCHO-treated to cow 110 caused the proportions of 18:0, 18:1 and 18:2 in the milk fat to rise ($p < 0.001$ all 3 acids, Figs. 3.8 and 3.9) compared with cow 109 fed the barley meal supplement. The proportion of 18:2 increased from a normal level of about 1.5% to a maximum of 10.5% of the total fatty acids estimated in the milk fat. This increase followed a stepwise fashion following the rise, from 1 to 2 kg, in the level of the supplement of HCHO-treated oil-seed in the diet. The supplement of treated oil-seed also caused a small increase (not analysed) in the proportion of 18:3 in the milk fat of cow 110 (Fig.3.9).

The untreated supplement of oil-seed resulted in a similar but very much smaller increase (from 2 to 4%) in the proportion of 18:2 in the milk fat of cow 109. This value of 4% may not have been the maximum that could have been achieved. The fatty acids 18:0 and 18:1, however, rose substantially.

4.1 Introduction

The earlier experiments in this study indicated that treatment of dried grass with HCHO could protect 18:3 in the forage from biohydrogenation by rumen micro-organisms in vitro. This was not supported by subsequent work with a pair of identical twin cows although in a supplement of sunflower seed which had been HCHO-treated, 18:2 was partially protected. Explanations for these findings are advanced.

4.2 The protection of protein and 18:3 in dried grass against digestion by the micro-organisms in rumen liquor.

4.2.1 Protection of forage protein

The results obtained in sections 2.3.2 and 2.3.3 are largely in agreement with those obtained by Hemsley et al. (1970), Barry (1971) and Sharkey et al. (1972). Formaldehyde treatment of dried grass (1 g HCHO/100 g dried grass) resulted in a significantly lower NH_3 -concentration (compared with controls) following incubation with rumen micro-organisms. It can be concluded that either the forage protein was substantially protected against microbial digestion or that the activity of the micro-organisms in the rumen liquor was suppressed. The micro-organisms in control incubations were quite active; degradation of available protein having been nearly completed after 8 h of incubation.

That microbial activity was suppressed in incubations with HCHO-treated grass was unlikely as digestibility was not depressed compared with control incubations. This finding was in agreement with those of Sharkey et al. (1972) and Barry (1972, pers.comm.).

4.2.2 The optimal rate of HCHO-application for protection of protein in dried grass.

Hemsley et al. (1970) and Barry (1971) suggested that 4-6 g HCHO/100 g crude protein gave optimal protection of forage protein against microbial digestion. This is higher

than the rate of application (1 g HCHO/100 g crude protein) recommended for purer forms of protein such as casein (Ferguson, 1970). Barry (1971) observed that the degree of protection of protein in vitro increased rapidly with rates of application of HCHO up to 4 g HCHO/100 g crude protein but thereafter it increased more slowly. This is somewhat at variance with the results in the present study. In fact, protection of protein (Fig.2.2) increased almost linearly up to 8.24 g HCHO/100 g crude protein which was the maximum level used.

The above differences are possibly due to variations in the amount of HCHO lost while drying the treated material. In neither this study nor in the others cited above was HCHO retained in the forage determined.

Where it was not possible or convenient to concurrently measure the digestibility of the dry matter, pH measurements were used. Robertson (1968) had found a highly significant correlation coefficient of -0.911 between pH and total volatile fatty acid concentrations in rumen fluid. It is also known that volatile fatty acid production is highly correlated with digestible organic matter (Weston and Hogan, 1968). Hence, the high correlation between pH and digestibility found in this study (section 2.3.3) is not surprising.

4.2.3 The extent of biohydrogenation of 18:3 in dried grass by rumen micro-organisms.

The suggestion was made (sections 1.2.1 and 1.8.8) that due to the close association of plant lipid and protein, particularly in the chloroplasts, protection of the protein against digestion by rumen microbes would also result in protection of the lipid in the plant. Following on from this, (section 2.3.3) it was argued that the level of HCHO which gave most effective protection of protein (2 g HCHO/100 g dried grass) was also likely to be the most effective in preventing the biohydrogenation of 18:3 by the rumen micro-organisms. The results in section 2.3.4 supported this. They are unlikely to be derived from a depression

in the activity of the rumen liquor micro-organisms since previous experiments had shown that the dried grass which had been HCHO-treated at 2 g % still had a dry matter digestibility of 53%. Although a little lower than the control value of 67% the difference still would not account for the almost complete protection of 18:3 observed in section 2.3.4. Although pH measurements (Fig.2.6) indicated that digestion of the dried grass which had been HCHO treated was slower, it was apparently almost as complete as that of untreated dried grass after 24 h of incubation.

The next experiment (section 2.3.5) provided results which supported the contention that lower rates of HCHO-application gave less protection to both protein and 18:3 in dried grass when incubated in vitro.

Of interest is the lower level of protection of 18:3 observed even at the high rate of HCHO application compared with the previous experiment (section 2.3.4). This demonstrates the wide variation in results which must be expected between in vitro experiments. The above variation occurred despite the fact that the cow used received the same diet prior to both experiments. Sampling variation in the HCHO-treated (and untreated) dried grass was not likely to be great as the grass was consistently well mixed as a result of grinding during its preparation.

4.2.4 The effects of free HCHO on the activity of the micro-organisms in rumen liquor.

The experiment aimed at distinguishing between the effects of free and protein-bound-HCHO on the fermentation of dried grass by rumen micro-organisms (section 2.3.6) gave unsatisfactory results in some respects. Free HCHO resulted in an almost complete loss of activity in rumen liquor compared with all the incubations involving protein-bound-HCHO when assessed by visual and olfactory observations. But, this was not entirely borne out by NH_3 , pH and fatty acid composition measurements. Ammonia production results indicated that nitrogen metabolism by the micro-organisms in the rumen fluid in the incubations with free HCHO was very low even compared with incubations containing protein-bound-

HCHO. Free HCHO also depressed digestibility as measured by pH but it was difficult to distinguish between the effects of protein-bound-HCHO and treatments containing 0.6 g % free HCHO on this basis. Free HCHO at all levels prevented biohydrogenation of 18:3 in the grass but so also did protein-bound-HCHO applied at 2 g HCHO/100 g dried grass.

From the results in this experiment, it was unfortunately not possible to conclude that free HCHO, even at low levels inactivated the rumen liquor micro-organisms whereas protein-bound-HCHO merely made the protein and lipid components of the grass inaccessible to the micro-organisms.

As described in the results, a better assessment of microbial activity could probably have been made if dry matter digestibility and gas production had been measured.

4.3 The protection of unsaturated fatty acids in the diet against biohydrogenation in the rumen of the cow.

4.3.1 Feedstuffs used.

Application rates of HCHO were calculated on the basis of weight of dried grass rather than crude protein. This was because crude protein figures were not always available at the time of applying the HCHO. The rate of application should have been on a crude protein basis as the amount of HCHO bound depends on the protein content of the grass. This probably accounts for the difficulty in removing excess HCHO from the treated forage as the crude protein content of the grass was considerably lower than that in grass used in the in vitro experiments.

The ether extract value of 3.25% of the dry matter in the dried grass which was treated with HCHO meant that for an animal receiving 8 kg grass dry matter daily, lipid intake would be 260 g per day. This is lower than the 500 g of lipid fed daily to cows by Pan et al. (1972) in their experiments.

4.3.2 The influence of dried grass which was HCHO-treated and the effects of underfeeding on milk yield and composition.

No increase in the content of 18:2 and 18:3 was detected in the milk fat of cow 109 when fed the dried grass treated with HCHO. This occurred in spite of the favourable results obtained in the in vitro assays (section 3.1.2).

The changes which were observed in the milk of cow 109 were more likely to have been an underfeeding effect. Voluntary intake was reduced and cow 109 lost condition. This was accompanied by most of the effects of underfeeding on milk composition documented by Flux and Patchell (1957), Robertson et al. (1960) and Munford et al. (1964) - refer section 1.5. The responses exhibited by cow 110 when underfed were similar in most respects although less marked than those seen in cow 109 when fed the HCHO-treated forage.

Flux and Patchell (1957) and Robertson et al. (1960) noted that restricted feeding generally resulted in depressed yields of milk, milk protein, milk lactose and milk fat with the yield of milk fat falling less than the other constituents. These effects, except perhaps those for lactose yield, were observed in both cows although less severely in cow 110. Cow 110 did not really show a significant fall in the yield of milk fat. This was probably because her intake was not as low as had been that of cow 109 when fed the dried grass treated with HCHO (Fig.3.1).

The lactose content of the milk of both cows did not alter much. This is not surprising as lactose is the major osmole of milk (Davies and White, 1960).

The content of fat in the milk did not change in the present study in either cow despite the fact that underfed cows often show an increase in butterfat content (Flux and Patchell, 1957; Robertson et al. 1960). This increase is derived from an increase in the mobilisation and use of fatty acids from depot fat for the synthesis of milk fat

(Robertson et al. 1960; Munford et al. 1964). However, most underfeeding experiments have been carried out on cows in early lactation when tissue fat is being actively metabolised for the production of milk fat. Near the end of lactation, as were the twins used in this trial, cows are more likely to be depositing depot fat (Moe et al. 1971). Hence, when underfed, the tendency to mobilise depot fat for milk production may be less than earlier in lactation.

If mobilised body fat was making an increased contribution to the production of milk fat then a rise in the proportions of 18:0 and 18:1 but not 18:2 and 18:3 in the milk fat could be expected - since the depot fat of ruminants contains a high proportion of 18:0 and 18:1 but not 18:2 and 18:3 (Shorland, 1953). Furthermore, the increased mobilisation of depot fat coupled with a lower availability of blood acetate (from the diet) could explain the depression in the proportions of the short chain fatty acids and the increase in the degree of unsaturation (as measured by iodine value) in underfed cows, (Munford et al. 1964). These effects were observed in cow 109 apart from 4:0 and 6:0 which did not change significantly when the cow was fed the dried grass which had been treated with HCHO. The effects were reversed when intake improved again on the control diet. Underfeeding cow 110 did not depress the proportions of the short chain fatty acids in the milk probably because the degree of underfeeding was not severe enough.

The behaviour of the fatty acids of medium chain length (12:0, 14:0 and 16:0) in the milk fat on underfeeding would depend on whether their increased supply from depot fat exceeded their decrease in production within the mammary gland from acetic and β OH butyric acids. The supply of these from blood decreases on underfeeding (Wilson, et al. 1967). From the results, the proportions of the fatty acids of medium chain length in the milk fat fell in both cows - more so in cow 109 than in cow 110.

4.3.3 The influence of supplements of oil-seed and barley-meal on milk yield and composition.

The supplement of oil-seed which had been treated with HCHO was quite acceptable to cow 110. Hence, the addition of barley meal to the oil-seed was discontinued and the oil-seed supplement fed on its own with the roughage. This finding contrasted with information provided by Evans (1973, pers.comm.) who had some difficulty in convincing goats to accept a similar supplement of sunflower seed.

On feeding a supplement of oil-seed which had been treated with HCHO, to cows, Pan et al. (1972) obtained an average increase of 15 and 6% in the contents of fat and protein in milk respectively. Yields of fat and protein were also increased and lactose yield fell. In the present study, fat content rose as also did the yield of milk fat and milk protein (over the last four days of feeding the treated supplement). Lactose yield, although not analysed, did not appear to decrease. The reason for this discrepancy compared with the work of Pan et al. (1972) is not readily explained as the energy content of the diet of roughage plus oil-seed supplement was estimated to be equivalent to that of the preliminary diet of roughage only. The rise in the yields of fat and protein in the milk of cow 110 when fed the supplement of HCHO-treated oil-seed was undoubtedly due to the high levels of these components in the supplement which comprised on a dry matter basis, 42 and 26% fat and protein respectively (Table 3.2).

That feeding the oil-seed supplement which had been HCHO-treated caused a depression in the fatty acids of short and medium chain length in the milk was in agreement with the results of others e.g. Mattos and Palmquist (1973) and Cook et al. (1972b). Mattos and Palmquist (1973) suggested that the increased supply of long chain fatty acids from the diet results in an increase in the concentration of these fatty acids and their acyl CoA derivatives in the mammary gland. These derivatives could inhibit acetyl CoA carboxylase and hence the synthesis within the mammary gland of the fatty acids of short and medium chain length, Brumby et al. (1972).

The result is in effect not unlike that observed earlier when the cows were underfed.

The fact that the proportion of 4:0 in the milk fat did not fall is evidence that 4:0 may be synthesised by a pathway other than that involving malonyl CoA. It is also possible that β OH butyrate, rather than being incorporated into fatty acids of longer chain length by the malonyl CoA pathway is instead released in greater amounts on to the milk fat triglyceride as 4:0.

Feeding the HCHO-treated supplement of oil-seed to cow 110 caused changes in the proportions of the fatty acids of long chain length which were partly in agreement with those observed by Cook *et al.* (1972b). They fed particles of safflower oil-casein (1/1 : w/w) which had been HCHO-treated and found that 18:2 from the diet was incorporated with up to 40% efficiency* into the milk fat of Jersey cows. This compared with an efficiency of 17-42% when a similar supplement was fed in a study by Bitman *et al.* (1973). An efficiency of only 2-8% was obtained when they fed a preparation of particles of soybean which had been treated with HCHO. Hutjens and Schultz (1971) fed ground soybeans which had been treated with HCHO to goats and could not distinguish between the effects of HCHO-treated and untreated supplements.

In the present study, cow 110 incorporated the 18:2 component of the HCHO-treated supplement of oil-seed with an efficiency of up to approximately 9%[†] when fed 2 kg of the supplement per day. When fed 1 kg of supplement per day, the efficiency of transfer was about 10%.

* efficiency of incorporation of 18:2

$$= \frac{\text{kg 18:2 in milk fat per day}}{\text{kg 18:2 eaten per day}} \times 100\%$$

[†] Based on the assumption that 18:2 made up 60% of the total fatty acid component of the sunflower seed - from Hilditch and Williams (1964).

Cook et al. (1972b) probably achieved a higher efficiency of incorporation of 18:2 from the diet because of a greater degree of encapsulation of the oil by casein in the oil-casein product produced as described by Scott et al. (1971). The oil in the oil-seed supplement used in this study was probably not as thoroughly coated with protein and so would not have been as well protected from biohydrogenation in the rumen. Hence, its low efficiency of incorporation and the relatively small increase in the proportion of 18:2 in the milk fat of cow 110. Instead, some 18:2 in the supplement after hydrogenation in the rumen appeared as 18:0 and 18:1 in the milk fat - a response which had not been observed by Cook et al. (1972b). They in fact observed a decrease in the proportion of 18:2 in the milk fat on feeding their treated oil supplement. Oleic acid in this study may also have increased due to desaturation of 18:0 to 18:1 in the mammary gland (McCarthy et al. 1965; Annison et al. 1967 and Bickerstaff and Annison, 1968).

Although the results derived from feeding the untreated supplement of oil-seed could not be analysed statistically it appears that most of the 18:2 in the supplement fed to cow 109 was hydrogenated in the rumen. A similar observation was made by Cook et al. (1972b) in their work with a supplement of safflower oil-casein (1/1:w/w) which had not been treated with HCHO. Biohydrogenation of 18:2 in the untreated oil-seed used in this trial was reflected in a large increase in the proportions of 18:0 and 18:1 in the milk fat of cow 109. The decreases in the proportions of fatty acids of short and medium chain length in the milk fat of cow 109 were no doubt a result of the increased uptake of 18:0 and 18:1 by the mammary gland as has been described for cow 110 when fed the oil-seed supplement which was HCHO-treated. The usual effects of milk fat depression documented in a review by Davis and Brown (1970) were not observed in cow 109. This may well have been because cow 109 was on the untreated oil-seed for only three days and so the effects of milk fat depression would not have had time to appear.

The barley meal fed as a supplement to cow 109 while cow 110 was fed the oil-seed supplement which had been HCHO

treated was not a very satisfactory control largely because of the manner in which it can influence fat metabolism in the ruminant. Hence, the reason for using the untreated supplement of sunflower seed when it became available on April 16.

Analysis of the results reveals that the effects of "high grain diets" in causing milk fat depression (Davis and Brown, 1970) were not observed when the diet of cow 109 was supplemented with barley meal. In fact, fat content increased and fat yield may have also although not significantly. However, feeding barley meal as a supplement to dairy cows can alter the propionic to acetic acid ratio upwards in the rumen (Bath and Rook, 1963, 1965).

This is most likely due to a rise in propionic acid production (Davis, 1967) which causes an increase in the supply of lactate and glucose to the blood plasma (Davis and Brown, 1970). As explained by Opstveldt *et al.* (1967) and Baldwin *et al.* (1969) this can have a lipogenic effect and will restrict the supply of long chain fatty acids from the depot fat and from plasma for milk fat production from these fatty acids. Hence the composition of the milk fat can alter favouring an increase in the proportions of the fatty acids of short and medium chain length at the expense of the fatty acids of long chain length - an effect which is the reverse of that already described for both HCHO-treated and untreated supplements of the oil-seed.

4.4 Summary and conclusions

4.4.1 The protection of protein and 18:3 in dried grass against digestion by the micro-organisms in rumen liquor.

a) Treatment of dried grass with HCHO resulted in protection of the endogenous protein from degradation by the micro-organisms in rumen fluid in vitro. These results largely confirmed those of other workers.

b) Protection of the protein from digestion by the micro-organisms in rumen fluid increased with levels of application of HCHO up to 2 g HCHO/100 g dried grass (8.24 g

HCHO/100 g crude protein). Digestibility of the dry matter as determined by first-stage digestion in vitro at this level of application was a little less than that of untreated forage.

c) Treatment of dried grass with HCHO prevented the biohydrogenation of 18:3 in the grass when incubated with rumen fluid. As in (b), the highest level of HCHO application was the most effective.

d) A preliminary experiment indicated that the presence of free HCHO severely reduces the activity of micro-organisms in rumen fluid although this was not shown conclusively.

4.4.2. The protection of unsaturated fatty acids in the diet against biohydrogenation in the rumen of the cow.

a) The feeding of dried grass which had been treated with HCHO did not result in an increase in the proportions of 18:2 and 18:3 in the milk fat of cow 109. Instead, intake was reduced and an underfeeding response was observed. Cow 110 when underfed showed a similar but less marked response.

b) It may be speculated that if grass high in lipid and protein content was treated with HCHO and fed to a cow then an increase in the proportions of 18:2 and 18:3 in the milk fat could occur. This response would be dependent on an adequate intake of the treated material.

c) A supplement of sunflower seed which had been treated with HCHO resulted in milk fat which contained about 10 moles % 18:2. Reasons for discrepancies between this result and levels obtained by other workers using more refined supplements of HCHO-treated oil-casein were discussed. It was thought likely that these supplements contained oil which was more thoroughly encapsulated with protein upon which protection of the oil depends.

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APPENDIX

Appendix 1 Sample Gas Liquid Chromatograph Trace and Analysis of a Milk Fat Sample.

Table A.1. Gas liquid chromatograph correction factors.

Fatty acid	Chromatograph	
	Packard	Aerograph
4:0	1.50	1.36
6:0	1.17	1.12
8:0	1.00	1.04
10:0	0.98	1.00
12:0	1.00	1.00
14:0	0.91	0.97
16:0	0.93	0.96
18:0	1.16	1.00
18:1	1.00	1.00
18:2	1.00	1.00
18:3	0.61	1.00

Table A.2. Example calculation on milk fat sample from cow 110. date; 10/4.

Fatty acid	Peak Height (mm)	Width at $\frac{1}{2}$ Height (mm)	Correction Factor	Molecular weight	Product	Moles %
4:0	35.0	1.8	1.50	88	1.0739	17.3
6:0	18.0	2.0	1.17	116	0.3631	5.8
8:0	14.0	1.1	1.00	144	0.1069	1.7
10:0	28.0	0.9	0.98	172	0.1436	2.3
12:0	23.0	1.1	1.00	200	0.1265	2.0
14:0	62.5	1.5	0.91	228	0.3742	6.0
16:0	92.5	2.7	0.93	256	0.9073	14.6
18:0	58.5	4.5	1.16	284	1.0752	17.3
18:1	79.5	5.7	1.00	282	1.6069	25.7
18:2	18.0	6.0	1.00	280	0.3857	6.1
18:3	4.0	8.1	0.61	278	0.0711	1.1

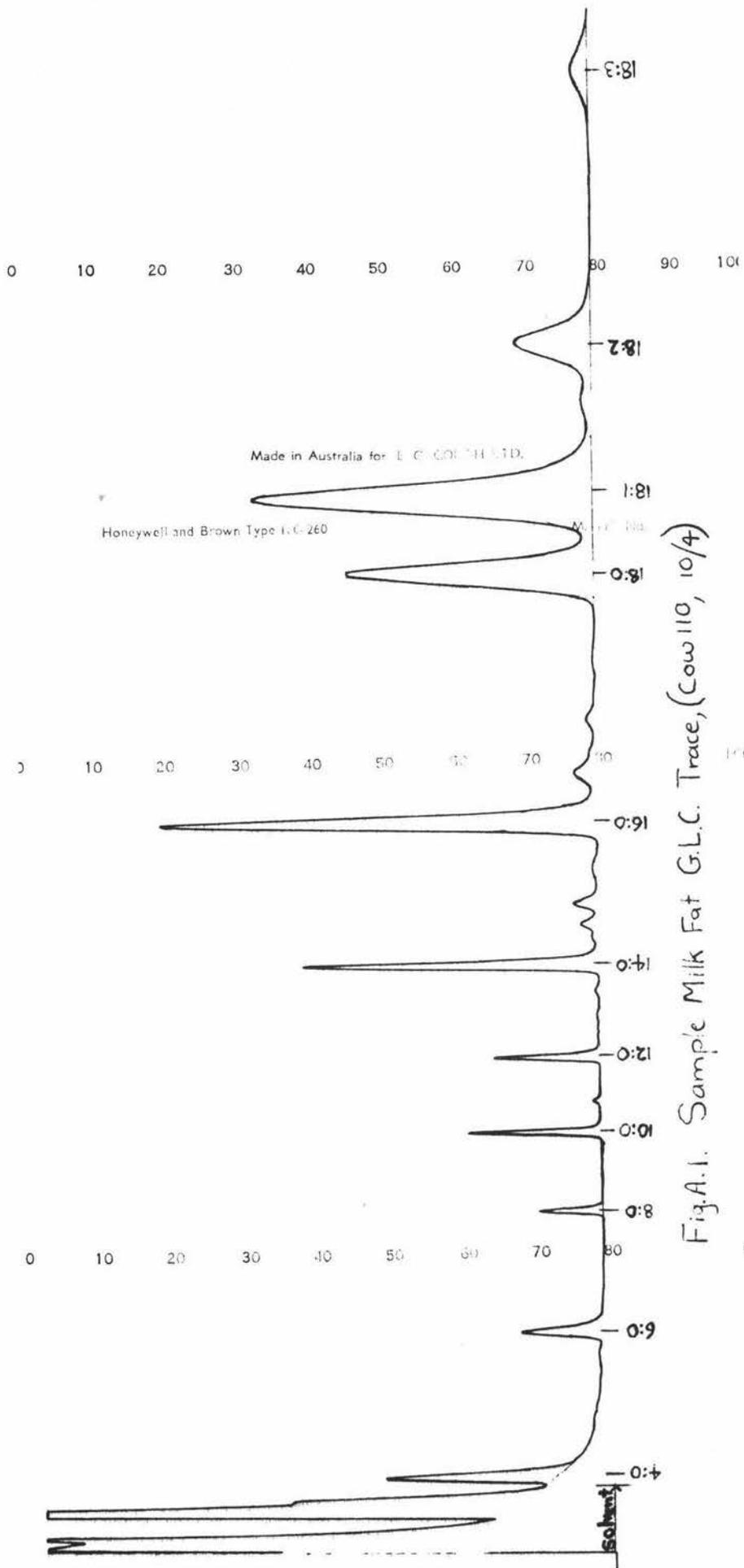


Fig.A.1. Sample Milk Fat G.L.C. Trace, (Cow 110, 10/4)