CHANGES IN BOVINE MAMMARY ENZYME ACTIVITIES AND MILK COMPOSITION

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

Massey University

December 1969

ACKNOWLEDGEMENTS

I wish to acknowledge my gratitude to the following who made the presentation of this thesis possible:

Professor R.E.Munford for his interest and guidance throughout the whole study, for organising the production testing and for his participation in the processing of the results;

J.E.Ormsby for his contribution to the development of the surgical method and regular assistance with the sampling;

T.J.Lynch and the farm staff for their cooperation throughout the experiment in the care of the experimental animals;

The technical staff of the Dairy Husbandry Dept. who contributed to the production testing;

Dr J. Kirkman and Dr R. Hodges who made equipment available for infra-red spectroscopy;

Dr N. Walker and Mr I.K.Gray, New Zealand Dairy Research Institute, who made their laboratory and equipment available for gas-liquid chromatography;

Professor D.S Flux and the staff of the Dairy Husbandry Dept. who made facilities available and provided useful discussion during the experiment;

Mary Bishop and my wife Jean for their help in the final presentation of the thesis;

The New Zealand Milk Board who made a grant to the author covering the cost of presentation of this thesis.

PREFACE

Concurrent study of mammary biochemical parameters and changes in the composition of the secreted product allows an examination of possible causal relations and is fundamental in identifying sites of action of physiological factors influencing the secretory activity of the gland. Changes in biochemical parameters accompanying various physiological states are now well documented for rodent species, e.g. Baldwin & Milligan (1966), but the relationships these bear with trans-lactation changes in the composition of milk have not been studied. The bovine provides ample material of sufficient diversity to characterise changes in milk composition and the species importance as a food producer justifies any attempt to determine how milk production is controlled and seek possibilities of artificial regulation.

Prerequisite to such a study is a technique for repeatedly obtaining tissue from lactating udders without seriously influencing future production. The present experiment was undertaken to develop such a sampling method and use it in a first attempt to study milk production at the synthetic level throughout whole lactations in dairy cattle.

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Chapter One. REVIEW

Introduction

Despite the wealth of recent experimental studies on the lactating mammary gland, few attempts have been made to survey the biochemical and physiological events leading to the formation of milk. Since this review was initially prepared, two major contributions have been published which has lead to the more restricted nature of this presentation. Jones (1969) has attempted to discuss the whole field of recent biochemical progress in light of the physiological studies made on the rodent gland. A symposium, "Physiological limitations on milk production" introduced by Emery (1969) attracted three other papers which when taken with Jones provides a more complete picture. In no case however is there a fully descriptive account of the synthesis of the major components of milk especially in the metabolic environment peculiar to the ruminant.

This present review gives a detailed biochemical account of the synthetic processes and discusses some physiological studies which assist an appreciation of the control systems. A short discussion of changes in milk fat composition is entered into as the author considers measurements of their changes the most potent tool currently available to integrate the biochemical and physiological processes of the lactating ruminant.

Milk Fat Synthesis

Because of the prodigious synthesis of butterfat and its unusually high content of short chain fatty acids, the mammary gland and its lipid secretion has been subjected to extensive biochemical study. The reviews of Hele & Popják (1958), Folley & McNaught (1961) and Fritz (1961) provide an adequate background to the more recent studies to be reviewed here.

The earlier studies established the potent lipogenic capacity of mammary tissue; that the fat comprised fatty acids synthesised both within the gland and elsewhere; that the fatty acid composition was unique and thus the possibility that there was more than one fatty acid synthetic mechanism.

1.1 Fatty Acid Synthesis

Since the above reviews were compiled, extensive study in a variety of tissues has largely characterised the so-called "soluble" fatty acid synthetic pathway. A single cycle of the chain elongation process, which uses NADPH as proton donor, is illustrated in Fig. 1.1. There is ample evidence for the existence of this pathway in mammary tissue and, as in other tissues, the rate-limiting step is in the carboxylation of acetyl CoA (Wakil, 1961). The enzyme acetyl CoA:CO_2 ligase (AMP) (EC 6.4.1.2) is under precise control in mammary tissue and so is one of the foci of lipogenic regulation.

Fatty acid synthesis is progressively diminished following natural or artificial weaning. Levy (1963, 1964) demonstrated that the addition of fresh rat milk to actively synthesising cell-free preparations from rats, similarly depressed synthesis. Furthermore he showed that the depressed lipogenesis resulted from an inhibition of acetyl CoA carboxylase *in vivo* and also *in vitro* where the addition of fresh milk to partially purified enzyme markedly reduced its activity. Levy proposed that fresh milk contained an inhibitor, normally secreted with the milk, and if not removed from the gland it operated a feedback control on lipogenesis acting at the rate-limiting step. He identified the inhibitor as

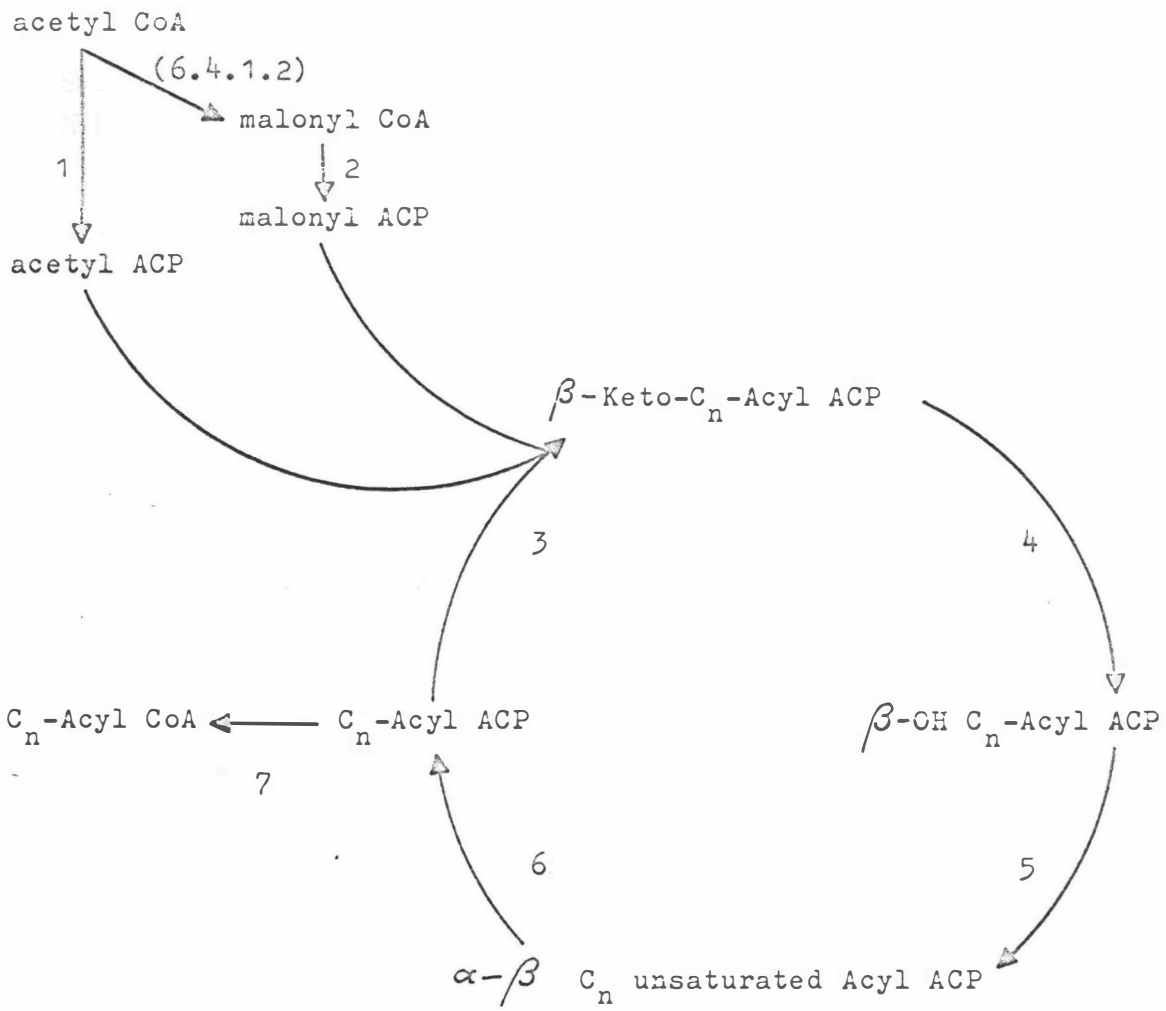


Fig. 1.1 Representation of a cycle of the Acyl Carrier Protein bound fatty acid synthetic system of cytoplasm.

- The seven component enzyme complex termed 'fatty acid synthetase' comprises reactions 1 - 7.
- The first cycle uses 1 - 6 producing butyryl-ACP (n = 4).
- The second cycle uses 2 - 6 producing capryl-ACP (n = 6).
- The m^{th} cycle uses 2 - 7 producing C_n-acyl CoA (n = 2m + 2)

microsomal bound free fatty acids of chain length 10 - 14, and suggested that their mode of action was through interfering with the essential activation of the enzyme by di- and tri-carboxylic acids.

Early studies on cofactor requirements for lipogenesis (see Hele & Popják, 1958) indicated participation of both NADH and NADPH in the reductive steps accompanying chain elongation. Hele, Popják & Lauryssens (1957) suggested that NADH was used in the synthesis of short chain fatty acids using enzymes of the β-oxidative pathway. The major product synthesised by mammary preparations supplied with NADH was crotonic acid. Lachance, Popják & de Waard (1958) found a microsomal enzyme which irreversibly reduced crotonyl CoA to butyryl CoA. This enoyl reductase uses NADPH as a proton donor and if it also accepts ACP esters, it is 6 in Fig. 1.1. The use of NADH in mammary lipogenesis, possibly with the aid of a transhydrogenase (EC 1.6.1.1) is now well established (Bogin & Katz, 1967), but the involvement of a reversal of β-oxidation is more equivocal.

In the presence of avidin, the egg white biotin-binding protein, the enzyme acetyl CoA carboxylase cannot transfer CO_2 and thus the "soluble" pathway is inactivated unless an alternative source of malonyl CoA is provided. Bogin & Katz used a cell-free rat preparation to show that the synthesis of short chained acids, chiefly butyrate, could proceed from acetyl CoA and NADH but not in the presence of avidin. This indicated a requirement for malonyl CoA - a non-participant in β-oxidation. In rabbit mammary preparations, Lachance & Morais (1965) found the enzyme crotonyl reductase quite labile on storage. Its addition to mammary preparations *in vitro* led to acetate being incorporated mainly into butyrate. This presumably explains the accumulation of crotonic acid in the experiment of Hele *et al* (1959). Added crotonyl CoA was recovered only as butyrate or butyryl CoA which suggests that there was no crotonyl transferase (crotonyl CoA ⟶ crotonyl ACP) analogous to reactions 1 and 2 as in Fig. 1.1. In rabbit and goat mammary preparations, Kumar, Singh & Keren-Paz (1965) found little effect of avidin on butyrate synthesis but their results show that

the synthesis of longer chain acids required either malonyl CoA or acetyl CoA carboxylase. Whilst there is some reason to suspect species differences in the enzyme complements and substrates normally available, the results of Kumar et al are confused by the finding that citrate, an activator of acetyl CoA carboxylase, enhanced butyrate synthesis. Citrate can contribute to the extramitochondrial pool of acetyl CoA via the "citrate cleavage enzyme" and acetyl CoA levels might be limiting in the rabbit, but in the goat the enzyme ATP:citrate oxaloacetate lyase (EC 4.1.3a) is absent (Hardwick, 1966) and acetate itself is hardly likely to limit synthesis.

In summary these findings suggest that butyrate synthesis may involve more than one pathway. One possibility is for a reversal of β-oxidation with the irreversible enoyl CoA:cytochrome c oxidoreductase (EC 1.3.2.1) being replaced by microsomal crotonyl reductase. A simple demonstration of crotonic acid synthesis from acetate using isolated mitochondria would resolve this. Butyric acid can also arise from the soluble pathway but competition between reactions 3 and 7 (Fig. 1.1) favour chain elongation making this a minor source. Clearly any differences in the importance of the two pathways of butyrate synthesis will cause variable responses to added avidin which inhibits only the soluble system.

Recent advances in methodology has enabled the study of a variety of factors on the nature of the products of lipogenesis. Most interesting, in light of the foregoing discussion, was the finding of Coniglio & Popják (1963) that rat mammary tissue incubated with 1 - C^{14}-acetate resulted in label concentration in short chain acids, but if incubated with 1 - C^{14}malonyl CoA, label was concentrated in the longer chain acids up to and including palmitate. Smith & Dils (1966) found a direct relationship between the concentration of malonyl CoA and the chain length of acids formed by rabbit mammary preparations. In the rat, the activity of acetyl CoA carboxylase and fatty acid chain length were related (Bartley, Abraham & Chaikoff, 1967). These studies all support the hypothesis that the concentration of malonyl CoA, the product of the rate-limiting step, is a major determinant of the

extent of lipogenesis.

The substrate used for fatty acid synthesis by the non-ruminant mammary gland is largely acetyl CoA arising from pyruvate decarboxylation and β-oxidation coupled with formation of intramitochondrial citrate. Citrate is transported to the cytoplasm and cleaved as above. In addition, although all species thus far studied have the cytoplasmic enzyme acetate:CoA ligase (AMP) (EC 6.2.1.1), the low levels of acetate presumably available in monogastric species makes it an unlikely source of acetyl CoA. On the other hand in ruminants there are data collected by the arterio-venous difference technique, reviewed by Barry (1964), and Linzell (1968), indicating extensive mammary utilization of acetate, acetoacetate and β-OH butyrate. Radioisotope studies confirm that these substrates are used in lipogenesis, the four carbon units in part without prior cleavage to acetyl CoA (Luick & Kameoka, 1966; Palmquist, Davis, Brown & Sachan, 1969). There are possibly ligase (- CoA synthetase) reactions followed by transferases similar to 1 in Fig. 1.1. The ACP esterified acids could then enter the cycle with reactions 4, 5 & 6 or 5 & 6 and undergo chain elongation. The significance of Kumar et al noting the capacity to use four carbon units without cleavage in rabbits as well as goats is obscure though because of caecal fermentation, the rabbit may use VFA as metabolic substrates.

1.2 Microsomal Regulation of Synthesis

It was noted earlier that Levy located the cause of inhibition of acetyl CoA carboxylase to be fatty acids bound in non-esterified form to microsomes. When sub-cellular fractions were first studied (see Hele & Popják, 1958), it was generally accepted that microsomes afforded a stimulation to fatty acid synthesis. This dichotomy was studied by Matthes, Abraham & Chaikoff (1960) in supernatants from liver where fatty acid synthesis was stimulated or depressed according to the level of added microsomes. The microsomes are the intracellular sites of triglyceride synthesis from both free acids

and their CoA esters. Their enzymatic complement includes acyl CoA:L-∝-glycerol phosphate acyltransferase (EC 2.3.1.15) and the thiokinase - acid:CoA ligase (AMP) (EC 6.2.1.3). In addition (see 1.5) the 'microsomes' of milk are considered to be the site of glyceride synthesis in the freshly secreted product.

The stimulatory effect of glycerol phosphate on fatty acid synthesis (Fritz, 1961) is considered to operate at the microsomes through increased esterification of fatty acids and hence removal of their feedback inhibition on acetyl CoA carboxylase. Howard & Lowenstein (1965) note that the effect of added glycerol phosphate is less in mammary gland than in liver and despite the lack of evidence for acyl carrier protein *per se* in mammary tissue, suggested that this might be limiting for lipogenesis.

Further study in the rat mammary gland by Smith, Easter & Dils (1966) indicated that microsomes stimulated fatty acid synthesis partly through removing the feedback inhibition but also through increasing the level of acetyl CoA carboxylase in the system. They found the enzyme in microsomes at specific activities equal to or greater than in the particle-free supernatant. Easter & Dils (1968) cite unpublished results that the rat enzyme is confined to the cytoplasm and checked that the distribution in the rabbit was not an artifact of preparation. The results indicate that the rabbit enzyme is indeed found as earlier reported by Smith *et al* (1966). The microsome bound and free enzymes had different molecular properties and when dissociated, the microsomal enzyme adopts the characteristics of the other. The authors were cautious not to claim exactly which form existed and was active *in vivo*.

Thus microsomes *in vitro* appear to offer both a stimulation and an inhibition to fatty acid synthesis. Both actions are focussed on a regulation of acetyl CoA carboxylase. Little is known of the *in vivo* situation but the following is offered in speculation to help explain the experimental results.

> Microsomes bind fatty acids awaiting esterification to glycerol. Should conditions favour this - viz.

adequate glycerol phosphate, ATP etc. then triglyceride synthesis proceeds. If, however, the interaction of glycerol phosphate, ATP and fatty acids leads to an accumulation of the latter, the feedback inhibition operates and fatty acid synthesis is halted awaiting restoration of glyceride synthesis. The microsomal content of acetyl CoA carboxylase in some species may provide a favourable juxtapositioning to enable precise control.

The significance of the species differences in microsomal properties is not clear.

1.3 Desaturation of Long Chain Fatty Acids

Glascock, Duncombe & Reinius (1956) reported a substantial conversion of H^3-stearic acid to unsaturated long chain acids in the cow's mammary gland. Lauryssens, Verbeke & Peeters (1961) perfused udders with albumin bound 1-C^{14}-stearic acid and found that of the octadecanoic acids incorporated into milk fat, 50% were unsaturated. Pursuing studies on extracellular glyceride synthesis (see 1.5), McCarthy, Ghiardi & Patton (1965) determined the fate of 1-C^{14}-stearic acid added to fresh milk. Radioactivity was recovered in both stearic and oleic acids in neutral lipids, phospholipids and as free fatty acids. The extent of conversion was greatest in the esterified fraction and the authors claimed that whereas that fraction had definitely been in contact with enzyme systems, the free fatty acids because of dispersion difficulties, may not have. The desaturase system was more active in milk from earlier than later stages of lactation. West, Annison & Linzell (1967) and later, Annison, Linzell, & Fazakerley & Nichols (1967) in examining the mechanism of uptake of blood lipids by the gland, found that arterial 1-C^{14}-stearic acid was recovered in mammary venous blood as both stearic and oleic acids, the latter having a specific activity 64% that of stearic compared with 2% in the arterial blood. If desaturation occurs outside the vascular bed and in the parenchyma, these results indicate that free fatty acids traverse the endothelial-epithelial

membrane in both directions. The similarity of specific activity curves obtained from glands perfused with 1-C^{14}-stearic and 1-C^{14}-palmitic acids suggests that desaturation involves only the preformed acids and not those synthesised de novo within the gland. If so, the results indicate a functional separation of these two classes of acids.

Bickerstaffe & Annison (1968) briefly described a microsomal desaturase system in goat mammary gland. The enzyme(s) preferentially oxidised stearate although palmitate was used at about 20% the rate of the former. The reactions were confined to the production of monoethenoid acids. Added glycerol phosphate stimulated the reactions, possibly through the removal of reaction products (c.f. 1.2).

1.4 Mitochondrial Chain Elongation

Chain elongation of hexadecanoic acid in tissues that have been examined appears to involve condensations with acetyl CoA by a reversal of β-oxidation in a manner similar to that proposed for short chain acid synthesis in mammary tissue. It is surprising then to find in data discussed by Folley & McNaught (1961) and that given by Annison et al (1967) near or complete absence of such reactions in the mammary gland. Close scrutiny of the figures and tables presented show a time dependent increase in specific activity of the longer chain acids (14:0 & 16:0) following injection of 1-C^{14}-acetate to lactating goats. Such an increase is not seen in vitro where fatty acids produced are not mixed with preformed long chain acids from the blood. The change with time probably reflects increased specific activities of the blood acids, also formed from the labelled substrate but at sites distant from the mammary gland. Shortly after injection the preformed acids could cause 'isotope dilution' (e.g. Palmquist et al 1969) but with time the specific activities would be comparable. In addition, the studies discussed in 1.3 where labelled stearic and palmitic were perfused through glands, did not indicate any label recovery in acids of longer chain length than those injected.

This apparently unimportant type of reaction casts some doubt on the ability of mammary mitochondria to participate in a reversal of β-oxidation. Such a dichotomy presents a valuable lead in the whole question of alternative lipogenic pathways and has not yet received the study it merits.

1.5 Triglyceride Synthesis

Fatty acyl CoA esters are esterified to L-α-glycerol phosphate producing, as intermediates, 1'2'diglyceride phosphate (phosphatidic acid) 1'2'diglycerides and finally triglycerides. This is the well known Kennedy pathway of synthesis and has benn identified in extracts of mammae of guinea-pigs (McBride & Korn, 1964 b), rat (Dils & Clarke, 1962) and goat (Pynadath, 1964).

Glycerol phosphate arises mainly from the reversible reduction of dihydroxyacetone phosphate by L-glycerol phosphate:NAD^+ oxidoreductase (EC 1.1.1.8). McBride & Korn (1964 a) have shown the presence of ATP:glycerol phosphotransferase (EC 2.7.1.30) in their guinea pig preparations. This could be of particular significance as it enables the use of the free glycerol produced by lipases acting on glycerides of the blood or of the fat pad as the secretory epithelium develops into it. However, data obtained by Barry, Bartley, Linzell & Robinson (1963) in lactating goats showed that less than 0.1 mg% of glycerol is taken up by the gland which could not make a significant contribution to glyceride glycerol in milk fat. Barry *et al* showed further that during the uptake of blood glycerides, increased lipoprotein lipase was detected in the venous blood suggesting that lipolysis occurs in the immediate vicinity of the endothelium and that glycerol uptake by the secretory epithelium is probably in non-esterified form.

Evidence for the presence of Acyl CoA:L-glycerol phosphate acyltransferase (EC 2.3.1.15) in guinea pig mammae was obtained by Kuhn (1967). The enzyme was largely (76%) located in microsomes. Kuhn detected dephosphorylation of *in vitro* added phosphatidic acid, synthesis of triglycerides and synthesis of phosphatides from the

common intermediate. In addition, the fatty acid compositions of milk triglycerides and phospholipids are similar (Patton, Durdan & McCarthy, 1964).

Glyceride synthesis also occurs outside the secretory cell. McCarthy & Patton (1964) reported fatty acid esterification in freshly secreted milk and later, Patton, McCarthy & Dimick (1965) found that these reactions had occurred in a particulate fraction comprised of lipoprotein and having properties similar to mammary gland microsomes.

1.6 Milk Fat Secretion

The milk microsomes responsible for lipogenesis in fresh milk display a phospholipid composition similar to the other phosphatide fractions of milk. Patton et al (1964) proposed that these were part of a membrane, possibly that of the fat globule, and possibly derived from the apical membrane of the secretory cell during the process of fat droplet secretion. Parsons & Patton (1967) separated the polar lipids from milk and mammary tissue and found considerable differences in their compositions. The higher content of phosphatidyl ethanolamine, cerebrosides and sphingomyelin in the milk lipid suggested to them that the phosphatides represented material picked up by the droplet specifically as it left the cell. Evidence from electron micrographs of lactating rat mammae obtained by Bargmann & Knoop (1959) indicated that fat secretion involved an enveloping of the fat droplet by the apical membrane of the cell followed by a pinching off into the alveolar lumen. There was some evidence of intracellular material escaping along with the droplet. Similar photographs can be seen in articles by Feldman (1961) in bovine tissue, Sekhri, Pitelka & DeOme (1967) and Stein & Stein (1967) in mouse tissues. Stein & Stein claim that the droplets form in cisternae of the endoplasmic reticulum and that remnants of the reticulum with attached and free microsomes were visible in the alveolar lumen. If this was not an artifact of preparation, it firmly established the identity of the milk microsomes with those of the endoplasmic reticular system.

1.7 Summary

Milk fat contains fatty acids of long chain length derived from blood lipids, and others of shorter chain length which are synthesised within the gland.

Synthesis within the gland appears to involve two major pathways although differences between the two are not fully resolved.

Microsomes are the major regulatory units participating in glyceride synthesis and the control of fatty acid synthesis. They contain enzymes involved in desaturation reactions of fatty acids and some evidence indicates that such reactions may be confined to the acids reaching the cell preformed.

Chain elongation of palmitic acid, either preformed or synthesised de novo is insignificant.

Milk glycerides are synthesised by microsomal enzymes within and without of the secretory cell.

The process of fat secretion is accompanied by a loss of cytoplasmic material, including enzymes, into the alveolar lumen.

Of all the components secreted in milk, least is known of the proteins. McKenzie (1967), in an historical treatment of the study of milk protein has suggested that difficulties in this field are due in part to the lack of standardization of preparative and analytical techniques and thus to no systematic nomenclature until the present decade.

1.8 Protein Synthesis

The proteins of milk include fractions transferred from the blood along with those synthesised de novo within the gland. Most obvious of the former group are the immunoglobulins, serum albumin and the transferrins. In addition, lactollin and some of the milk enzymes including one of the ribonucleases may belong to this group. The proteins synthesised de novo include α-lactalbumins, the β-lactoglobulins, α_{s1}, β, γ, & κ caseins and another group of the milk enzymes. Evidence for the presence of genetic variants of the proteins, has given support to the theory of genetic direction of protein structure and prompted the development of rapid analytical techniques. Although initially qualitative, these electrophoretic methods have been modified such that individual fractions can now be measured quantitatively - this represents a first step in studying the regulation of synthesis of the milk proteins. (see e.g. Larson 1969).

The only aspect of de novo synthesis of milk proteins known with any certainty is that blood free amino acids are the principle substrates. Barry (1964) provides a tabulation of blood amino acid levels and a list of the 'essential' and 'non-essential' acids on which mammary arterio-venous differences have been measured. Other experiments with labelled substrates (Kronfeld, 1963) indicate that non amino compounds can contribute carbon skeletons to milk proteins, probably after synthesis of 'non-essential' amino acids. The biochemistry of protein synthesis within the gland has not been fully characterised although studies employing antibiotics (see below) suggest that the process in mammary tissue is similar to the better understood microbial system. The steps which have been recognised

deserve description because they may offer suitable assays for protein synthesis.

Enzyme bound amino acids are activated by an adenosyl monophosphate transesterification from ATP to the carbonyl function of the acid. Obligate requirement for ATP and Mg^{++} has been demonstrated. The stable complex formed undergoes a reversible transfer reaction involving the carbonyl group of the activated acid and the 2' or 3' hydroxyl of the terminal adenosine of the appropriate transfer ribonucleic acid (t-RNA). This yields AMP derived from the original ATP employed. Turba & Hilpert (1961 a, b & c) have identified these steps in lactating mammary glands from guinea pigs. The attraction by ribosomes for t-RNA-amino acids and subsequent incorporation to proteins was found to increase 20 fold from low levels in pregnancy to mid lactation (Baird & Herriman, 1968).

1.9 Initiation of Protein Synthesis

Recent advances in the study of milk protein synthesis have been made with the use of organ and tissue cultures of mammary glands. Juergens, Stockdale, Topper & Elias (1965) found p^{32} incorporation into casein to be 4 - 8 times greater in tissue from virgin or mid-pregnant mice when cultured in media containing insulin, prolactin and a corticoid than when cultured in media containing the hormones singly or in pairs. Turkington & Topper (1966) showed that human placental lactogen could substitute for prolactin and with pulse labelling with tritiated thymidine found the response in casein synthesis to be independent of additional DNA synthesis. Tissue from pregnant mice was more responsive than that from virgins. The study of Stockdale & Topper (1966) demonstrated that non-lactating mammary tissue was required to undergo mitotic proliferation in the early stages of culture before prolactin could stimulate casein synthesis. Whilst mitoses occur during culture in medium containing insulin alone, the ultimate differentiation of daughter cells, as adjudged by histological examination and the capacity to respond to prolactin with increased casein synthesis was absolutely dependent on exposure to the corticoid during the cell divisions (Lockwood,

Stockdale & Topper, 1967).

In similar cultured preparations, Turkington (1968) found the synthesis of 'lactalbumin' and 'lactoglobulin' isolated by fractional precipitation with $(NH_4)_2 SO_4$ to be similarly controlled. Daughter cells formed under the influence of insulin and a corticoid responded to prolactin or human placental lactogen by doubling the synthesis of RNA. Actinomycin D completely inhibited this response and casein synthesis fell to levels lower than in tissues exposed to insulin and corticoid throughout. Colchicine added in the initial culture period blocked cell division and hence any response to prolactin, but if added in the post-mitotic phase was without effect on the response to added prolactin.

These studies have provided a basis for further more careful work using both the culture technique and studies in vivo. Methods are now available to measure individual RNA species and milk proteins and if coupled with labelled substrates would enable the measurement of synthetic rates of all the components. Strains other than the C3H which cannot be considered a representative mouse strain (Nandi & Bern, 1960; Rivera, 1966), and even other species of mammals should be studied.

1.10 Summary

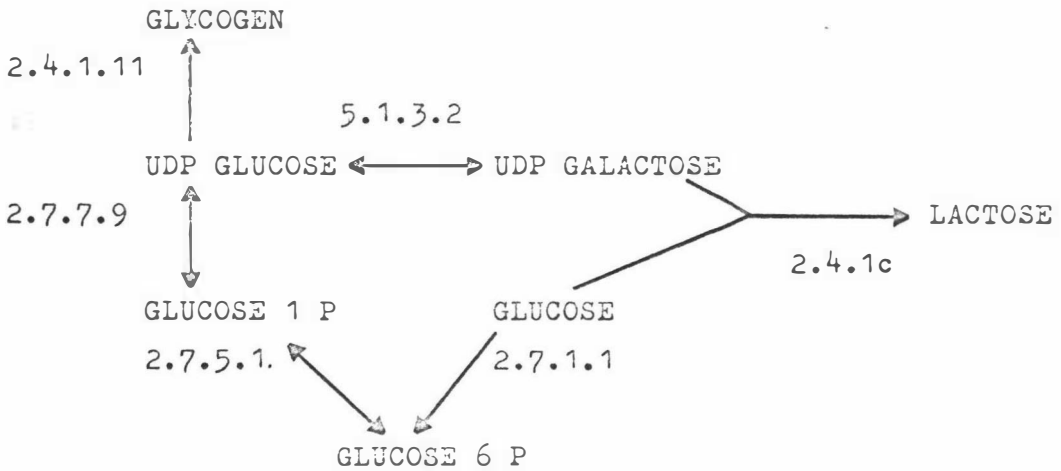
Milk proteins include some serum proteins and others synthesised de novo by the mammary gland.

The synthetic mechanisms appear similar to those of microbial species.

Non lactating tissue cultured under appropriate conditions differentiates into cells capable of de novo protein synthesis.

1.11 Lactose Synthesis

Mammary tissue contains little glycogen (Twarog & Larsen, 1964) yet the full enzymatic complement including UDPglucose: glycogen--4-glucosyltransferase (EC 2.4.1.11) necessary for its synthesis is present (Mendicino & Pinjani, 1964). The synthesis of glycogen and lactose follows a common route to the metabolite UDPglucose.



The presence of UDPglucose-4'-epimerase (EC 5.1.3.2) producing UDPgalactose competes with glycogen synthetase for available UDP-glucose. The absence of glycogen in the lactating gland may simply reflect this competition.

Lactose is synthesised by a galactosyl transfer from UDPgalactose to glucose. That glucose and not glucose-1-P was the acceptor lay in dispute (Leloir & Cardini, 1961) until Watkins & Hassid (1962) found no evidence of the formation of lactose-1-P an intermediate postulated for the pathway of Gandar, Peterson & Boyer (1957). Watkins & Hassid found greatest synthesis of lactose occurred when homogenates of bovine or guinea pig mammae were incubated with UDP-galactose and glucose. The same authors found galactosyl transfer to N-acetyl-D-glucosamine producing a compound they tentatively identified as N-acetyl lactosamine. Bartley, Abraham & Chaikoff (1966) have confirmed that glucose is the galactosyl acceptor in lactating rat mammary tissue.

UDPgalactose:D-glucose-1-galactosyltransferase (EC 2.4.1c) or lactose synthetase has been extensively studied since 1962. Watkins & Hassid (1962) located the enzyme in microsomes and Babad & Hassid (1964) as a soluble enzyme in milk. Brodbeck & Ebner (1966 a) established that the enzyme comprised two proteins termed by them A and B. Palmiter (1969) claims he has identified a third component (Z) which contributes to the stability of the A-B complex but this awaits confirmation. Brodbeck & Ebner further found that the microsomal enzyme was stimulated by adding B protein from milk and proposed that, in microsomes, the B protein was limiting. Brodbeck & Ebner (1966 b) tabulate the distribution of the two proteins in bovine and rat mammary extracts and in bovine milk. The A protein is 70% microsomal and the B protein 30 - 40% microsomal. The bulk of the remainder was soluble. In milk, the enzyme (A + B) is 99% soluble and a trace can be located in milk 'microsomes'. Studies on the B protein were stimulated by the finding that α-lactalbumin could substitute for it in activating the A protein (Ebner, Denton & Brodbeck, 1966). On the basis of several protein analytical criteria, Brodbeck, Denton, Tanahashi & Ebner (1967) established that the B protein and α-lactalbumin were identical.

Further studies on the A protein revealed that in the absence of the B, it catalyses the galactosyl transfer to N-acetyl-glucosamine (Brodbeck & Ebner, 1966 a) producing N-acetyl-lactosamine as suggested earlier by Watkins & Hassid (1962). Brew, Vanaman & Hill (1968) note that this enzyme is distributed widely in mammalian tissues including the prepartum mammary gland and that its normal function is in glycoprotein synthesis. Glycoproteins containing lactosamine moieties are found in colostrum.

1.12 Initiation of Synthesis

Prior to the routine measurement of lactose synthetase in mammary tissue extracts, the enzyme UDPglucose-4'-epimerase was considered rate limiting for lactose synthesis. However all enzymes of the pathway (see above) save the synthetase are present at levels able to be measured <u>in vitro</u> before parturition (Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967; Heitzman, 1968). In view of this, Kuhn &

Lowenstein were first to state:

> "Unless a very rapid removal of lactose is postulated, alternate reasons are required to explain its absence before parturition."

Kuhn (1968) was unable to detect sufficient lactose hydrolysing activity to account for such a turnover, he did however find the terminal enzyme - lactose synthetase- to be rate limiting both before and after parturition. In such tissue there was a consistent response *in vitro* to added α-lactalbumin, this being maximal on the day of parturition. The disparity noted suggested that the B protein itself was limiting, especially at parturition.

Lockwood, Turkington & Topper (1966) found that the hormonal conditions essential for lactogenesis in hypophysectomised rodents *in vivo* (Lyons, Johnson & Li, 1957) and in pregnant tissue *in vitro* (Jeurgens, Stockdale, Topper & Elias, 1965) were also responsible for controlled synthesis of α-lactalbumin in mouse pregnancy mammary tissue cultured *in vitro*. Turkington, Brew, Vanaman & Hill (1968) determined the levels of the A & B proteins in tissue developed *in vivo* and found the disparity in favour of the A protein throughout the lactation cycle but most marked just prior to the day of parturition. At parturition there was a rapid induction of the B protein to 75% that of the A, thereafter both fractions increased at a similar rate to the fifteenth day of lactation.

Turkington *et al* determined the kinetics of induction of the two proteins *in vitro* and found that as for other milk proteins, induction in response to prolactin occurred only if prior mitoses had occurred under the influence of a corticoid. Prolactin added to such preparations induced both proteins of the enzyme, and over the range of concentrations used, the A protein responded to a greater extent than the B. No plausible explanation for the rapid and specific induction of the B protein was offered.

Recent independent work with tissue developed both *in vivo* and *in vitro* suggested that progesterone levels in blood may participate

in the events initiating lactation. Turkington & Hill (1969) found addition of progesterone to media containing differentiated mammary cells blocked the normal induction of α-lactalbumin by prolactin. The treatment was without effect on the A protein of lactose synthetase except at extremely high dose levels. RNA synthesis was reduced and the authors consider the results show a specific effect of the hormone acting on nucleic acid transcription. Oestradiol, over a wide range of doses did not affect either of these actions of prolactin.

Kuhn (1969) determined that the appearance of lactose in mammary tissue coincided with a prepartum fall in blood progesterone associated with increased ovarian 20 α-hydroxysteroid dehydrogenase activity about 30 hours before parturition. Furthermore, progesterone blocked the appearance of lactose caused by ovariectomy, hysterectomy or ovarhysterectomy of pregnant rats.

These studies show progesterone to have a local action at the mammary gland in blocking the lactogenic response to prolactin. The author is not aware of any evidence for progesterone interfering with prolactin's mammogenic actions, and mammary development during pregnancy bears witness to the secretion of the hormone. The initiation of lactation may then result from the withdrawal of progesterone's antagonism of the lactogenic action of prolactin without necessarily influencing the secretion rate of prolactin as in the theories of Meites (1954) and Folley (1956). Whatever the stimulis for the induction of α-lactalbumin, the presence of a specifier protein and the change in galactosyl acceptor it effects, resulting in the biosynthesis of lactose, represents at present a unique biological control mechanism.

1.13 Summary

Lactose synthesis involves free glucose as a galactosyl acceptor from UDPgalactose.

The rate-limiting step at least at lactogenesis is the activity of lactose synthetase.

Lactose synthetase contains two proteins, one of which is the milk protein αlactalbumin. This protein is limiting for the synthesis of lactose and when present causes a change in galactosyl acceptor from N-acetyl glucosamine to glucose.

Initiation of lactose synthesis follows the pattern of events described for non-enzyme milk proteins.

A fall in blood progesterone levels has been suggested as a specific lactogenic signal *in vivo*.

Changes in the Composition of Milk Fat

This section is concerned with developing a theme for the discussion of milk fat fatty acid compositions in light of physiological and biochemical changes within the animal. The numerous chemical studies on milk fat will not be discussed but reference is made to the classical work of Hilditch (1956) for an exhaustive coverage of early research in this field, to Garton (1963) for a summary of early gas chromatography studies presented along with a listing of all fatty acids positively identified in milk fat and to Jensen & Sampugna (1966) for a review on the triglyceride structure of milk fat and a fair criticism of the methods in use for its study.

Little data on whole lactation changes in milk fat properties of individual cows are available. In New Zealand, information is restricted to composition data on bulked factory material and to a variety of studies on fat properties from part lactations conducted at the Dairy Research Institute.

1.14 Nutritive Factors

Hansen & Shorland (1952) collected fat samples from factories over a dairy season and found a distinct pattern of compositional changes. There was a gradual rise in the content of short and medium chain saturated acids from spring through early summer, a plateau and a fall to the end of the season. Long chain unsaturated acids varied in the reverse direction. Bulked data such as this does not permit a separation of seasonal and stage of lactation effects. Hawke (1963) determined the fatty acid compositions of butterfat from twin cows fed H1 ryegrass at two stages of maturity. Cows fed mature herbage had a lower content of long chain unsaturated acids and the saturated acids 14:0 and 16:0 were higher compared with cows fed young vigorously growing grass. The pattern was consistent at three stages of the season studied. Milk fat changes largely reflected changes in the content of long chain unsaturated acids in the lipids of the herbage.

An attempt to distinguish seasonal from stage of lactation effects was made by McDowall (1962). He measured butterfat properties in fat collected from early and late calving twin pairs grazed together during the experimental period. Results were obtained for individuals but averaged for presentation. The changes observed in all properties - iodine value, refractive index, saponification value, Reichert value and softening point indicated to the author that they arose mainly from seasonal causes and he suggested that these were probably variations in quantity and quality of herbage available to the cattle. High iodine and low Reichert and saponification values from the earlier calving cattle suggested considerable use of body reserves for fat production. This effect was consistent throughout the lactation and similar in three successive seasons. McDowall, without the aid of statistical analysis, discounted stage of lactation effects in favour of direct nutritive effects and did not consider other indirect causes which could affect all animals on a given day of sampling. The "stress" effects discussed in 4.2.3 could bias results in a manner that appears related to season. Other indirect effects operating through lipolytic mechanisms possibly interacting with stage of lactation can give rise to changes in fat properties which might be confused with direct effects from herbage lipid composition. The changes observed in cattle fed "fat depressing diets" contributes one of these indirect effects. Milk fat from these cattle is characterised (McCarthy, Dimick & Patton, 1966) by a high content of short chain fatty acids and low levels of acids derived from blood (see 1.7). Blood lipids showed generally low levels of stearic acid. Confirmation of these results was provided by Opstevdt, Baldwin & Ronning (1967) and Baldwin, Lin, Cheng, Cabrera & Ronning (1969) who found increased adipose glyceride synthesis and a net shift in fatty acid equilibrium away from lipolysis. A possible explanation arises from the "fat depressing diet" raising blood glucose and/or insulin levels (McClymont & Vallance, 1962) and inhibiting lipolysis, resulting in a "deficiency" of stearic acid at the mammary gland and reduced milk fat content.

Fatty acid compositions of milk fat bulked from nine cows fortnightly through a whole lactation are provided by Decaen and Adda (1966). Cows were housed and fed a consistent diet varying in quantity according to production. Their results were tabulated as weight percentages and for comparison with this experiment, a selection of the acids are presented as molar percentages in Fig. 1.2. The steadiness of the changes in components probably results from the controlled conditions of feeding and from the bulking of fat from several animals. Falls in the molar content of octadecanoic (18:0) and octadecenoic (18:1) acids follow closely the loss in bodyweight. On the other hand, the proportional rise in all acids of carbon chain length less than 16 parallels the rise in total D.M. intake. The results suggest that, at least over the first weeks of lactation, the long chain acids reflect body fat mobilization and the shorter acids an increase in the availability of substrates to the mammary gland. The authors calculated that about 50% of the fatty acids in milk fat were derived from the blood lipids or body reserves. This ratio agrees with an average value of 1 lb body weight loss for every 2 lbs extra milk fat in monozygous twin studies at Ruakura (Phillips, pers comm). The possibility that post partum mobilization of depot fat may be a causal factor in the physiological depression of appetite at this time has not yet received serious study.

Stull, Brown, Valdez & Tucker (1966) measured fatty acid proportions in milk fat bulked from four cows and fitted polynomial regression lines to the individual components. The proportions of total variance accounted for by the lines were generally low and the absence of either the raw data or a satisfactory discussion of their findings prevents a consideration of why their results differed from those of Decaen & Adda.

1.15 Endocrine Factors

The lack of information on the effects of hormones on milk fat composition makes difficult any attempts to separate direct and indirect (see above) nutritive effects. The possibility of

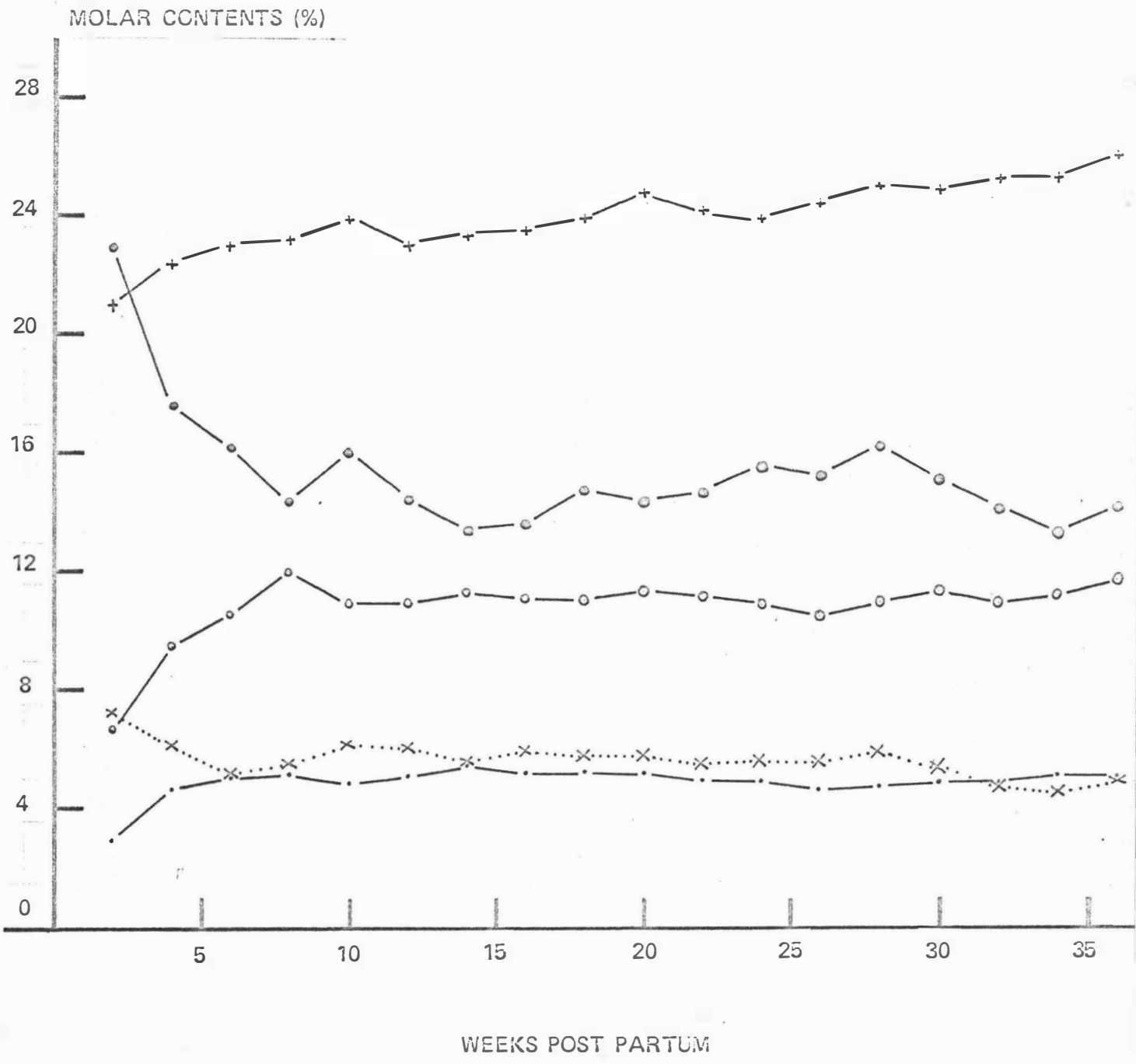


Fig. 1.2 Milk Fat Fatty Acid Molar Proportions from the data of Decaen & Adda (1966).

o 18:1, × 18:0, + 16:0, o 14:0, • 12:0

pregnancy and its associated hormonal changes influencing milk fat composition has not been studied. However, Aurand, Singleton, Ulberg & Britt (1968) observed changes during an oestrous cycle and were able to mimic them in an ovariectomised cow by injections of oestrogen but not progesterone. They found that oestrous and oestrogen increased the content of unsaturated and branched chain acids in milk and concluded that the effects noted result from an influence on the blood lipids. Hutton (1958) suggested that changes in milk composition in late lactation could reflect a gradual increase in the level of circulating oestrogen. The effects on milk fat properties or fatty acid compositions were not studied in either of the experiments where oestrogen (Hutton, 1958) or growth hormone (Hutton, 1957) caused changes in milk fat secretion. Cattle fed thyroprotein (Stanley & Morita, 1967) lost body weight and produced milk with elevated fat contents. High contents of stearic and oleic acids in the fat reflected body fat mobilization. The only effects of ACTH on milk fat composition yet reported come from the data of Campbell, Davey, McDowall, Wilson & Munford (1964). ACTH treatment of lactating cattle caused an increased fat content which was accompanied by a drop in iodine value. Milk yield was depressed to a greater extent than was fat yield suggesting little change in total fat synthesis despite a change in fat composition.

1.16 Interpretation

The major difficulty in the use of determination of fatty acid compositions to explain differences in metabolism lies in the statistical problems of analysing multiple components showing a high degree of interdependence. Unless provision is made for the correlations among components, quite misleading inferences could be drawn from changes in one or more acids. The difficulties can be overcome if an independent variable can be introduced as in the case of daily yields of fatty acid components. Changes in one or more yields could then be related more reliably to metabolic changes. Where insufficient data are available to clearly establish the changes in fatty acid yields, individual components could be analysed as single variables and all components considered together in drawing inferences from the analyses.

1.17 Summary:

When variations in herbage lipids are reduced by standardising diets, there are still long term changes in milk fat composition probably arising from physiological changes within the animal with advancing lactation.

Long term changes appear to involve mobilization of body lipid reserves immediately post partum and a less well defined effect of steroid hormones with advancing pregnancy.

Short term variation under field conditions probably reflects changes in the composition of dietary lipids although other factors of the diet can influence milk fat composition possibly through physiological adjustments within the animal.

Care is required in the analysis and interpretation of differences in composition. When daily yields of fatty acids are considered, there is some improvement in the validity of comparisons as the interdependence of components is removed.

Chapter Two. METHODS

2.1 Design

The experiment was designed to provide information on variation in mammary gland biochemical parameters, milk yield and composition and milk fat composition arising from differences between cows, stages of lactation and seasons of the year. Preliminary work indicated that a maximum of four cows could be handled in this study so in order to separate stage of lactation and seasonal effects, two early and two later calving cows were chosen. Milk samplings were planned to coincide with routine 'herd testing' and surgical samplings were allocated at 28 day intervals such that production was measured 7 days before and after each set of operations.

Six mammary enzymes were chosen for study (Fig. 2.1). Acetate thiokinase (1) regulates the activation of acetate and ruminant mammary tissue preferentially uses acetate as a lipogenic substrate. There is no known physiological regulation of this enzyme. Acetyl CoA carboxylase (2) is the principle focus of lipogenic control (see 1.1). Hexokinase (3) participates in glucose metabolism and hence indirectly in glyceride, protein and lactose synthesis. Since glucokinase is not present in ruminant tissue, hexokinase alone regulates the entry of glucose into the cell. Glucose - 6 - phosphate dehydrogenase (4) contributes to the control of glucose - 6 - phosphate utilization between lactose synthesis, Embden-Meyerhoff glycolysis and oxidation in the pentose cycle. The latter provides reduced nicotinamide coenzymes involved particularly in lipogenesis. UDP glucose - 4' - epimerase (5) was considered rate limiting for lactose synthesis at the time this study began. The enzyme regulates the penultimate step in lactose synthesis (see 1.11) and is now regarded as being secondary to lactose synthetase in importance. Lactose synthetase could not be conveniently assayed with the facilities available. Glycerol phosphate dehydrogenase (6) serves in the provision of glycerol phosphate for glyceride synthesis (see 1.5) and through its regulation of triose phosphate levels, it may participate in the control of glycolysis and energy metabolism.

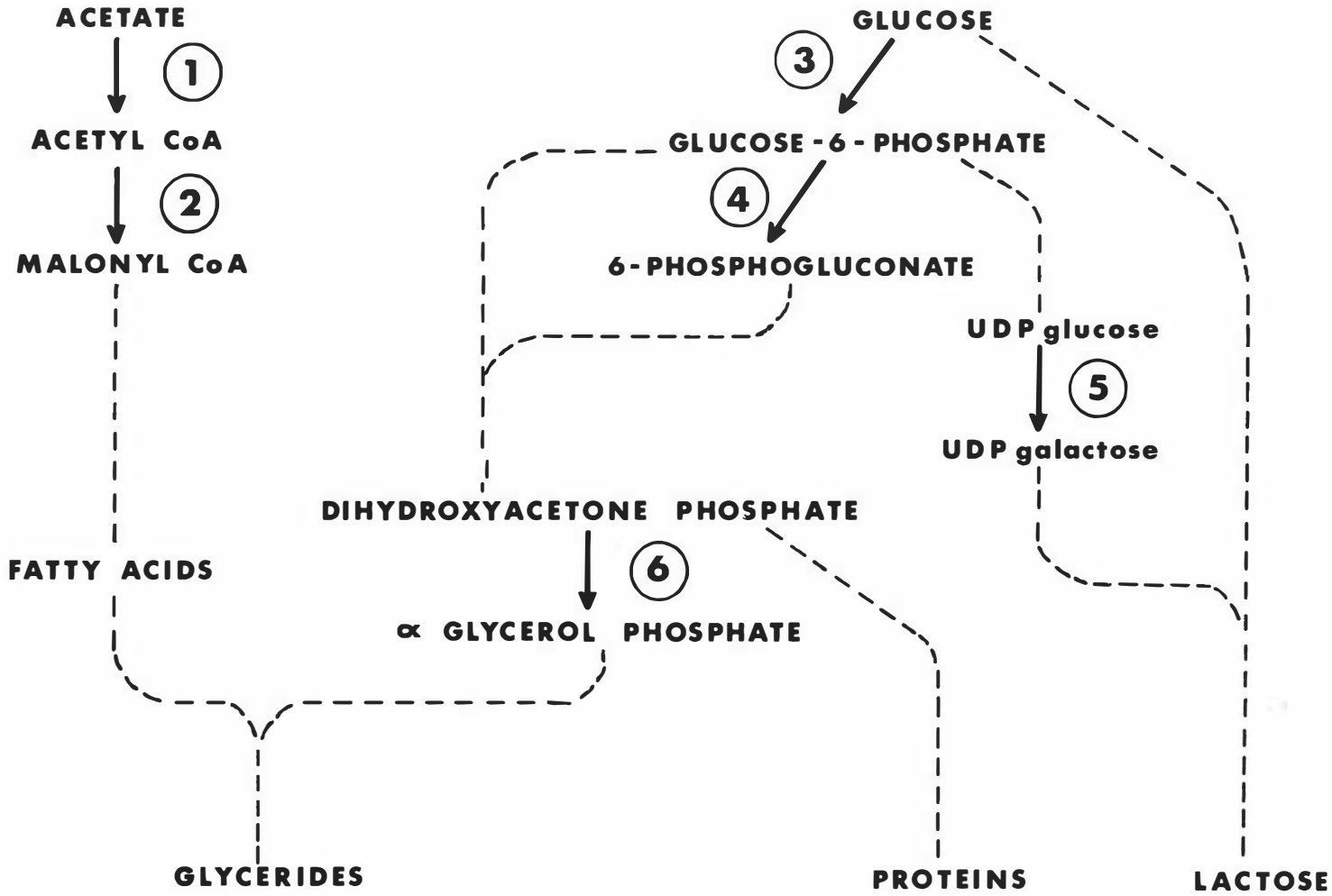


Fig. 2.1 Schematic representation of the synthetic pathways and the enzymes chosen for study in bovine mammary tissue.

1. Acetate:CoA ligase (AMP) - (EC 6.2.1.1)
2. Acetyl CoA:CO_2 ligase (ADP) - (EC 6.4.1.2)
3. ATP:D-hexose-6-phosphotransferase - (EC 2.7.1.1)
4. D-glucose-6-phosphate:$NADP^+$oxidoreductase - (EC 1.1.1.49)
5. Uridyldiphosphoglucose-4'-epimerase - (EC 5.1.3.2)
6. L-glycerol-3-phosphate:NAD^+oxidoreductase - (EC 1.1.1.8)

In this type of study, it was desirable that variations within the udder at any one time should be accounted for. However, multiple samplings both within and between quarters were not possible in the present experiment, (a) because the time involved in surgery and biochemical analyses limited the total number of samples that could be handled and (b) because reliable milk data was essential. Whilst the biopsy operation had a transitory effect on production in the given quarter, overall production was little influenced. This could not be guaranteed with multiple samplings at one period in time repeated throughout the lactation.

2.2 Cows and Management

Four cows were made available for the experiment from the Reserve Herd of pedigree Jerseys at No. 3 Dairy Unit, Massey University. Cows 19 and 118 were three year olds and calved on 26-7-68 and 24-7-68; cow 5, a six year old, on 18-9-68 and cow 26, a four year old, on 30-9-68.

The four experimental animals grazed with their herd throughout the lactation spending 24 to 48 hours in paddocks grazed rotationally. Although the seasonal supply of pasture was erratic, the only period of critical underfeeding occurred over the last two months of the experiment.

Cows were inseminated from mid October; 19 held to the third service (26-12-68), 118 to the first (17-10-68), 5 and 26 were inseminated three times but were not pregnant when tested in the Autumn.

The experiment was conducted from August 1968 until March 1969 when the grazing situation deteriorated and the herd was dried off.

2.3 Surgical Biopsy

2.3.1 Surgical Method

Cows were restrained in the bails with a leg-rope and tranquilized with Acetylpromazine (Boots) administered intramuscularly at 25 - 40 mg acetopromazine base per 100 lbs. liveweight. The udder and hindleg on the selected side were thoroughly washed with Zephiran (Bayer) and the operative site was shaved. All biopsies were performed on forequarters in a region dorsal to the gland cistern, located by palpation (Fig. 2.2).

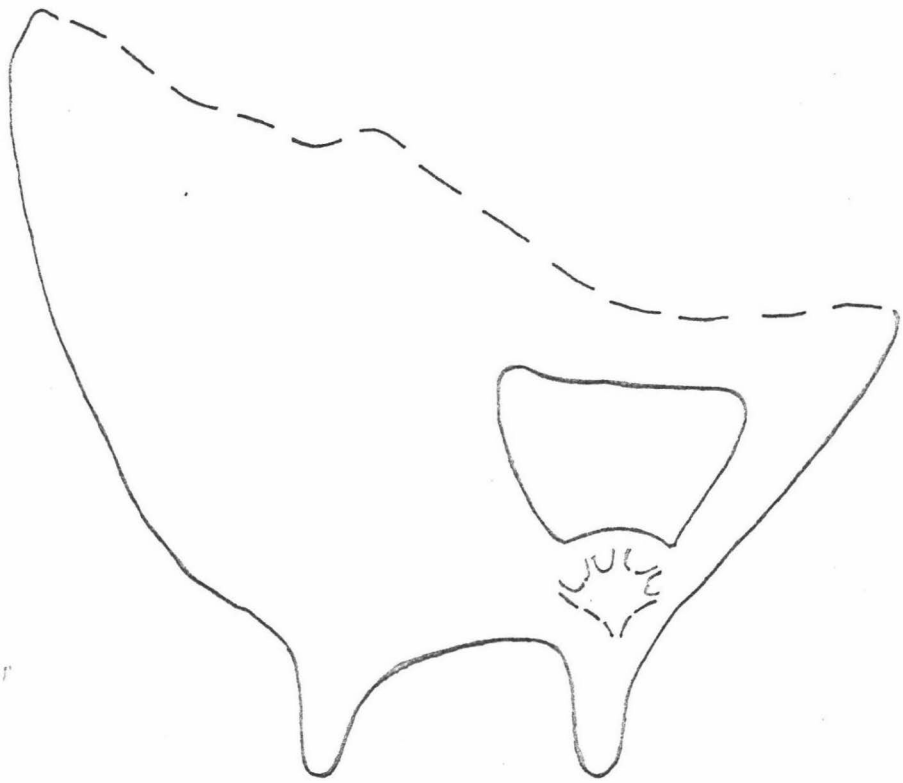


Fig. 2.2 Representation of the right udder surface showing the region sampled in relation to the forequarter cistern.

The dorso-rostral periphery was avoided as large vessels were encountered in this region in preliminary work. (The large anterior ventral mammary vein - Swett & Matthews (1949) traverses this area).

The shaved area was painted with tincture of Zephiran and local anaesthesia induced by subcutaneous injection of Xylocaine (Astra), a 2% (w/v) lignocaine solution free of adrenaline.

A vertical skin incision (4 - 5 cm) was made and the fascia (lateral suspensory ligament) exposed by dissection with sharp pointed scissors. Large subcutaneous vessels were easily avoided and small bleeders ignored. A purse-string gut suture (Atraumatic T-3 Davis & Geck) was used to anchor the connective tissue to the fascia on the periphery of a retracted circle about 1.5 cm in diameter. Care was taken not to suture the immediate subcutaneous connective tissue as this interfered with closure of the skin incision. A small vertical cut was made in the fascia within the retracted area and the sharpened end of a tungsten-chromium-steel canula (0.8 cm i.d. x 15 cm long), sharpened with fine emery paper, was applied to the exposed parenchyma. The canula was advanced 5 - 6 cm into the gland using a twisting action with gentle pressure. Upward traction on the canula followed by its withdrawal usually resulted in the tissue core being removed in the instrument (Plate I). If not, the core was readily grasped with forceps and severed at the base with fine pointed scissors.

At this stage there was mild bleeding which was routinely controlled by packing the wound tract with an absorbable cellulose haemostat (Surgicel - Johnson & Johnson). The haemostat and any residual bleeding were contained within the secretory mass by tying off the purse-string suture. Occassionally, an additional gut suture was tied across the knot of the former as reinforcement.

The wound was lightly sprayed with Rikospray (Riker), the incision closed with 4 - 5 michel clips (19 mm) and liberally dusted with Aureomycin powder (Cyanamid) (Plate II).

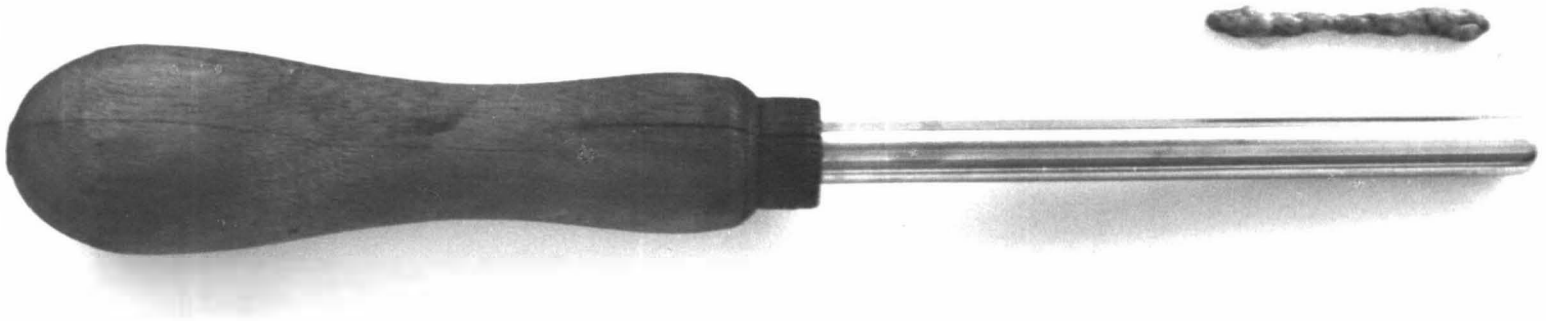


Plate I The canula used and a freshly obtained tissue sample.

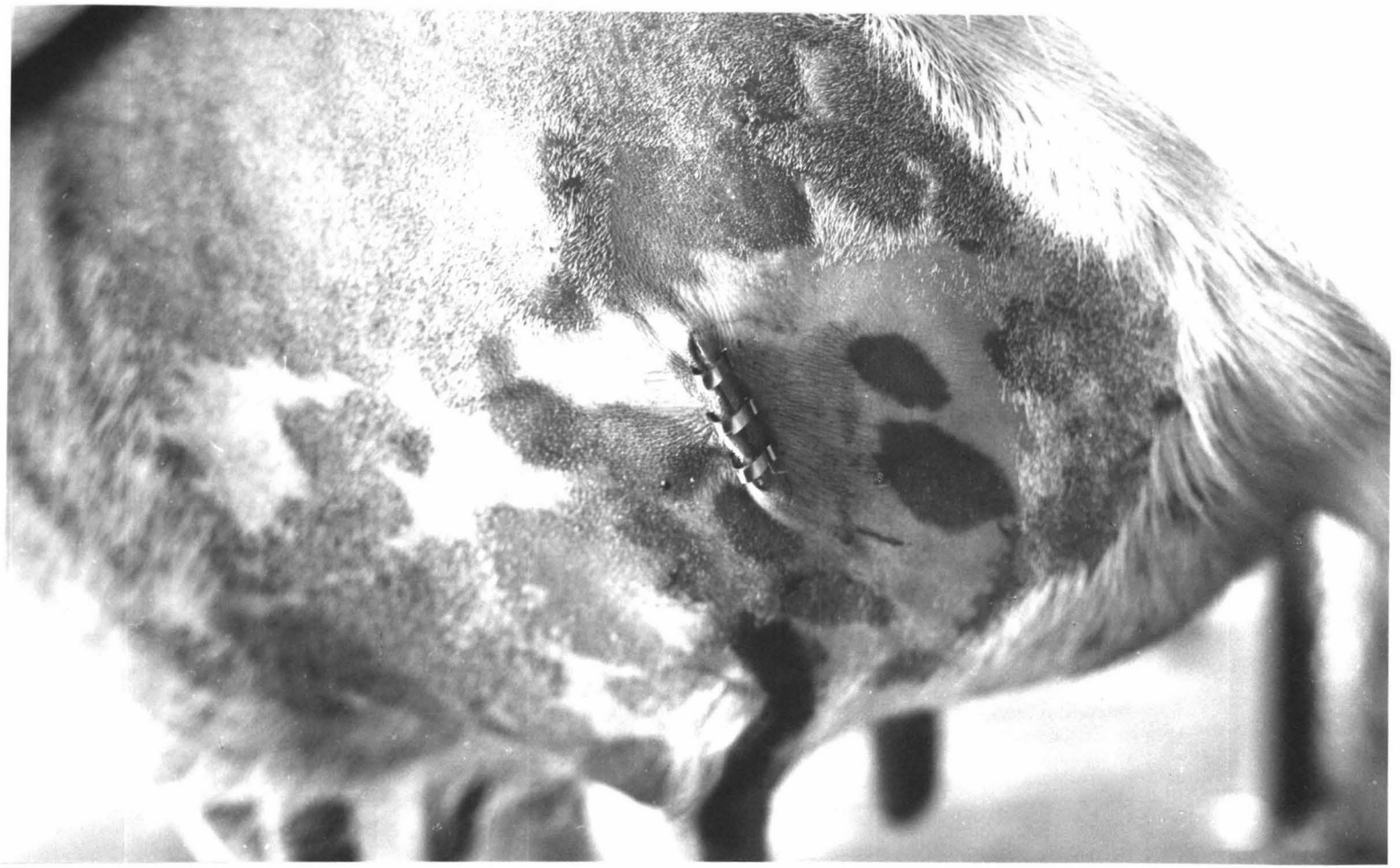


Plate II The closed incision showing two previous sites above.

Mixed duration antibiotic supplying 2 megaunits of benethamine benzylpenicillin, 2 megaunits of sodium benzylpenicillin and 1 megaunit of procaine penicillin (Triplopen - Glaxo) was injected intramuscularly.

Animals were returned to pasture and joined with their herd at the afternoon milking.

2.3.2 Post Operative Care

The cows were machine milked with sampling buckets for the three days when antibiotics might be expected in the milk. Normal milking was then resumed unless any evidence of bleeding or infection was noted and in these cases, a quarter milking bottle was used on the machine. Mastitis was treated with commercial penicillin preparations for intramammary application.

Clips were removed and the wounds dressed with Aureomycin powder 10 - 12 days following operations.

2.3.3 Effect of Operations on Milk Production

Milk yields were recorded at PM/AM milkings on January 15/16, 22/23 and 29/30 using testing equipment described in 2.5.1. Cows 19 and 118 were biopsied on January 23 and cows 5 and 26 on January 24. On January 26/27, yields were determined by weighing the contents of sampling buckets used for milking. Composite milk samples were collected on 15/16 and 29/30 and analysed as described in 2.5.1.

2.4 Biochemical Analyses

2.4.1 Tissue Preparation

The tissue cores were lightly blotted on paper surgical drapes and stored in glass vials under ice pending transport to the laboratory. Samples were weighed in chilled, tared vessels and finely minced with scissors into 10 ml. vortex beakers (M.S.E.) containing about 8 mls of cold homogenising medium (Shonk & Boxer, 1964) comprising 0.15 M KCl, 0.05 M $NaHCO_3$ and 0.006 M Na_2H_2EDTA in deionised water. The contents were homogenised at full speed for 6 minutes. Any material caught on the knife was removed to the medium after 2 - 3 minutes. A further 1 ml. of medium was used to rinse the impeller shaft and knife into the beaker and the total contents were filtered through a layer of butter muslin into a graduated test-tube in ice. The filtrate was made up to 10 mls with further rinsings of the beaker and muslin with medium.

After mixing by inversion, a 2 ml. aliquot of homogenate was removed for DNA determination and the remainder was transferred to polypropylene tubes and centrifuged for 20 minutes at 3000 x g and 4°C. Fat floated to the surface and was removed by aspiration then the supernatant was transferred to a clean glass tube, stoppered and held on ice for enzyme determinations.

2.4.2 Determination of DNA Content

The procedure used was essentially as described by Munford (1963). The 2 mls of homogenate was added to an equal volume of cold 0.5 M perchloric acid (PCA) and held on ice with occasional shaking for 60 minutes. After centrifuging for 10 minutes at 2000 x g, the supernatant (acid soluble nucleotides) was decanted to a graduated glass tube. The precipitate was resuspended in 3 mls of PCA and held at 70°C with shaking for 25 minutes. The supernatant (acid insoluble nucleotides) was collected as above and the hot PCA extraction repeated twice again. The three 'hot' extracts were bulked, cooled and mixed. After noting the volume, the tubes were covered with Parafilm and stored at 4°C until colorimetric determinations were carried out.

Diphenylamine reactive material (Burton, 1956) was determined on single 2 ml. aliquots of the 'cold' and on duplicates of the 'hot' extracts. In addition, duplicate determinations were made on standards containing 0, 20, 40, and 80 μg calf thymus DNA (Koch-Light) / ml. 0.5 M PCA. Four mls of diphenylamine reagent was added to each tube, mixed and held at 37°C for 18 hours. The reagent was prepared immediately before use by adding 0.5% (v/v) 0.364 M acetaldehyde to a stock solution comprising 0.089 M diphenylamine and 1.5% (v/v) conc. H_2SO_4 in glacial acetic acid.

Colour development was measured at 600 mμ against water. All values were adjusted for the zero reading and the standards were used to construct a calibration curve by the method of least squares. Slight differences in the regression coefficients of standard curves for different months, which arose from, amongst other things, a precipitate which formed in the stock solution on extended storage, were used by adjusted readings from a common calibration equation. The concentration of acid insoluble nucleotides (DNA) was expressed on a wet tissue weight basis and the DNA content of 0.2 mls of homogenate was calculated for the expression of enzyme activities.

2.4.3 Determination of Enzyme Activities

The assays of the enzymes were conducted using adaptations of methods designed for recording spectrophotometers. Such apparatus was not available so assays were devised so that the accumulation of reaction products from fixed time incubations gave valid estimates of comparative activity. This was established in preliminary work using varying levels of homogenates of rodent and bovine mammae run for various lengths of time. In order to minimise variation arising from differences in the reaction mixtures, all labile reagents were prepared with volumetric apparatus in the week of the determinations and stored at 4 °C in the dark.

Because there were detectable differences in the extent of homogenisation and filtration, all assays were performed on cell free supernatants prepared by centrifugation.

Measurement of activity in four cases involved the development of an absorption peak at 340 mμ due to the reduction of nicotinamide coenzymes involved either directly in the reaction of interest or indirectly in a coupled indicator reaction. In the fifth case the reaction product was trapped chemically and measured as a complex in the reaction stopping mixture. Assay mixtures were preincubated without substrate for 5 minutes at 37°C, reactions were started by substrate addition at 30 second intervals and terminated as described at 30 second intervals. All assays were duplicated and blanks were provided by substituting additional buffer for coenzyme solution.

Activities were calculated in arbitary units based on optical density changes of 0.001 per minute per mg. DNA.

Acetate Thiokinase:

Acetate:CoA ligase (AMP) - (EC 6.2.1.1)

(Adapted from Jones & Lipmann, 1955)

Thickinase was assayed by the accumulation of acetyl CoA. The ester was trapped with hydroxylamine as acetohydroxamate and measured by the absorbance of the complex formed in acidified $FeCl_3$.

Assay tubes contained:

*Tris-HCl buffer pH 7.6	15 μ moles
$MgCl_2$	6 μ moles
Glutathione (GSH)	5 μ moles
ATP	20 μ moles
*CoASH	0.25 μ moles
**Hydroxylamine	100 μ moles
Supernatant	200 μ l.

* CoASH was omitted from blank tubes and buffer increased to 20 μ moles.

** Hydroxylamine reagent was prepared immediately prior to use by mixing equal volumes of 2 M KOH and 2 M NH_2OH - HCl.

Reactions were started with 20 μ moles of CH_3COONa giving a total volume of 1.0 ml. After 30 $\pm$ $\frac{1}{4}$ minutes at 37°C, 1.0 ml. of the stopping reagent (10% (w/v) $FeCl_3.6H_2O$ + 3% (w/v) CCl_3COOH in 0.66 M HCl) was added, the tubes mixed and then centrifuged for 15 minutes at 3000 x g. Colour development was complete in this time. The O.D. of the supernatants were determined at 540 mμ against a blank of 1.0 ml. of homogenising medium + 1.0 ml. of stopping reagent prepared directly after the assay reactions were halted.

UDPglucose epimerase:

Uridyl diphosphoglucose-4'-epimerase - (EC 5.1.3.2)

(Adapted from Maxwell, Karahashi & Klackar, 1962)

The epimerase was assayed by driving in reverse, coupling the production of UDPglucose to added UDPglucose:NAD^+oxidoreductase (EC 1.1.1.22) and measuring the accumulation of NADH + H^+ in the spectrophotometer

Assay tubes contained:

*Tris-HCl buffer pH 7.6	15 μmoles
*NAD^+	1.125 μmoles
**UDPglucose dehydrogenase	9 units (Strominger)
Supernatant	200 μl.

* The coenzyme was omitted from blank tubes and buffer increased to 30 μ moles.

** Indicator enzyme was prepared just before use by diluting a concentrate (600 units/ml. of 3 M $(NH_4)_2SO_4$) to 60 units/ml. with buffer.

Reactions were started with 0.45 μ moles of UDPgalactose giving a total volume of 1.15 mls. After 10 $\pm \frac{1}{4}$ minutes at 37°C, 0.1 ml. of M PCA was added, the tubes mixed and centrifuged for 15 minutes at 3000 x g. The O.D. of the supernatants were determined at 340 mμ against water. There was no detectable loss of NADH + H^+ under these conditions for at least 60 minutes.

Glycerol Phosphate Dehydrogenase:

L-glycerol-3-phosphate:NAD^+ oxidoreductase - (EC 1.1.1.8)

(Adapted from Baldwin & Milligan, 1966)

The dehydrogenase was assayed by the accumulation of NADH + H^+ which accompanies the oxidation of glycerol-phosphate to dihydroxyacetone-phophate.

Assay tubes contained:

*Tris-HCl buffer pH 7.6	30 μ moles
*NAD^+	1.125 μ moles
Supernatant	200 μl.

* In blank tubes the coenzyme was omitted and buffer increased to 45 μ moles.

Assays were started by adding 5 μ moles of DL α-glycerol phosphate giving a total volume of 1.3 mls. After 10 $\pm \frac{1}{4}$ minutes at 37°C, 0.1 ml. of M PCA was added, the tubes mixed and centrifuged for 15 minutes at 3000 x g. The O.D. of the supernatants were measured at 340 mμ against water. There was no detectable loss of NADH + H^+ under these conditions for 90 minutes.

Hexokinase:

ATP;D-hexose-6-phosphotransferase - (EC 2.7.1.1)

(Adapted from Bortz, Abraham & Chaikoff, 1963)

Hexokinase was assayed by coupling the production of glucose-6-phosphate to excess added D-glucose-6-phosphate:$NADP^+$ oxidoreductase - (EC 1.1.1.49) and measuring the accumulation of $NADPH + H^+$.

Assay tubes contained:

*Tris-HCl buffer pH 7.6	20 μ moles
$MgCl_2$	12 μ moles
ATP	40 μ moles
*$NADP^+$	0.6 μ moles
**Glucose-6-phosphate dehydrogenase	3 units (Kornberg)
Supernatant	200 μl.

* In blank tubes the coenzyme was omitted and buffer increased to 30 μ moles.

** Indicator enzyme was prepared on the day of use by diluting a concentrate (200 units/ml. of 3 M $(NH_4)_2SO_4$) to 20 units/ml. in buffer.

Reactions were started by adding 60 μ moles of glucose to give a final volume of 1.45 mls. After 10 $\pm \frac{1}{4}$ minutes at 37°C, 0.1 ml. of M PCA was added, the tubes mixed and then centrifuged for 15 minutes at 3000 x g. The O.D. of the supernatants were measured at 340 mμ against water. There was no detectable loss of $NADPH + H^+$ under these conditions for 90 minutes.

Glucose-6-Phosphate Dehydrogenase:

D-glucose-6-phosphate:$NADP^+$ oxidoreductase - (EC 1.1.1.49)

(Adapted from Glock & McLean, 1953)

The dehydrogenase was assayed by the accumulation of $NADPH + H^+$ which accompanies the oxidation of glucose-6-phosphate to 6-phosphogluconate.

Assay tubes contained:

*Tris-HCl buffer pH 7.6	25 μ moles
*$NADP^+$	0.75 μ moles
$MgCl_2$	12 μ moles
Supernatant	200 μl.

* The coenzyme was omitted from blank tubes and buffer increased to 37.5 μ moles.

Reactions were started with 2.0 μ moles of glucose-6-phosphate giving a final volume of 1.25 mls. After 10 $\pm \frac{1}{4}$ minutes at 37°C, 0.1 ml. of M PCA was added, the tubes mixed and then centrifuged for 15 minutes at 3000 x g. The O.D. of the supernatants were determined at 340 mμ against water. In preliminary investigations covering the range of activities encountered in the experiment, no reaction provided sufficient 6-phosphogluconate to cause elevated NADPH + H^+ arising from further oxidation by phosphogluconate dehydrogenase. This was found by running identical assays and substituting glucose phosphate with 6-phosphogluconate at levels calculated from NADPH + H^+ production assuming 1 μ mole of coenzyme was reduced for every 1 μ mole of substrate oxidised.

Under the conditions described there was no loss of NADPH + H^+ for 60 minutes.

Note on Acetyl CoA Carboxylase:

Acetyl CoA:CO_2 ligase (ADP) - (EC 6.4.1.2)

The assay of this enzyme was attempted with a modification of the original method of Waite & Wakil (1962), using the incorporation of C^{14} bicarbonate into malonyl CoA. The method had to be adapted to facilitate radioactivity counting in the liquid scintillation equipment available in the laboratory.

The following was used extensively in preliminary work:

Tris-HCl buffer pH 7.0	10 μ moles
Na citrate	20 μ moles
$MnCl_2$	0.3 μ moles
Homogenate	200 μ l

This was preincubated for 5 minutes at 37° and the assay reaction initiated by adding:

Acetyl CoA	40 μ moles
ATP	1 μ mole
^{14}C-$NaHCO_3$ (2 x 10^{-5} dpm/μ mole)	12 μ moles

The final volume was 0.8 ml. After 15 minutes incubation, reactions were stopped by adding 0.1 ml. PCA (varying molarity - 0.5M - 6 M). Tubes were placed in a 70°C bath for 10 minutes with shaking, chilled on ice and 0.2 ml. KCl (molarity as for the PCA) added to precipitate excess perchlorate ions. After centrifugation for 10 minutes at

3000 x g, the supernatants were treated in the following manner. Aliquots were either transferred to scintillation vials, to Millipore filters (type HAWP 13) or to 12 mm diameter discs of Whatman No. 5 filter paper and dried in a stream of hot air from a hair drier. Samples were then washed with 0.05 ml. M HCl and dried as above. The Millipore filters were rendered transparent in 0.5 ml. of Dioxane prior to the addition of Brays scintillation fluid but the other preparations were added directly.

There were major difficulties throughout these tests with excessive blank (no acetyl CoA) values, often exceeding the assay samples. In addition there was no regular pattern of activity with different amounts of homogenate. After a series of tests involving water, buffer and C^{14} bicarbonate treated as above, the problem was found to be due to incomplete removal of free bicarbonate with the treatments used.

No satisfactory method was found for preparing the supernatant for counting, but the most promising procedure involved pre-irrigating the Whatman filters with 0.05 ml. M HCl prior to the addition of 0.1 ml. of supernatant, followed by drying in a stream of hot air.

There was insufficient time to continue developing this approach, verify that residual activity was due to C^{14}-malonyl CoA, establish the linearity of the assay and organise the method for routine application. Hence acetyl CoA carboxylase was not assayed at all in the actual experiment.

2.5 Milk Production and Composition

2.5.1. Determination of Milk Production.

Milk production of the experimental cows was measured fortnightly during routine departmental herd testing. Milk was passed through a TRU TEST (Consolodated Plastics) milk meter and milk yield determined from the sample collected. Sample volume was directly proportional to total milk volume hence 24 hour composites were obtained by bulking the evening and morning samples. Compositions of the 24 hour samples were determined 3 - 5 hours after the morning milkings using a Mk 1 Grubb Parsons Infra Red Milk Analyser, calibrated to standard chemical methods (Munford, 1968).

2.5.2 Milk Fat preparation

A 25 mls aliquot of milk gently mixed at 40°C was added to 20 mls of absolute ethanol and thoroughly shaken. Fat was then extracted by shaking with 2 x 20 mls additions of peroxide-free diethyl ether. When the phases had separated, the upper etherial layers were decanted and bulked. After evaporating the ether under vacuum, the residue comprising fat, ethanol and water was transferred to a separating funnel with washings of petroleum ether (b.p. 40°C - 60°C). After gently mixing, the ether layer was separated and evaporated to near dryness, the fat washed into 25 mls polyethylene bottles with 5 mls of petroleum ether and stored at 4°C until analysis.

2.5.3 Milk Fat Fatty Acid Compositions

Total milk fats were analysed as methyl esters of the fatty acids prepared by the boron-trifluoride transesterification method of Metcalfe, Schmitz & Pelka (1966) as modified by van Wijngaarden (1967). Methyl esters in n Heptane were dried over anhydrous Na_2SO_4 and analysed on a Varian Aerograph Series 1520 gas liquid chromatograph using an 8' x $\frac{1}{8}$" stainless steel column packed with 11.5% diethylene-glycol succinate (Analab) on Chromosorb W (DCMS) 60/80 (Varian) with dry N_2 as carrier gas. Detection was by hydrogen flame ionisation.

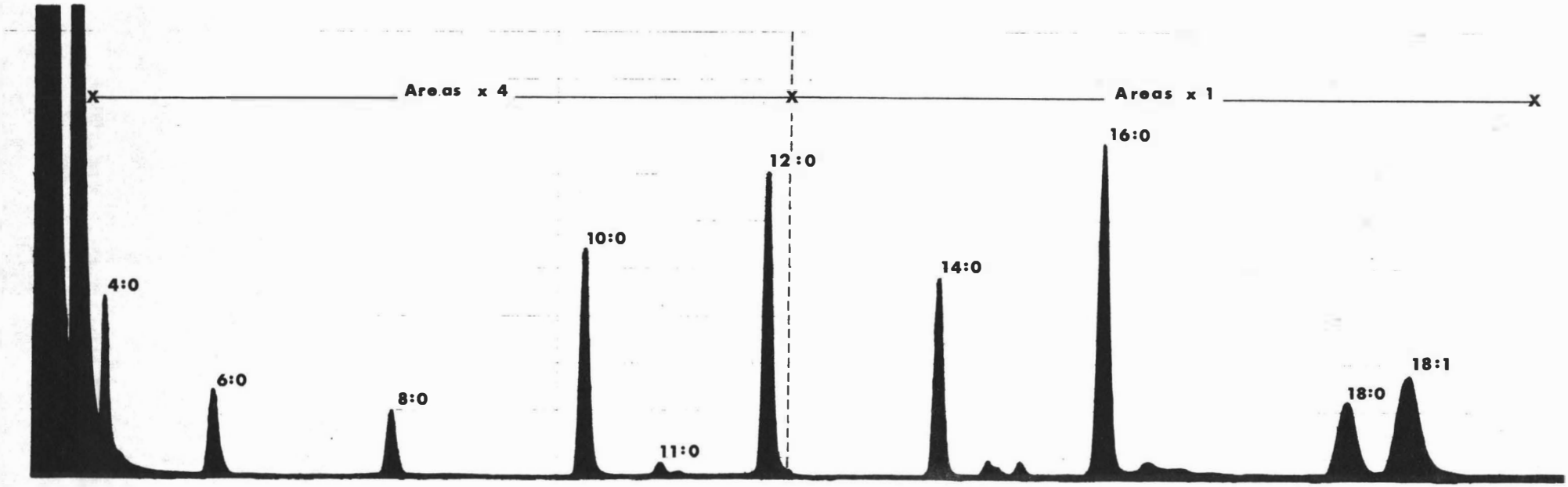


Fig. 2.3 Chromatogram of milk fat fatty acid methyl esters separated on diethyleneglycol succinate.
(Sample : Cow 19, 7 weeks post partum.)

Conditions were as follows:

Injection		235°C
Detection		200°C
Column	Initial	65°C
	Matrix programmed	2°C/min. for 6 min.
		4°C/min. for 27 min.
	Isothermal	185°C
Gas flow rates:	N_2	24 ml./min.
	H_2	29.5 ml./min.

For accuracy of measurement, recorder response was attentuated during each run (see Fig. 2.3) with due correction being applied in the calculations. Peak areas were measured by peak height x width at half height. Compositions were determined on a molar basis with no adjustment made for the slight differences in molecular weights between the methyl esters and the free fatty acids.

2.5.4 Determination of Isolated trans - Unsaturation

The procedure followed was essentially Tentative Method Cd 14 - 61 of the American Oil Chemist's Society (see Firestone & LaBouliere, 1965). Approximately 200 mg. melted fat was weighed and made up to 10 mls with spectrophotometric grade CCl_4. Solutions were injected into NaCl cells of 1 mm lightpath and scanned from 4,000 cm^{-1} against CCl_4 on a Shimadzu IR-27G infra red spectrophotometer. A baseline for measurement was scribed on charts from O.D. at 995 cm^{-1} to 937 cm^{-1}. The differences in O.D. at the peak (966 cm^{-1}) and the baseline at that wavenumber were determined and converted to absorbtivities (Fig. 2.4). These were referred to a 'Trielaidin'* standard with an isolated trans content of 79.4% scanned with each series of unknowns. For a description of the analysis employed to determine this value, see Appendix A-1.

* Kindly provided by Dr F.B. Shorland, Division of Food Chemistry, D.S.I.R., Wellington.

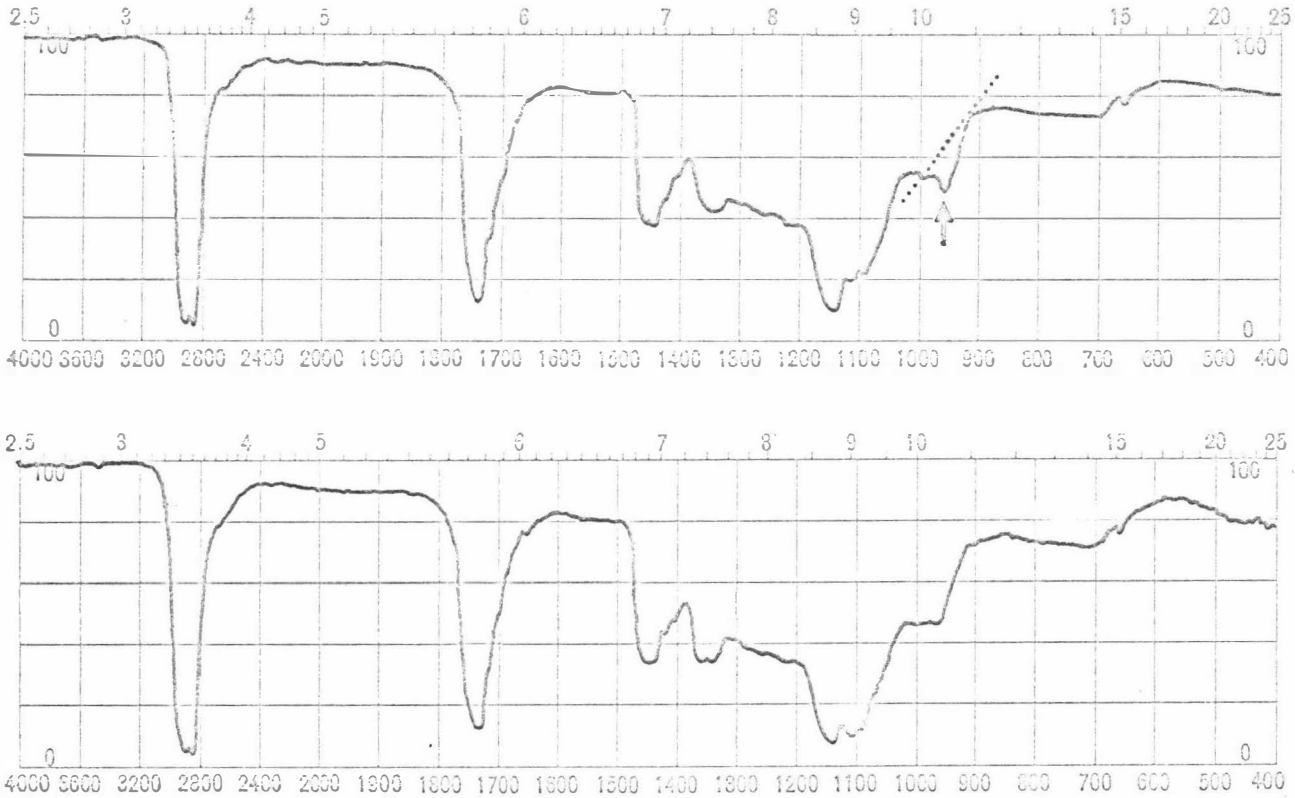


Fig. 2.4 Infra Red Spectra

Upper: Trielaidin reference with baseline indicated and absorption peak arrowed.

Lower: Milk fat sample (Cow 19, 7 weeks post partum)

2.6 Statistical Methods

Analyses of variance for unequal subclass numbers were performed by the least squares method of fitting constants (Harvey, 1960). The University Computing Centre's IBM 1620 computer and a program (Cockrem, 1968) were used for these analyses. The program enabled solution of the following three classification models and all single classification parts thereof:

$$Y_{ijkl} = \mu_{ijkl} + IA_i + K_j + L_k + e_{ijkl}$$

IA_i = effect of the i^{th} cow; $i = 1,2,..\ 4$

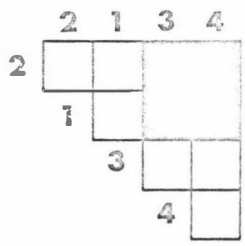
K_j = effect of the j^{th} stage of lactation; $j = 1,2,..\ 10$

L_k = effect of the k^{th} seasonal period; $k = 1,2,..\ 9$

The indices i,j, and k are identified in Table 2.1 and the Y_{ijkl} are described preceeding each tabulation of results.

To meet the program restriction of j and k not exceeding 10, pairs of milk data collected at fourteen day intervals were considered as replicates (l = 1,2) within 28 day periods for both the K and L classifications in analyses of variance.

Differences between means were determined by a modification of Duncan's (1955) Multiple Range Test. Standard errors of individual means were computed since the number of observations in each varied. Differences between pairs of means were tested using the average of the respective standard errors. Since unequal numbers can give rise to assymetrical patterns of significance between ranked means, the underscoring presentation suggested by Duncan cannot be used. A schematic presentation is used as an alternative, any shaded square indicating a difference between means which reaches the 95% level of probability.



In the example given 2 and 1 are significantly ($P < 0.05$) greater than 3 and 4. No other differences reach significance.

Linear regressions were calculated and tested by usual procedures. Differences among slopes of regressions were tested by analyses of residuals after fitting individual and pooled within lines. Where slopes did not differ significantly and variances were homogeneous by Bartlett's (1937) test, differences among intercepts were tested. Under these conditions, the hypothesis that $\hat{a}_1 = \hat{a}_2 = \hat{a}_3 = \hat{a}_4$ is identical to the hypothesis that means of Y_i adjusted for regression, do not differ. Residuals, after fitting a common line over all data and the pooled within regression above, were compared as in the analysis of adjusted means following covariance.

Table 2.1 Description of indices used in the general statistical model

Classification	Index	Code
IA (cows)	i	1 = cow 19 2 = cow 118 3 = cow 5 4 = cow 26
K (stage of lactation)	j	1 = 0 - 28 days p.p. 2 = 29 - 56 days p.p. 3 = 57 - 84 days p.p. 4 = 85 - 112 days p.p. 5 = 113 - 140 days p.p. 6 = 141 - 168 days p.p. 7 = 169 - 196 days p.p. 8 = 197 - 224 days p.p. 9 = 225 - 252 days p.p. 10 = 253 - 280 days p.p.
L (seasonal period)	k	1 = 1. 8.68 - 29. 8.68 2 = 30. 8.68 - 26. 9.68 3 = 27. 9.68 - 24.10.68 4 = 25.10.68 - 21.11.68 5 = 22.11.68 - 19.12.68 6 = 20.12.68 - 16. 1.69 7 = 17. 1.69 - 13. 2.69 8 = 14. 2.69 - 13. 3.69 9 = 14. 3.69 - 10. 4.69

Chapter Three. RESULTS

3.1 Experimental Methods

3.1.1 Surgical Biopsies

Cows 19 and 118 were each biopsied nine times and cows 5 and 26 each six times in the lactation under study. Operations took between 10 and 15 minutes from incision to closure although preparation of the animals varied considerably, sometimes involving 60 minutes. Wounds healed rapidly with no incidence of milk oozing from the incisions at milking. Of the thirty operations, only one did not yield a tissue sample. In this case (cow 118 - 23.1.69), on exposing the parenchyma a small fibrous region obscured the secretory tissue. An attempt to avoid this and sample the surrounding area from the same incision resulted in a haemorrhage and as the cow appeared stressed, the wound was closed and the animal released from restraint. This was the only serious case of blood residues persisting in the milk for longer than 2 - 3 days after an operation. In most cases, clots were present in the foremilk for 1 - 3 milkings after a biopsy but if more than slight haemorrhage occurred during the surgery, clots and tinted milk were present for 2 - 3 days. Twice following biopsies of cow 19, small (4 cm dia.) swellings appeared near, but not at, the incisions some 8 - 10 days later. After a further 10 days one of these erupted and healed, and after a similar period the other disappeared. There was only one case of mastitis in an operated quarter. Identified 4 days after the operation, the infection responded to local treatment within 3 days.

3.1.2 Effects of Operations on Milk Production.

A noticeable depression in the yields of operated quarters occurred following each biopsy. Milk yields and compositions were determined before and after the January operations (Fig. 3.1, Table 3.1). A small depression in production was observed 2 - 3 days after surgery but normal yields were recovered in 7 days. The slight changes in composition, particularly increases in fat content, were consistent with the longer term changes which occurred over these stages of lactation (see 3.3).

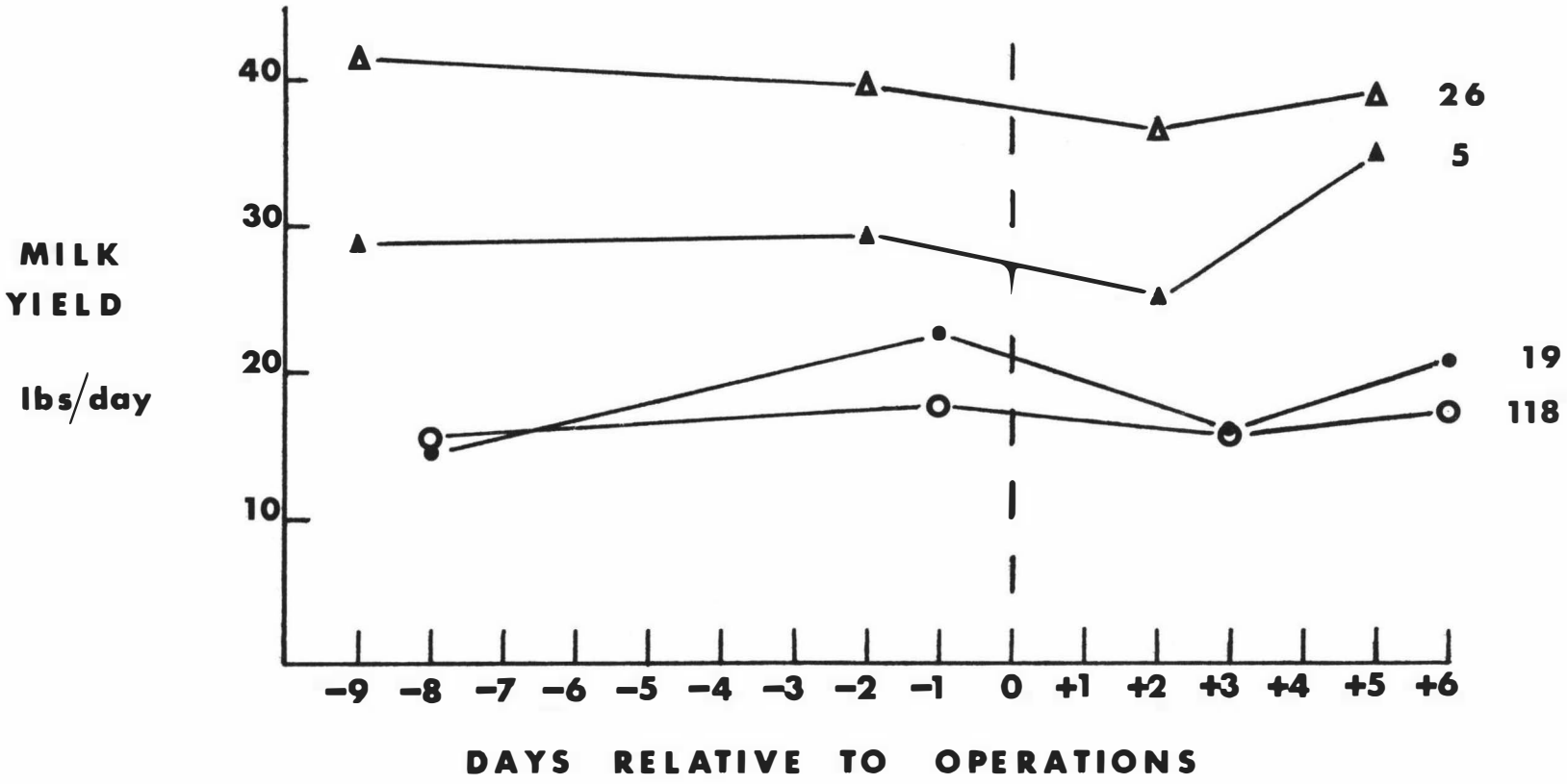


Fig. 3.1 The effect of surgical biopsies on milk yields in mid-lactation.

● cow 19 (i = 1), ○ cow 118 (i = 2),
▲ cow 5 (i = 3), △ cow 26 (i = 4).

Table 3.1 Composition of Sample Milks taken Before and After Biopsy in Mid-lactation.

Cow Number	Date of Sampling	Days Post-partum	M.Y. lbs	Fat%	Protein%	Lactose%
19 *	15/16 Jan.	174	14.5	6.24	4.62	5.42
	29/30 Jan.	188	20.7	6.95	4.76	5.27
118 *	15/16 Jan.	176	16.0	6.28	4.95	5.19
	29/30 Jan.	190	17.3	6.57	4.80	4.98
5 **	15/16 Jan.	120	28.9	5.92	4.13	4.67
	29/30 Jan.	134	34.9	5.90	4.08	4.78
26 **	15/16 Jan.	108	41.0	5.13	3.53	5.14
	29/30 Jan.	122	38.5	4.85	3.78	5.25

* Biopsied 23 Jan. ** Biopsied 24 Jan.

The sharp increase in protein contents of milk from cows 19 and 118 following the fourth operations (Fig. 3.6 c, 13 - 15 weeks p.p) were examined as possible effects of the surgery. Samples collected from individual quarters showed little variation in protein content, all being in excess of 5%. There was a possibility that the operations, or some factor associated with them (e.g. the tranquilizer), influenced milk production through a more general mechanism and hence affected all quarters in a similar manner.

3.1.3 Biochemical Methods

The colorimetric determination of calf thymus deoxyribonucleic acid was linear over the range 0 - 160 μg. Duplicates agreed to within 3% and the colour developed was stable between 16 and 20 hours at 37°C (Fig. 3.2). Three extractions with perchloric acid at 70°C removed all acid insoluble nucleotides from three bovine mammary homogenates (Table 3.2).

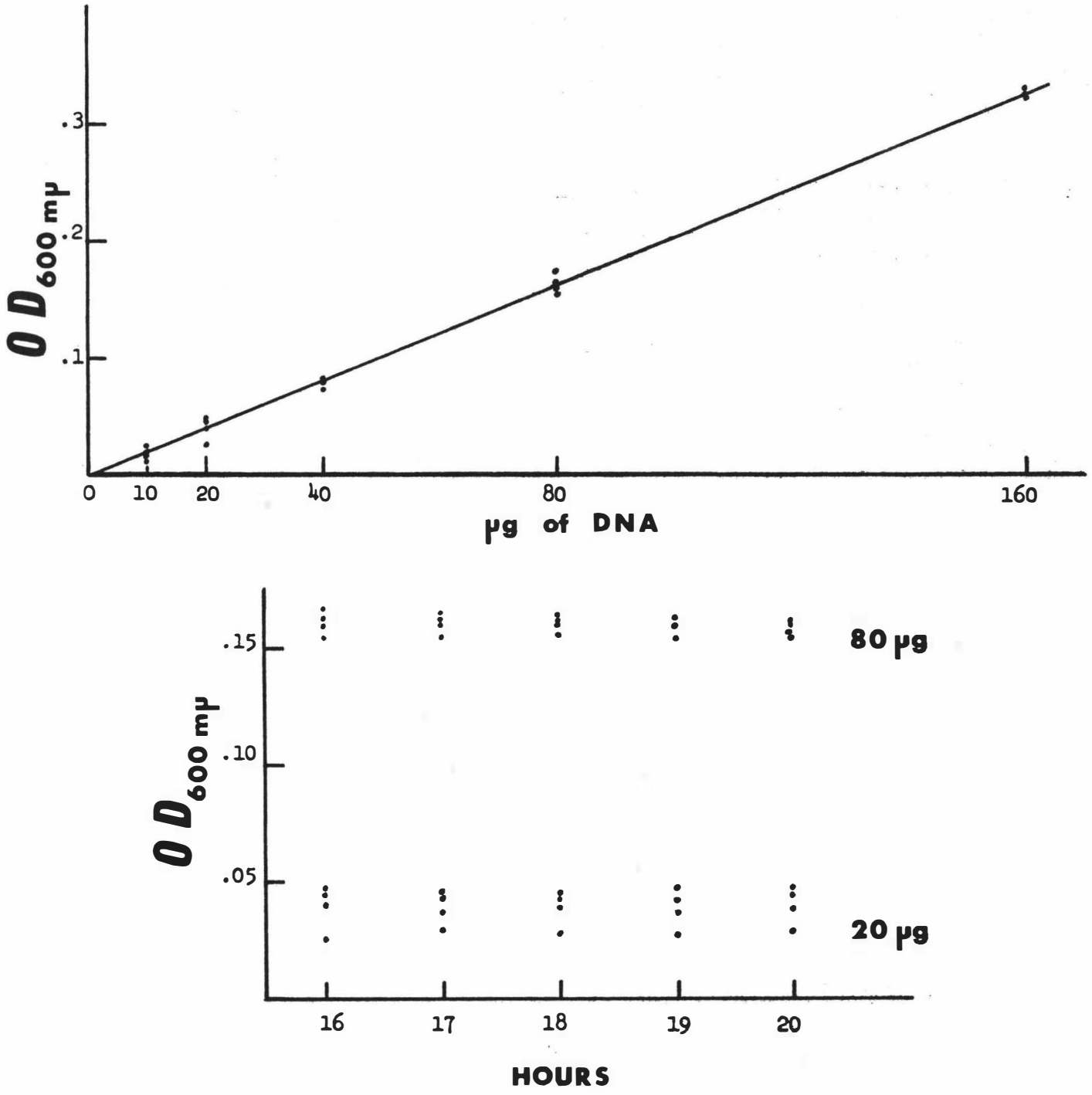


Fig. 3.2 Diphenylamine reaction for deoxyribonucleic acid.
Upper: Standard curve for the determination.
Lower: Stability of the colour at 37°C.

Table 3.2 Determination of DNA in repeated extracts of mammary homogenates with perchloric acid at 70°C.

Extract \ Homogenate	A		B		C	
	DNA	%	DNA	%	DNA	%
1	42.25	96.2	28.1	96.2	52.5	96.6
2	1.65	3.5	1.0	3.4	1.65	3.0
3	0.15	0.3	0.1	0.3	0.2	0.4
4	0	-	0	-	0	-
Total	47.05		29.2		54.35	

Acetate thiokinase proved the most difficult enzyme to assay and results from the preliminary work are presented in full (Fig. 3.3). The assay involved the chemical trapping of acetyl CoA as aceto-hydroxamate and the development of a coloured complex in acidified $FeCl_3$. The assay was linear for 0 - 400 µls of bovine mammary supernatant and by using 200 µls routinely during the experiment, all activities were within the O.D. range given here. Activity in the supernatant remained constant for 60 minutes but fell with prolonged storage on ice. The enzymes in 400 µls of supernatant were denatured by 1.0 but not 0.75 mls of stopping reagent and colour development was maximal in 10 - 20 minutes after its addition, remaining stable for at least a further 40 minutes. During experimental assays, samples were centrifuged for 20 minutes after halting the reactions to prevent the measurement of optical densities before colour development was complete.

The other enzymes were assayed using absorbance changes at 340 mµ. No serious difficulties were met in these determinations when substrate levels were modified from the original methods as described in 2.4.3. Assays were linear for 0 - 400 µls of supernatant and, when corrected for blank values, the lines passed through the origin. Optical densities remained constant for 60 - 90 minutes after reactions were halted provided the tubes were kept in the dark. There was no detectable loss in activity when supernatants were stored for up to 5 hours on ice.

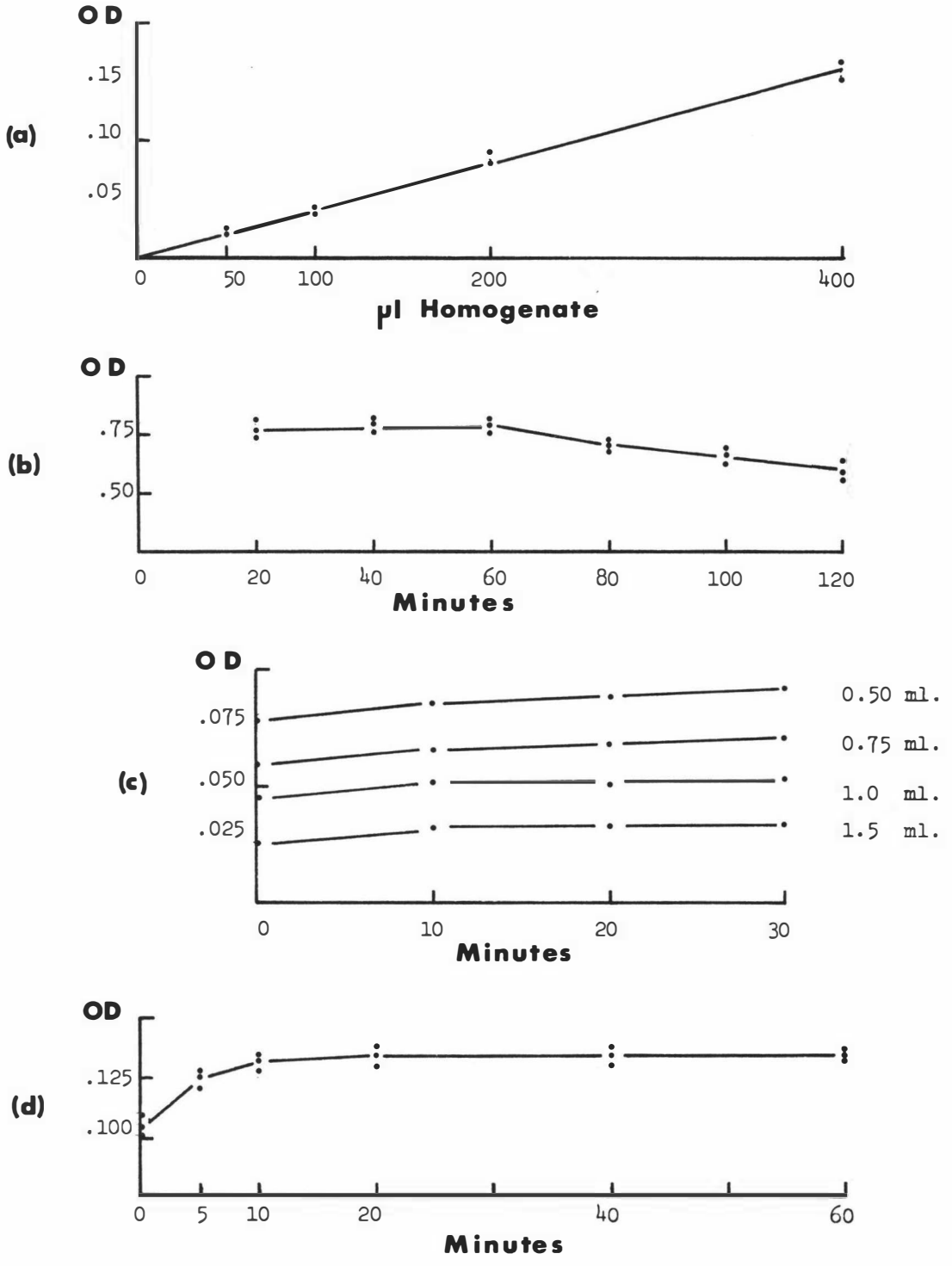


Fig. 3.3 Acetate thiokinase assay.

(a) Standard curve for varying levels of added bovine mammary supernatant.

(b) Activity in supernatant stored on ice.

(c) Effect on activity and Optical Density of varying levels of stopping reagent.

(d) Colour development after addition of stopping reagent.

3.2 Biochemical Results

3.2.1 Deoxyribonucleic Acid Levels

The contents of DNA, expressed on a wet weight of tissue basis as in Fig. 3.4, show consistent falls following calving and irregular patterns of rises thereafter. Since the contents are ratios, changes could have arisen from either the numerator or denominator. Changes in the numerator could reflect variations in tissue composition involving cell types, cell numbers or cell sizes. The denominator possibly reflects variations in retained milk in the biopsy samples although if this was the case, the lowest DNA contents would be expected to coincide with the peak milk yields. As no determinations of retained milk were made (see 4.1) nor any histological examinations on the biopsy samples, the reasons for the variation in DNA content could not be resolved. The low value for sample 3 from cow 118 has probably contributed to the very high enzyme activities in this sample (see below - 3.2.2).

3.2.2 Mammary Enzyme Activities

Enzyme activities at various stages of lactation are plotted in Figs 3.5 a and 3.5 b. Although the data are discrete determinations, the points have been connected with lines to aid visual separation of the activities in the four cows. The differences in activities of the five enzymes were examined by analyses of variance corresponding to the following models (Table 3.3):

ENZ 1 $\quad Y_{il} = \mu_{il} + IA_i + e_{il}$

ENZ 2 $\quad Y_{jl} = \mu_{jl} + K_j + e_{jl}$

ENZ 3 $\quad Y_{ijl} = \mu_{ijl} + IA_i + K_j + e_{ijl}$

(where classifications and indices are described in 2.6 and Table 2.1).

When analysed for variation among cows, ignoring stages of lactation (ENZ 1), significant differences between cows were identified for the activities of UDPglucose-4-epimerase ($P < 0.05$), glycerol phosphate dehydrogenase ($P < 0.05$) and glucose-6-phosphate dehydrogenase ($P < 0.05$). Differences in the activities of acetate thiokinase ($0.25 > P > 0.1$) and hexokinase ($P > 0.25$) failed to reach significance.

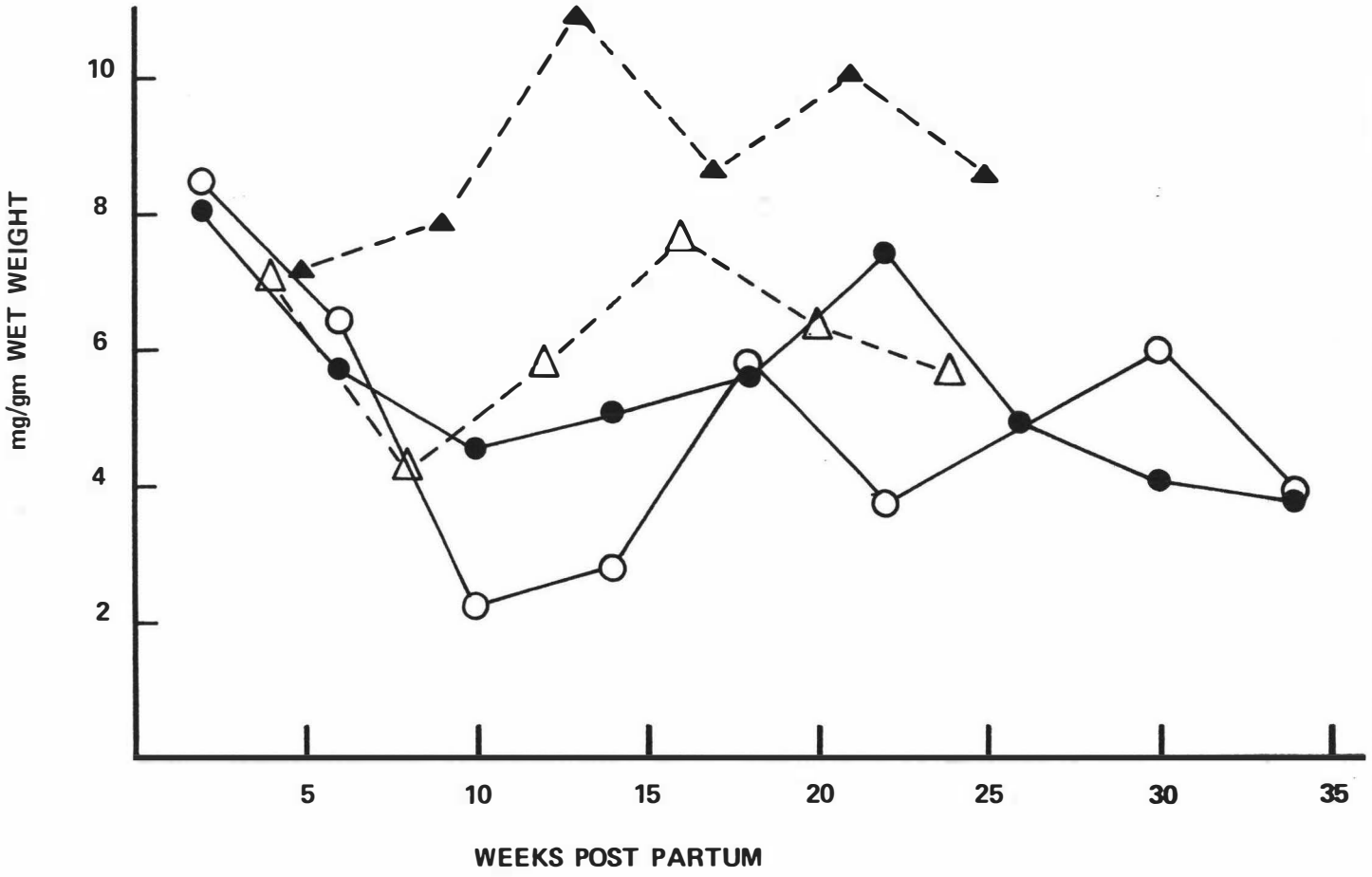


Fig. 3.4 Concentration of DNA in biopsy samples taken at different stages of lactation.
(Symbols as in Fig. 3.1)

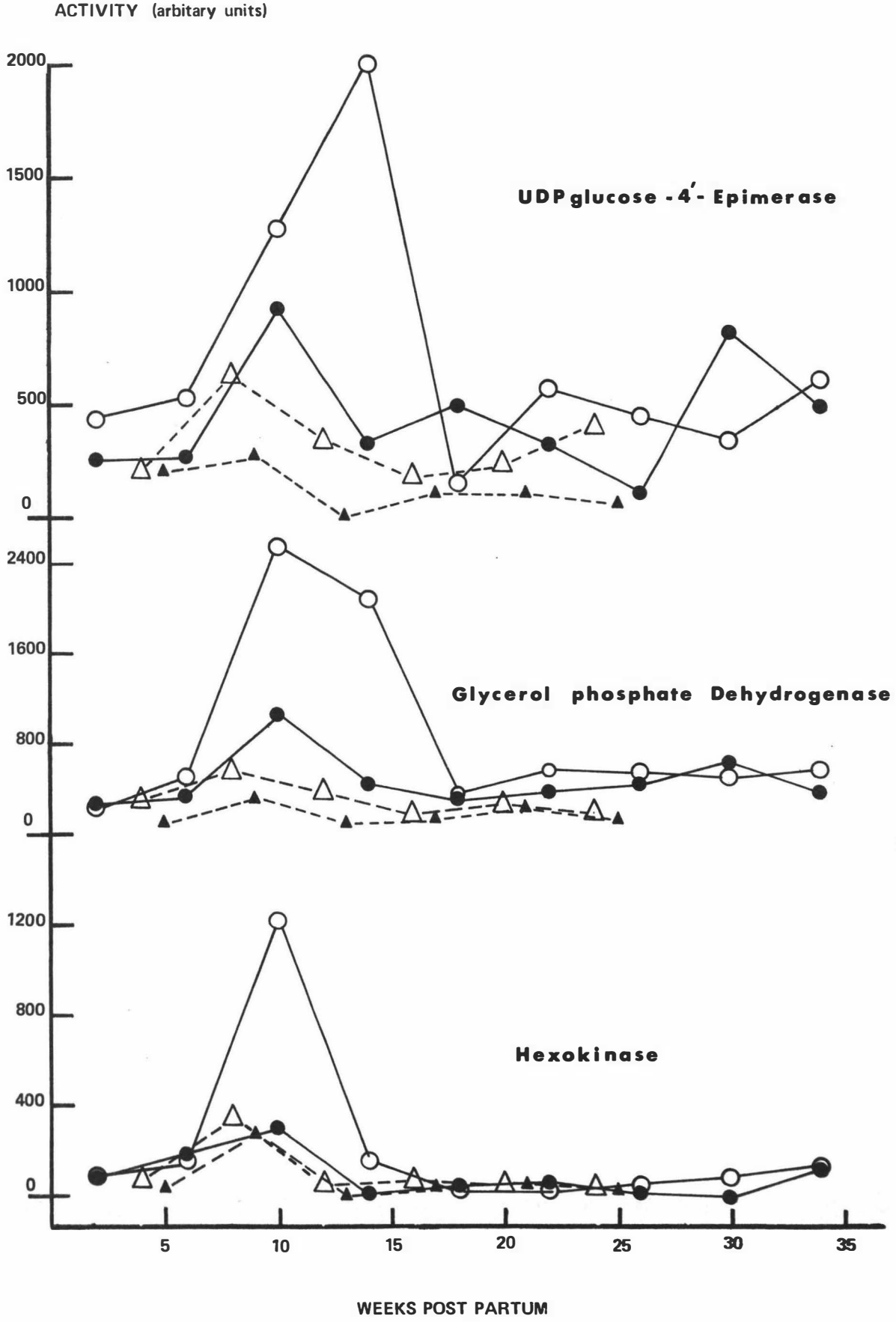


Fig. 3.5a Changes in enzyme activities with stage of lactation.
(Symbols as in Fig. 3.1)

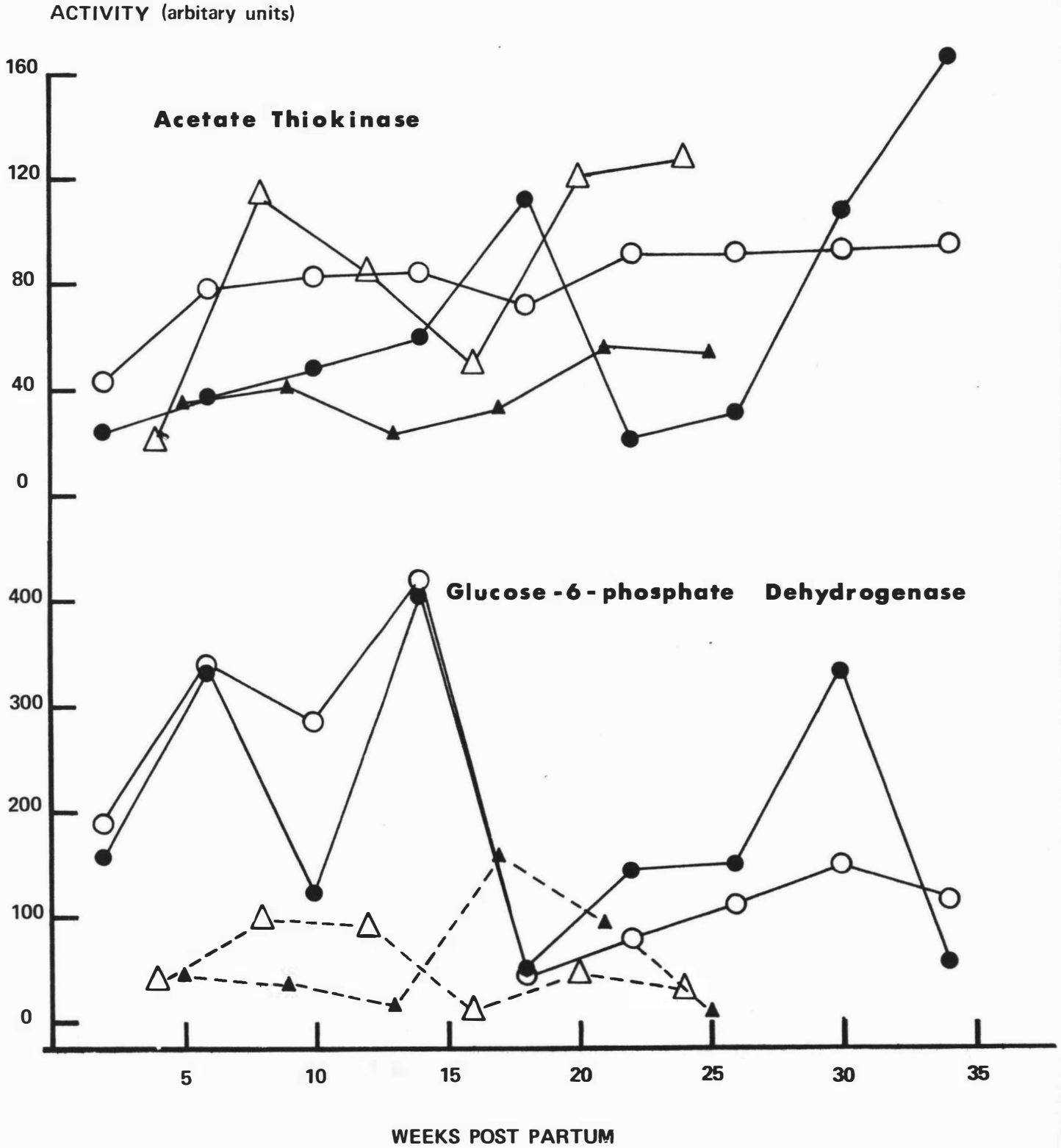


Fig. 3.5b Changes in enzyme activities with stage of lactation.
(Symbols as in Fig. 3.1)

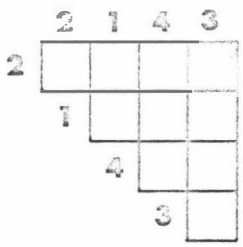
Table 3.3 Summary of Analyses of Variance for Mammary Enzyme Activities

(Mean Squares and Variance Ratios)

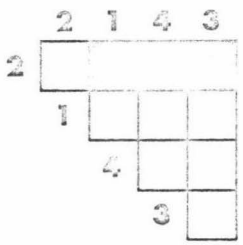
Model	Source	d.f.	Acetate Thiokinase	UDPglucose epimerase	Glycerol P Dehydrog.	Hexokinase	Glucose 6 P Dehydrog.
ENZ 1	Bet. Cows	3	2552.88	465554.53	831451.00	44665.12	49037.97
	Within	25	1324.93	134301.36	228180.88	53923.86	11449.48
	$F_{3,25}$		1.93ns	3.46*	3.64*	0.83ns	4.28*
ENZ 2	Bet. Stages	8	1960.24	155578.57	304326.50	101312.38	15785.64
	Within	20	1255.00	175478.45	288213.10	33579.64	15353.29
	$F_{8,20}$		1.56ns	0.89ns	1.06ns	3.02*	1.03ns
ENZ 3	Cows	3	2302.72	448867.76	933142.00	51698.03	46932.59
	Stages	8	1866.43	148867.32	342460.62	103949.73	14996.13
	Residual	17	1070.11	127446.70	174402.00	30382.26	9780.46
F Ratios:							
	$Cows_{3,17}$		2.15ns	3.51*	5.35**	1.70ns	4.80**
	$Stages_{8,17}$		1.74ns	1.17ns	1.96ns	3.42*	1.53ns

ns $P > 0.05$ * $P < 0.05$ ** $P < 0.01$

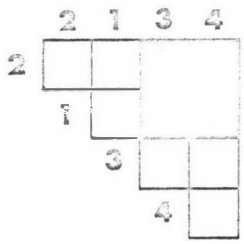
Cow means for enzyme activities are tabulated in Appendix A-2 and ranked means with significant ($P < 0.05$) differences shaded as described in 2.6 are given for the enzymes with activities varying significantly between cows (Table 3.3).



UDPG epimerase



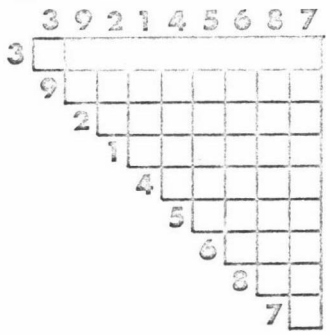
Glycerol P D'hase



Glucose 6 P D'hase

Cow 118 exceeded cow 5 in UDPglucose epimerase activity, all other cows in the activity of glycerol phosphate dehydrogenase and cows 19 and 118 both exceeded cows 5 and 26 in the activity of glucose-6-phosphate dehydrogenase.

In analyses of differences among stages of lactation, ignoring variation among cows (ENZ 2), hexokinase activity was the only variable found to reach significance ($P < 0.05$). Stage of lactation means are tabulated in Appendix A-3 and ranked means with significant ($P < 0.05$) differences for hexokinase activity are given below.



Hexokinase

The activity of hexokinase in stage 3 greatly exceeded all other stages and examination of Fig. 3.5 a shows the difference to be due largely to cow 118. The high activity in stage 3 may have resulted from the low DNA content of the third sample collected from that cow (see 3.3.1).

Analyses of variance of differences among cows and stages of lactation taken together (ENZ 3) indicated the same general pattern

as when analysed one classification at a time ignoring the other (Table 3.3). There were minor changes in the significance of the variance ratios but no changes in the differences among ranked means.

When all of the biochemical data were considered together (Figs 3.4,3.5 a, 3.5 b, Table 3.3), it was obvious that differences in the activities of UDPglucose epimerase, glycerol phosphate dehydrogenase and hexokinase arose from the high values for cow 118 particularly at stage 3. These values have influenced both cow and stage of lactation comparisons so they were examined further in case there was any real justification for omitting them in the analyses. Since the activities of acetate thiokinase and glucose-6-phosphate dehydrogenase were not abnormally high in samples 3 and 4 for cow 118, it appeared unlikely that the common factor, DNA content, was responsible for the high activities of the other enzymes. For this reason, all data were retained in the analyses as above.

3.3 Milk Production

Changes in the yields and compositions of milk from the experimental cows are presented in Figs 3.6 a, b, c, d. The changes were examined by analyses of variance corresponding to the following models (Table 3.4):

$$\text{MYC } 1 \qquad Y_{il} = \mu_{il} + IA_i + e_{il}$$

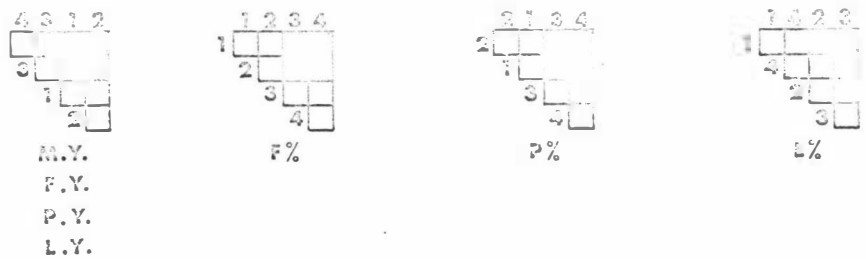
$$\text{MYC } 2 \qquad Y_{jl} = \mu_{jl} + K_j + e_{jl}$$

$$\text{MYC } 3 \qquad Y_{kl} = \mu_{kl} + L_k + e_{kl}$$

$$\text{MYC } 4 \qquad Y_{ijkl} = \mu_{ijkl} + IA_i + K_j + L_k + e_{ijkl}$$

(where classifications and indices are as in 2.6 and Table 2.1).

3.3.1 Significant ($P < 0.001$) between cow variance existed in all variables measured. Cow means are tabulated in Appendix A-4 and presented below in ranked form with significant ($P < 0.05$) differences as described in 2.6.



M.Y.
F.Y.
P.Y.
L.Y.

F%

P%

L%

All cows except 19 and 118 differed in milk yield. Milk from the two earlier calving cows (19 and 118) had higher fat and protein contents than did 5 and 26. In addition, the content of protein in the milk of cow 5 exceeded that from 26. Lactose contents in the milk from cows 19, 26 and 118 exceeded that from cow 5 with the lactose from 19 exceeding 118 (Fig. 3.6 d). As suggested by Figs 3.6 a, b, c, d, the patterns of differences among cows for the daily yields of milk components follow exactly the differences in milk yields.

3.3.2 Differences among stages of lactation, ignoring variation between cows (MYC 2), reached significance ($P < 0.001$) for all variables except lactose content. Stage of lactation means are tabulated in Appendix A-5 and presented below in ranked form with

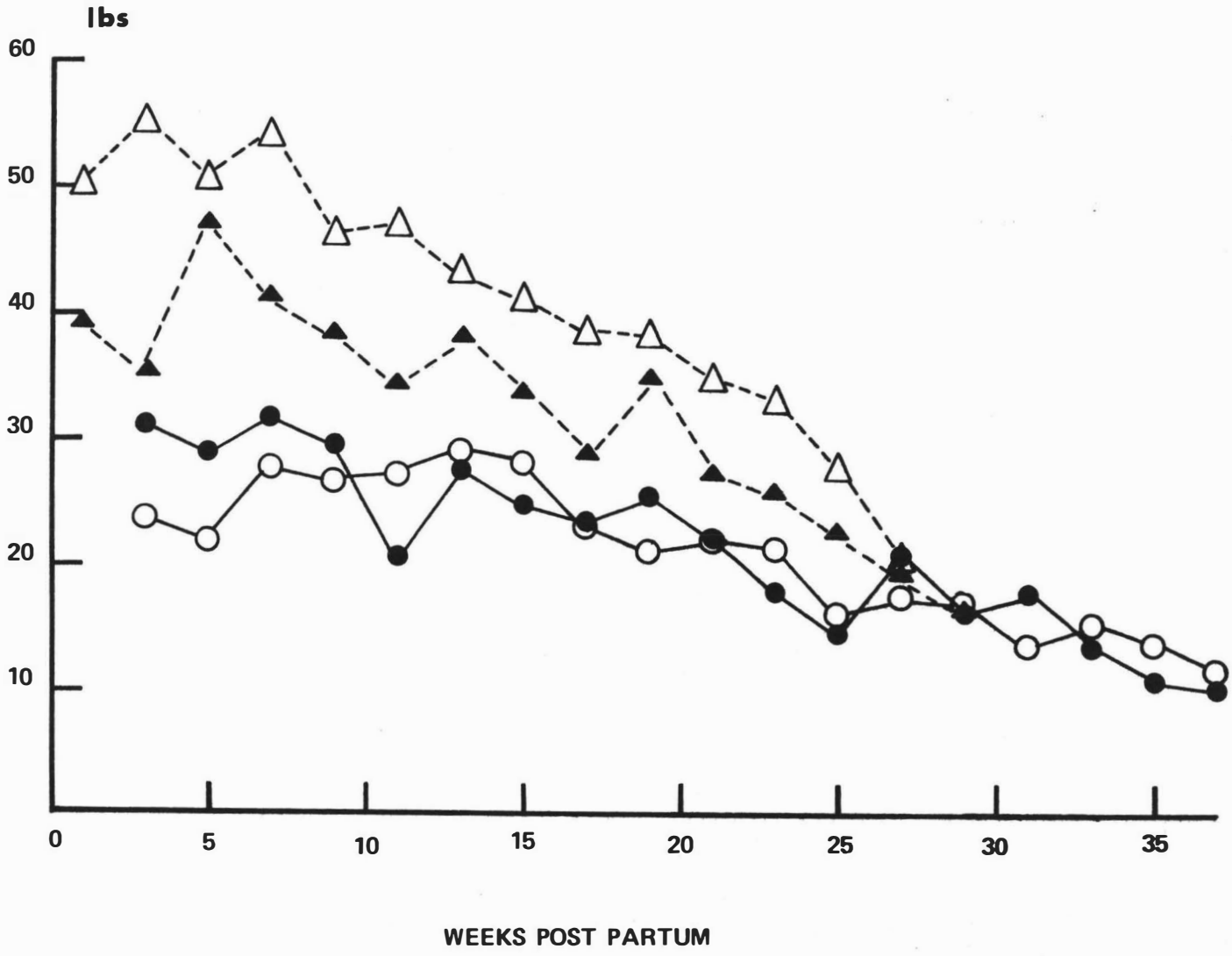


Fig. 3.6a Milk production: Daily Milk Yield
(Symbols as in Fig. 3.1)

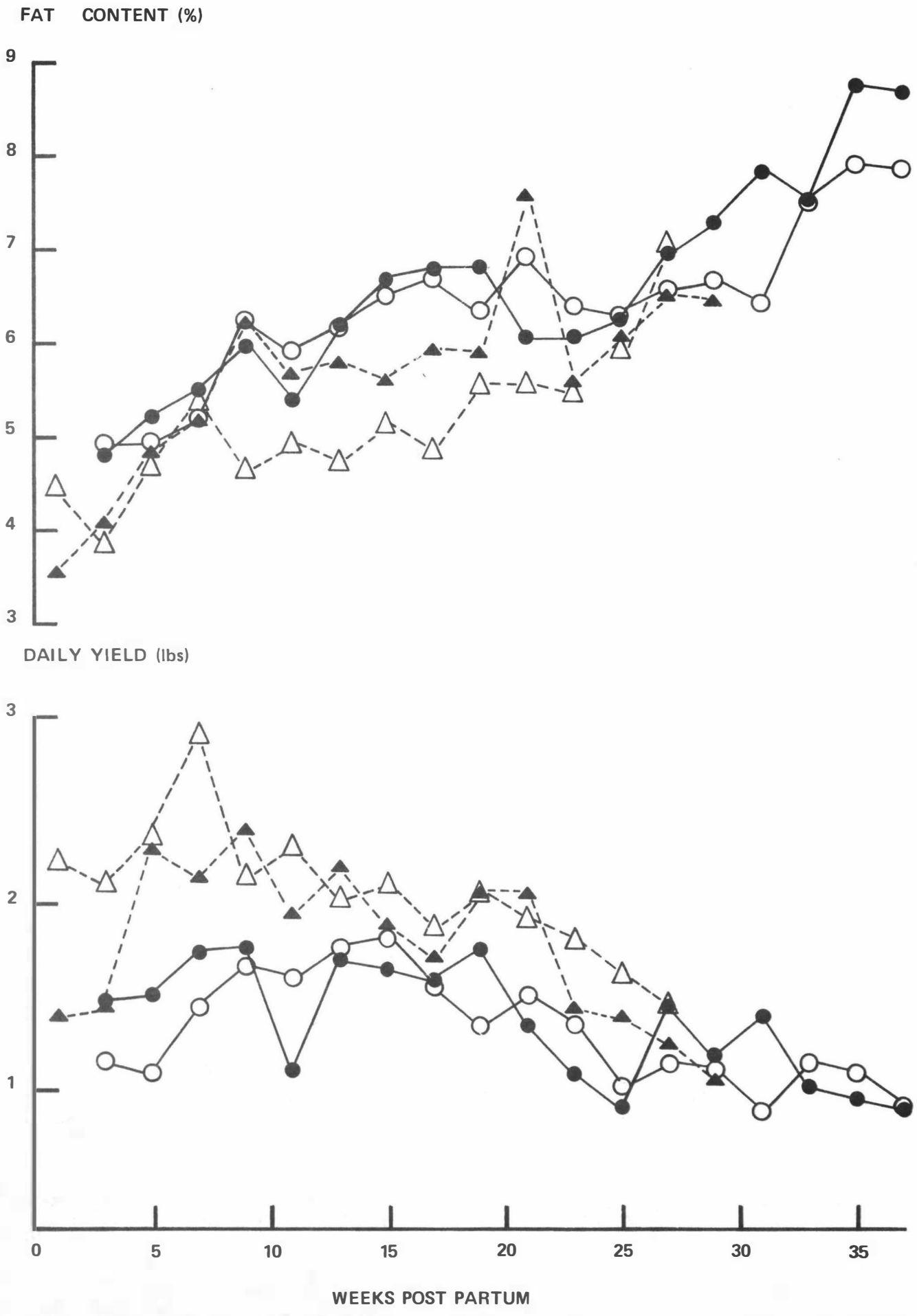


Fig. 3.6b Milk Production: Fat Content and Daily Yield.
(Symbols as in Fig. 3.1)

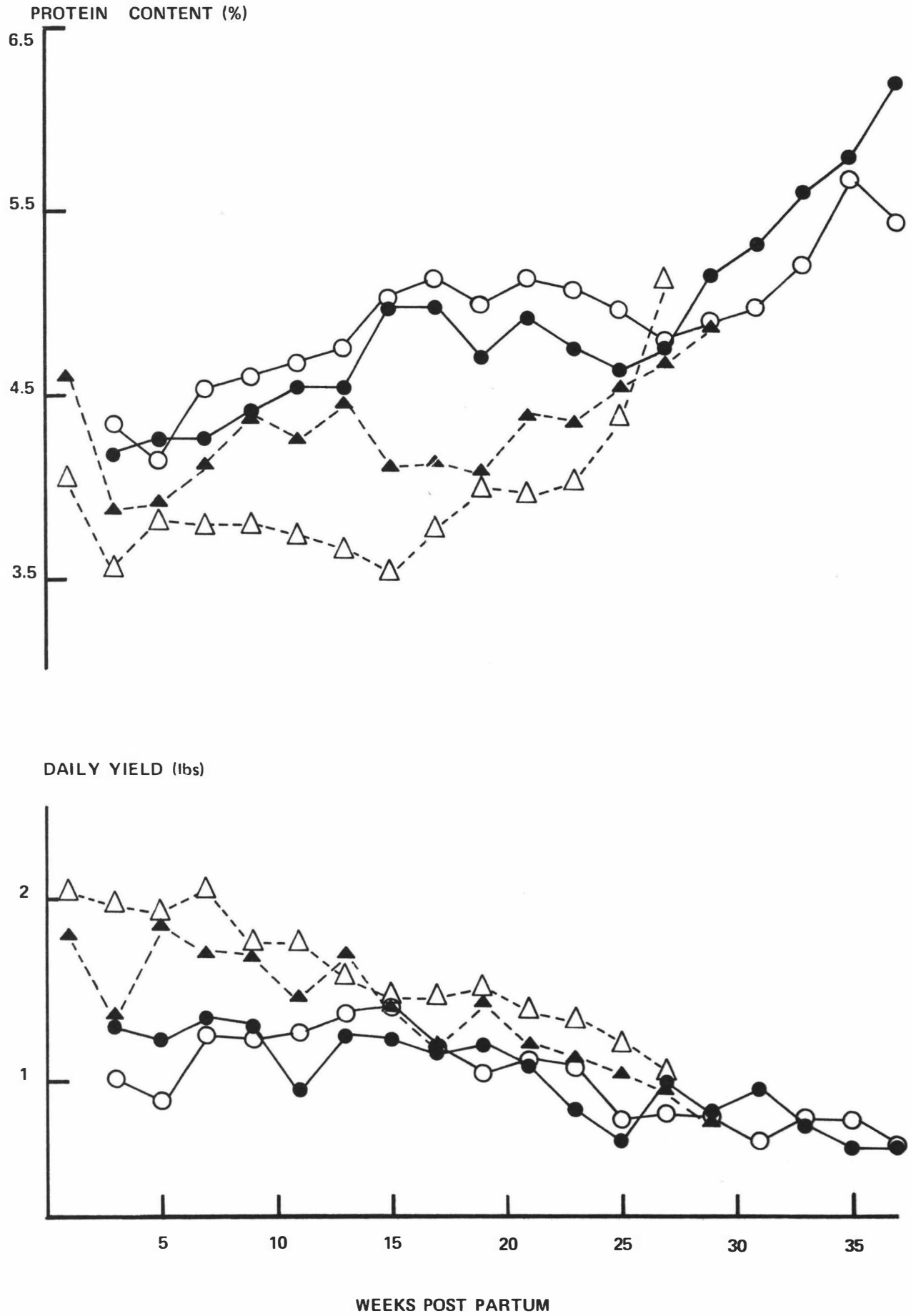


Fig. 3.6c Milk Production: Protein Content and Daily Yield.
(Symbols as in Fig. 3.1)

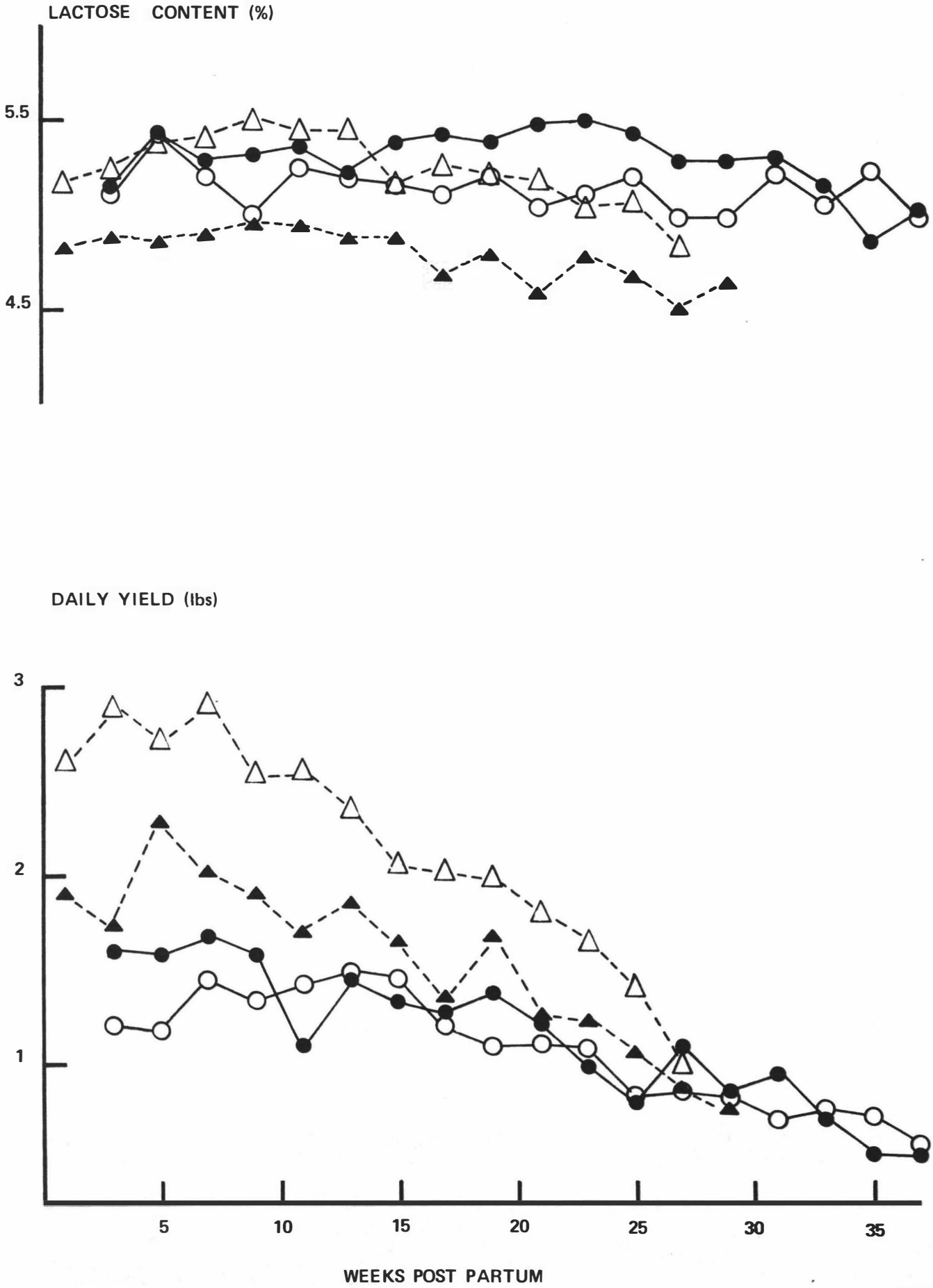


Fig. 3.6d Milk Production: Lactose Content and Daily Yield.
(Symbols as in Fig. 3.1)

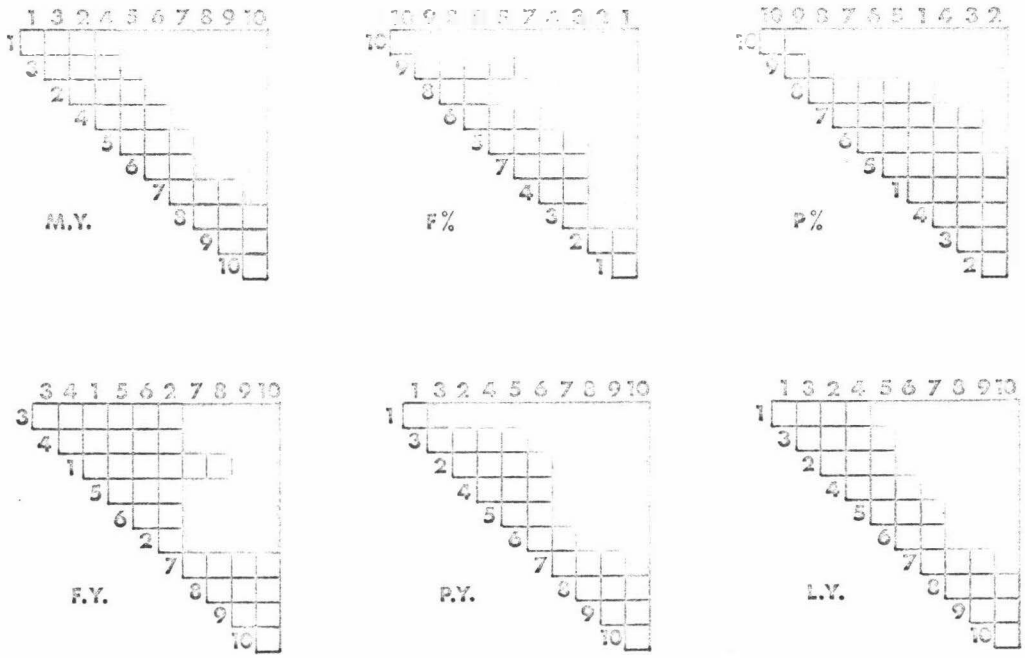
Table 3.4 Summary of Analyses of Variance for the Milk Production Variables

(Mean Squares and Variance Ratios)

Model	Source	d.f.	Milk Yield	Fat Content	Protein Content	Lactose Content	Fat Yield	Protein Yield	Lactose Yield
MYC 1	Bet. Cows	3	1479.25	7.08	3.43	0.81	2.01	1.29	3.91
	Within	61	60.46	0.90	0.18	0.02	0.12	0.08	0.18
	$F_{3,61}$		24.47***	7.89***	19.40***	35.03***	17.14***	16.12***	22.02***
MYC 2	Bet. Stages	9	527.97	6.55	1.40	0.06	0.74	0.63	1.46
	Within	55	61.35	0.31	0.15	0.06	0.12	0.06	0.17
	$F_{9,55}$		8.61***	21.12***	9.10***	1.00ns	6.14***	11.07***	8.60***
MYC 3	Bet. Periods	8	329.25	4.07	0.75	0.10	0.59	0.46	0.97
	Within	56	98.07	0.78	0.27	0.05	0.15	0.09	0.26
	$F_{8,56}$		3.36**	5.26***	2.80*	1.92ns	3.88***	5.02***	3.68**
MYC 4	Cows	3	147.23	0.43	0.19	0.65	0.20	0.10	0.65
	Stages	9	24.17	0.33	0.11	0.01	0.09	0.02	0.08
	Periods	8	51.99	0.28	0.18	0.04	0.13	0.07	0.16
		44	9.13	0.19	0.04	0.01	0.04	0.02	0.03
	F Ratios:								
	$Cows_{3,44}$		16.12***	2.20ns	5.20**	51.67***	4.78**	6.54**	24.92***
	$Stages_{9,44}$		2.65*	1.71ns	3.01**	1.10ns	2.22*	1.17ns	2.92**
	$Periods_{8,44}$		5.69***	1.43ns	4.96***	2.78*	3.04**	4.69***	6.02***

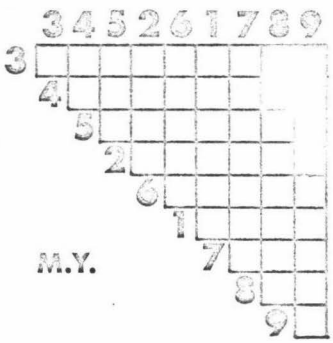
ns $P > 0.05$ * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

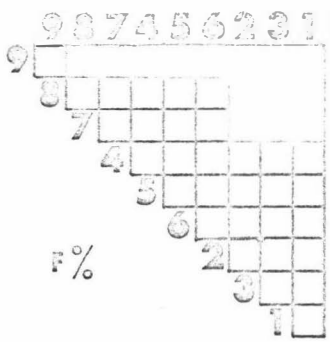
significant ($P < 0.05$) differences as described in 2.6.

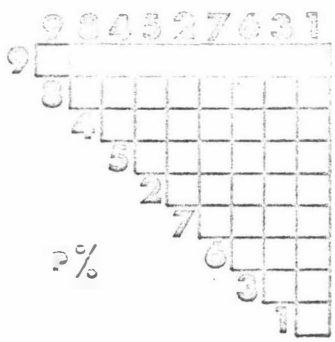


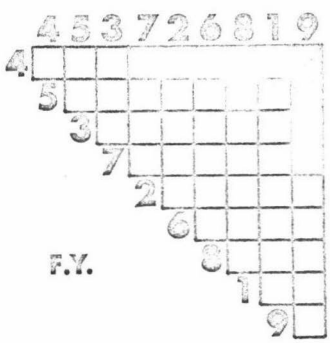
The steady decline in milk yield (Fig. 3.6 a) led to fairly symmetrical patterns of significant differences among stages of lactation. Fat content rose across the lactation with the last stage clearly in excess of all others. The lack of symmetry in the significant differences partly reflects variations in standard errors arising from the different number of observations contributing to mean values. Daily fat yields in the first six stages exceeded those in later stages. Differences in daily fat yields followed those in milk yield tempered by the sharp increases in fat content over the later stages of lactation (Fig. 3.6 a, b). The protein content of milk varied in a similar manner to fat content ($\hat{r}_{P\%F\%} = 0.85$) but the increase across the lactation was less marked (note differences in the ordinate scale in Figs 3.6 b, c). A regular, but not significant, depression in protein content occurred shortly after calving. The stage of lactation means for protein yield are ranked the same as those for milk yield but patterns of significant differences vary slightly, again reflecting a compensation for declining milk yield with increased protein content in the later stages. These 'drying off' changes were similar for all cows. Lactose content did not vary ($P > 0.25$) with stages of lactation but the non-significant depression which occurred in the last stage (Fig. 3.6 d, Appendix A-5) was probably part of the 'drying off' change noted above. Variations in lactose yield ($P < 0.001$) reflected entirely the changes in milk yield as the patterns of mean differences were identical for the two variables.

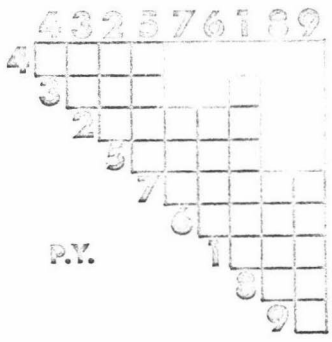
3.3.3 Variation arising from differences among seasonal periods (MYC 3) reached significance for all variables except lactose content ($0.1 > P > 0.05$). Period means are tabulated in Appendix A-6 and presented below in ranked form with significant ($P < 0.05$) differences as described in 2.6.











The seasonal variance in milk yield ($P < 0.01$) arose in part from the significantly lower yields in periods 8 and 9 (Feb - Apr). Fat content varied according to seasonal periods ($P < 0.001$) with the last three periods (Jan - Apr) being higher than the first three (Aug - Oct). Differences in fat yields among periods led to a significant seasonal variance ($P < 0.001$). Differences among fat yield means were similar to the milk yield means with the asymmetry reflecting variation in the number of observations comprising each value and the reciprocal movement of fat content and milk yield in the late summer. The seasonal variance in protein content ($P < 0.05$) arose from the general increase across the season. The protein content of milk from the last period was significantly greater than all other periods. Seasonal variance in the yield of protein ($P < 0.001$) arose from differences among means which were very similar to the milk yield differences. The limited variation in protein content was obviously

overridden by the differences in milk yields. Lactose yield followed milk yield very closely and the significant seasonal variation ($P < 0.01$) clearly arose through the variations in milk yield.

3.3.4 When differences among cows, stages of lactation and seasonal periods were considered together (MYC 4), there were slight changes in the extent to which the three main effects explained the variation observed in the components. The differences from the one classification analyses (Table 3.4) can be summarised:

The stage of lactation variation (MYC 2) in protein yield lost significance when considered along with the other sources of variance (MYC 4).

The seasonal variation (MYC 3) in the content of protein and lactose reached greater significance in the three way analysis (MYC 4).

Differences in fat content arising from all sources of variation when considered alone (MYC 1, MYC 2, MYC 3) all lost significance when the classifications were considered together ($0.25 > P > 0.1$).

Of the changes, those for the significance of variations in fat content are of greatest interest. Table 3.5 shows ranked means obtained from omitting one or more classifications in the fitting of constants during analyses of variance by least squares. There was little influence of the classifications fitted in the ranking of stage of lactation means, suggesting that the interactions IA x K x L, IA x K and K x L were not important. There was however an influence of the classifications on the ranking of seasonal period means. Having delected the three interactions above as being unimportant, the patterns of ranked means suggests the presence of an IA x L interaction.

Table 3.5 Ranked Means (highest on left) obtained from estimates during 3 way and 1 way analyses of variance for fat content.

Model	Classifications Fitted	Stages of Lactation									
MYC 2	K	10	9	8	6	5	7	4	3	2	1
MYC 4	IA K L	10	9	8	6	7	5	4	3	2	1
	K L	10	9	8	6	7	5	4	3	2	1
	IA L	10	9	8	6	5	7	4	3	2	1
		Seasonal Periods									
MYC 3	L	9	8	7	4	5	6	2	3	1	
MYC 4	IA K L	4	1	9	3	2	7	5	6	8	
	K L	1	4	2	3	5	6	7	9	8	
	IA K	9	8	7	4	5	6	3	2	1	

A further two classification analysis was undertaken to test the possible cow x season interaction. In order to meet the program restriction of not exceeding 30 equations for solution, milk fat percentage data were rearranged as three 14 day replicates within 42 day periods as an approximation. Since no data were available for the later calving cows in the first 42 day period, the analysis corresponded to the following model:

$$Y_{ikl} = \mu_{ikl} + IA_i + L_k + (IA \times K)_{ik} + e_{ikl}$$

where classifications and i are as in 2.6 and Table 2.1 and

$k = 2,3,\ldots 6$ (k = 1 deleted)

$l = 1,2,3$

Table 3.6 Two Way Analysis of Variance with Interaction for Fat Content

Source	d.f.	Mean Squares	F Ratios	P
Total	58			
Seasons	4	6.69	31.37	<0.001
Cows	3	10.44	48.97	<0.001
Interaction	12	0.38	1.79	<0.10
Residual	39	0.21		

The interaction in Table 3.6 just reaches significance at $P<0.10$ but this analysis cannot rule out a more significant interaction in the data as originally analysed ($k = 1,2,....9$; $l = 1,2$). Fig. 3.6 b shows that variations in fat content were somewhat dependent on individual cows suggesting that the interaction did in fact exist. Furthermore, the confounding of main effects, which was a consequence of the design employed, limits inferences drawn from any of the analyses of variation among stages of lactation or seasonal periods (see 4.1).

3.4 Milk Fat Fatty Acid Compositions

Changes in the proportions of a selection of the milk fat fatty acids are presented for all cows in Fig. 3.7. Visual evaluation is limited by the gross sample to sample variation so analyses of variance were performed (Table 3.7) corresponding to the following models:

$$\text{FA 1} \qquad Y_{il} = \mu_{il} + IA_i + e_{il}$$

$$\text{FA 2} \qquad Y_{jl} = \mu_{jl} + K_j + e_{jl}$$

$$\text{FA 3} \qquad Y_{kl} = \mu_{kl} + L_k + e_{kl}$$

$$\text{FA 4} \qquad Y_{ijkl} = \mu_{ijkl} + IA_i + K_j + L_k + e_{ijkl}$$

(where classifications and indices are as in 2.6 and Table 2.1 except that for FA 2, data for the first stage of lactation ($j = 1$) were included with the second ($j = 2$) to enable analysis).

3.4.1 Analyses of variance for model FA 1, ignoring stages of lactation and seasonal periods as sources of variation (Table 3.7), found that differences among cows in the contents of 18:1 ($P<0.05$) and 18:0 ($P<0.001$) reached significance. Cow means for all variables are tabulated in Appendix A-7 and ranked means with significant ($P<0.05$) differences, as described in 2.6, are given below for the two acids which varied significantly between cows.

18:1

1	4	2	3
1			
4			
2			
3			

18:0

4	1	2	3
4			
1			
2			
3			

The content of 18:1 in milk fat from cow 19 was significantly greater than in the fat from cows 118 and 5. The content of 18:0 was significantly lower in the fat from cow 5 than all other cows with fat from cow 118 having a lower content of the acid than fat from cow 26. When differences among cows were analysed along with the two other sources of variation (FA 4), the content of 16:0 in milk fat varied significantly ($P<0.05$) among cows in addition to the two 18C acids discussed above. The acids whose contents in fat varied between

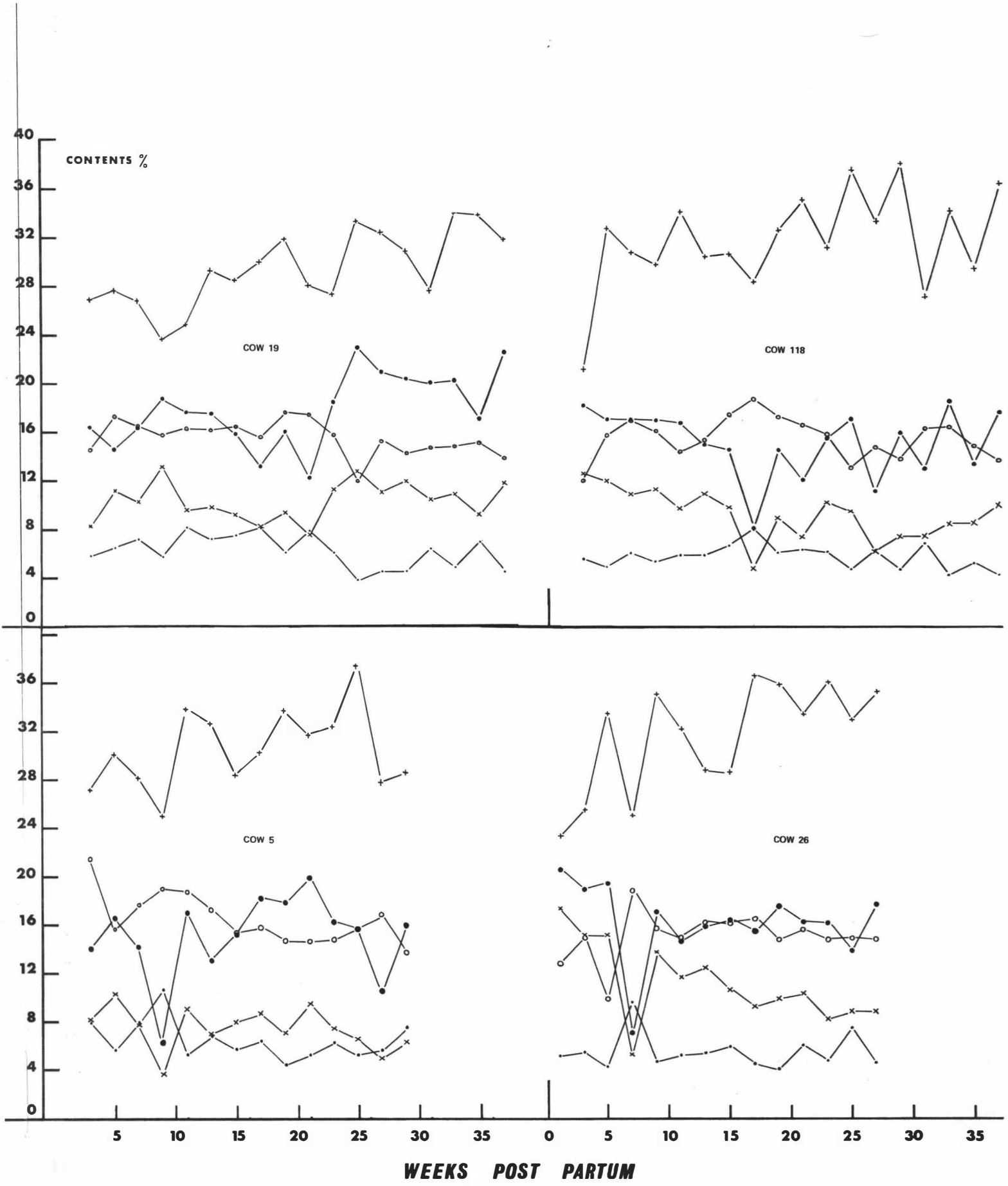


Fig. 3.7 MILK FAT FATTY ACID MOLAR COMPOSITIONS

(Symbols as in Fig. 1.2)

Table 3.7 Summary of Analyses of Variance for Molar Proportions of Fatty Acids in Milk Fat

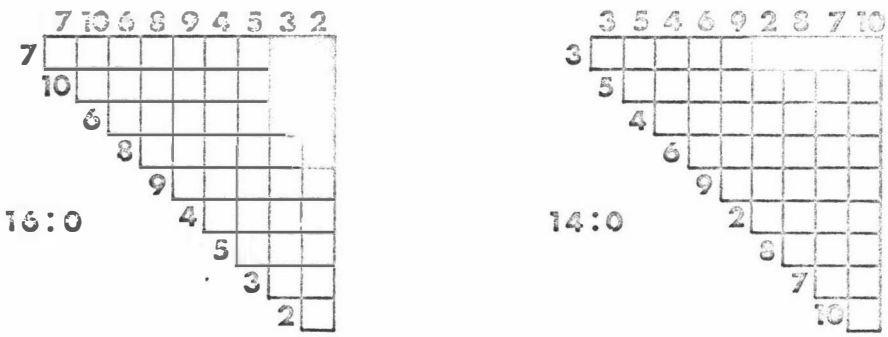
(Mean Squares and Variance Ratios)

Model	Source	d.f.	18:1	18:0	16:0	14:0	12:0	10.0	8:0	6:0	4:0
FA 1	Bet. Cows	3	27.83	38.02	23.32	5.15	2.59	3.55	0.30	1.19	0.65
	Within	60	9.42	4.91	14.13	3.21	1.83	2.08	0.50	0.99	2.68
	$F_{3,60}$		2.96*	7.74***	1.65ns	1.61ns	1.42ns	1.70ns	0.60ns	1.20ns	0.24ns
FA 2	Bet. Stages	8	11.31	11.80	35.86	6.14	2.82	3.79	0.80	0.90	2.15
	Within	55	10.15	5.71	11.47	2.89	1.73	1.92	0.44	1.01	2.64
	$F_{8,55}$		1.11ns	2.06ns	3.13**	2.13*	1.63ns	1.98ns	1.82ns	0.89ns	0.81ns
FA 3	Bet. periods	8	22.78	11.65	42.59	5.17	5.61	5.68	1.23	1.49	3.64
	Within	55	8.48	5.73	10.49	3.03	1.32	1.64	0.38	0.92	2.42
	$F_{8,55}$		2.69*	2.03ns	4.06***	1.71ns	4.25***	3.46**	3.23**	1.61ns	1.50ns
FA 4	Cows	3	22.16	29.79	27.80	3.70	1.84	2.66	0.48	1.64	4.72
	Stages	9	7.41	9.01	13.65	3.58	1.62	1.72	0.44	0.85	2.71
	Periods	8	19.20	8.60	16.57	2.75	4.49	3.40	0.91	1.63	4.96
	Residual	43	7.46	2.91	8.85	2.76	1.17	1.51	0.37	0.91	2.43
	F Ratios:										
	$Cows_{3,43}$		2.97*	10.25***	3.14*	1.34ns	1.58ns	1.76ns	1.29ns	1.80ns	1.95ns
	$Stages_{9,43}$		0.99ns	3.10***	1.54ns	1.30ns	1.39ns	1.14ns	1.17ns	0.93ns	1.12ns
	$Seasons_{8,43}$		2.57*	2.96**	1.87ns	1.00ns	3.85**	2.25*	2.44*	1.79ns	2.04ns

ns $P > 0.05$ * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

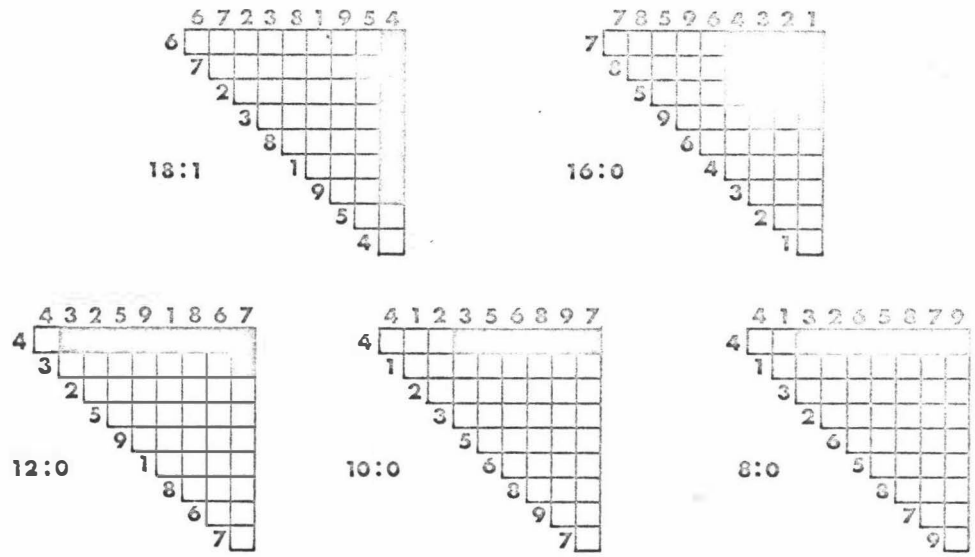
cows are those derived in part, if not entirely, from the blood lipids and probably the depot fat. The results indicated that the four cows differed in the contribution made by their blood lipids to the milk fat.

3.4.2 Variation arising from differences among stages of lactation, ignoring differences among cows and seasonal periods (FA 2), were significant for the contents in milk fat of 16:0 ($P<0.01$) and 14:0 ($P<0.05$). Stage of lactation means are tabulated in Appendix A-8 and ranked means with significant differences are presented below.



The content of 16:0 in milk fat rose across the lactation (Fig.3.7) but the ranked means indicate that the increase was not steady. Changes in the content of 14:0 follow no regular pattern with advancing lactation and no explanation can be offered for the four significant differences between the means above. When considered along with cows and seasonal periods (FA 4), the only component whose content changed significantly with stage of lactation was 18:0 ($P<0.001$). In view of the confounding of stages of lactation and seasonal periods, discussed in detail in 4.1, analyses using model FA 4 are subject to difficulties of interpretation.

3.4.3 Variation arising from differences among seasonal periods considered alone (FA 3) reached significance for the content in milk fat of 18:1 ($P<0.05$), 16:0 ($P<0.001$), 12:0 ($P<0.001$), 10:0 ($P<0.01$) and 8:0 ($P<0.05$). Period means are tabulated in Appendix A-9 and presented below in ranked form with significant differences.



The content of 18:1 in milk fat was significantly lower in period 4 than all other periods except 5. The content of 16:0 rose fairly steadily across the lactation with the first four periods (Aug - Nov) significantly lower than later periods. The remaining components - 12:0, 10:0 and 8:0 all have means for period 4 considerably in excess of other periods. These differences are inversely related to those for the content of 18:1. Despite the confounding between seasonal periods and stages of lactation, analyses using model FA 3 were valuable in demonstrating how the two classes of acids varied together. The relationships are described further with the aid of correlations in 3.4.4.

The low content of 18:1 in milk fat of period 4 was examined using data from infra réd spectrophotometric analyses of the fat samples. The fat contents of milk from cows 19 and 118 (17 weeks p.p.), 5 (9 weeks p.p.) and 26 (7 weeks p.p.) were higher than in samples taken 14 days before and after (Fig. 3.6 b). The differences were less obvious for the first two cows which appeared lighter in condition. Fig. 3.8 shows the contents in milk fat of total octadecenoic acids and the two geometrical isomers according to seasonal periods (date of sampling). The marked fall in 18:1 content observed in period 4, which led to the significant period differences in fat composition, was more associated with the <u>cis</u> than the <u>trans</u> fraction. This

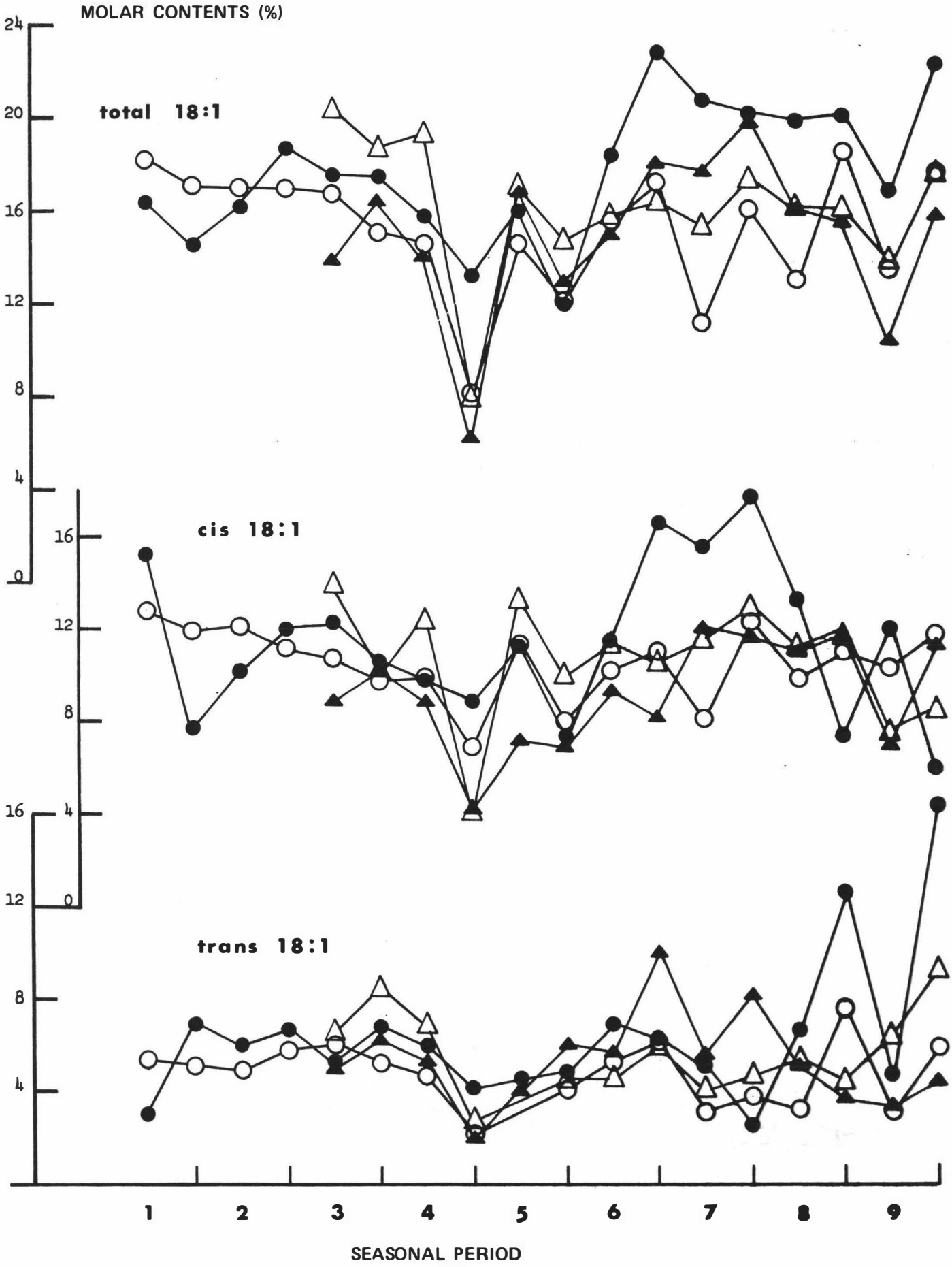


Fig. 3.8 Variations in the content of total, cis and trans octadecenoic acids in milk fat according to seasonal period.

(Symbols as in Fig. 3.1)

suggests an overall fall in the incorporation of octadecenoic acids in the gland which may have been related to hydrogenated polyunsaturated acids from the diet (trans), the depot 18:1 acids (cis and trans) or the dietary oleic acid and mammary desaturation of stearic acid (cis).

When analysed with cows and stages of lactation using model FA 4, there was no significant variation in 16:0 due to period differences although a significant ($P < 0.01$) variation in the content of 18:0 was identified.

3.4.4 Correlation coefficients between all pairs of fatty acid components were obtained from the linked statistical program described in 2.6 (Table 3.8).

Table 3.8 Correlation coefficients ($\hat{r}$) and % Coefficients of Determination* for pairs of milk fat fatty acids.

	18:0	16:0	14:0	12:0	10:0	8:0	6:0	4:0
18:1	.783 61.3%	.152 2.3%	-.566 32.0%	-.677 45.8%	-.701 49.2%	-.636 40.4%	-.778 60.6%	-.535 28.6%
18:0		.068 0.5%	-.566 32.1%	-.633 40.0%	-.616 37.9%	-.507 25.7%	-.609 37.0%	-.509 25.9%
16:0			-.147 2.2%	-.529 28.0%	-.615 37.8%	-.613 37.6%	-.588 34.5%	-.362 13.1%
14:0				.526 27.6%	.428 18.3%	.297 8.8%	.299 9.0%	.018 0%
12:0					.845 71.3%	.718 51.5%	.667 44.5%	.343 11.8%
10:0						.890 79.2%	.836 69.8%	.546 29.8%
8:0							.860 73.9%	.551 30.3%
6:0								.691 47.8%

* % Coefficient of Determination = $100 \cdot \hat{r}^2$

The correlation table is clearly divided into two parts. Acids of chain length 16C and 18C were negatively related to all shorter length acids and those of length less than 14C were all positively related by pairs. The two 18C were highly correlated but neither of them was related to 16:0 which was surprising considering the relationship all three held with the other acids. Correlations among the short and medium chain length acids 12:0, 10:0, 8:0 and 6:0 indicated that they varied essentially as a single component.

3.4.5 Summary

Taken together, the changes in fatty acid compositions indicated that:

> The content of long chain acids in milk fat varied between cows. This may have reflected differences in the contribution made by depot fatty acids to milk fat.
>
> Medium and long chain acids varied with stage of lactation. Palmitic acid content rose with advancing lactation whereas myristic acid content showed no regular change.
>
> Both the long chain length and short chain length acids varied among seasonal periods but patterns of differences among means and the correlations among pairs of components indicated considerable interdependence of the two groups of fatty acids. Whether the seasonal source of variation acted primarily on one group or the other could not be resolved statistically (see 4.2.3).

3.5 Relationships Between Milk Composition and Mammary Enzyme Activities.

A preliminary graphical examination of the milk and enzyme data indicated that the only obvious relationship was between the changes in milk fat content and the activities of acetate thiokinase.

3.5.1 Linear regressions were fitted on 28 day means of fat content (dependent variable) and estimates of the activities of acetate thiokinase (independent variable) taken from Fig. 3.5 b as described in 2.6. Reductions in total variation due to fitting regressions were variable and reached significance in three cases (Table 3.9).

Table 3.9 Simple linear regressions of milk fat content on acetate thiokinase activity (DNA).

Cow	n	$\hat{r}$	$\hat{b}$	$\hat{a}$	F	P
19	9	0.8524	0.0176	5.164	18.60	<0.01**
118	9	0.7414	0.0378	3.155	8.54	<0.05*
5	6	0.7103	0.0299	4.690	4.07	>0.10 ns
26	6	0.8678	0.0104	4.139	12.20	<0.05*
Common	29	0.5113	0.0125	5.093	9.91	<0.01**
Between Slopes					$F_{3,22}$ 1.80	>0.10 ns
Homogeneity of Variance					χ^2 = 2.6 (d.f. = 3)	>0.25 ns
Between Intercepts					$F_{3,25}$ 11.90	<0.01**

(For analyses of variance of regression - see Appendices A-10, A-11)

Differences between slopes were not significant so the common regression adequately described the relationship between changes in fat content and the activity of the enzyme. Highly significant differences in intercepts mean that the common line could not provide an estimate of fat content at zero enzyme activity. The distribution of enzyme activities (Fig. 3.9) was such that little error would be introduced by extrapolation to the ordinate. The differences in intercepts clearly arose from cow 26 which had the lowest fat content in milk throughout the experiment (see 3.3.1).

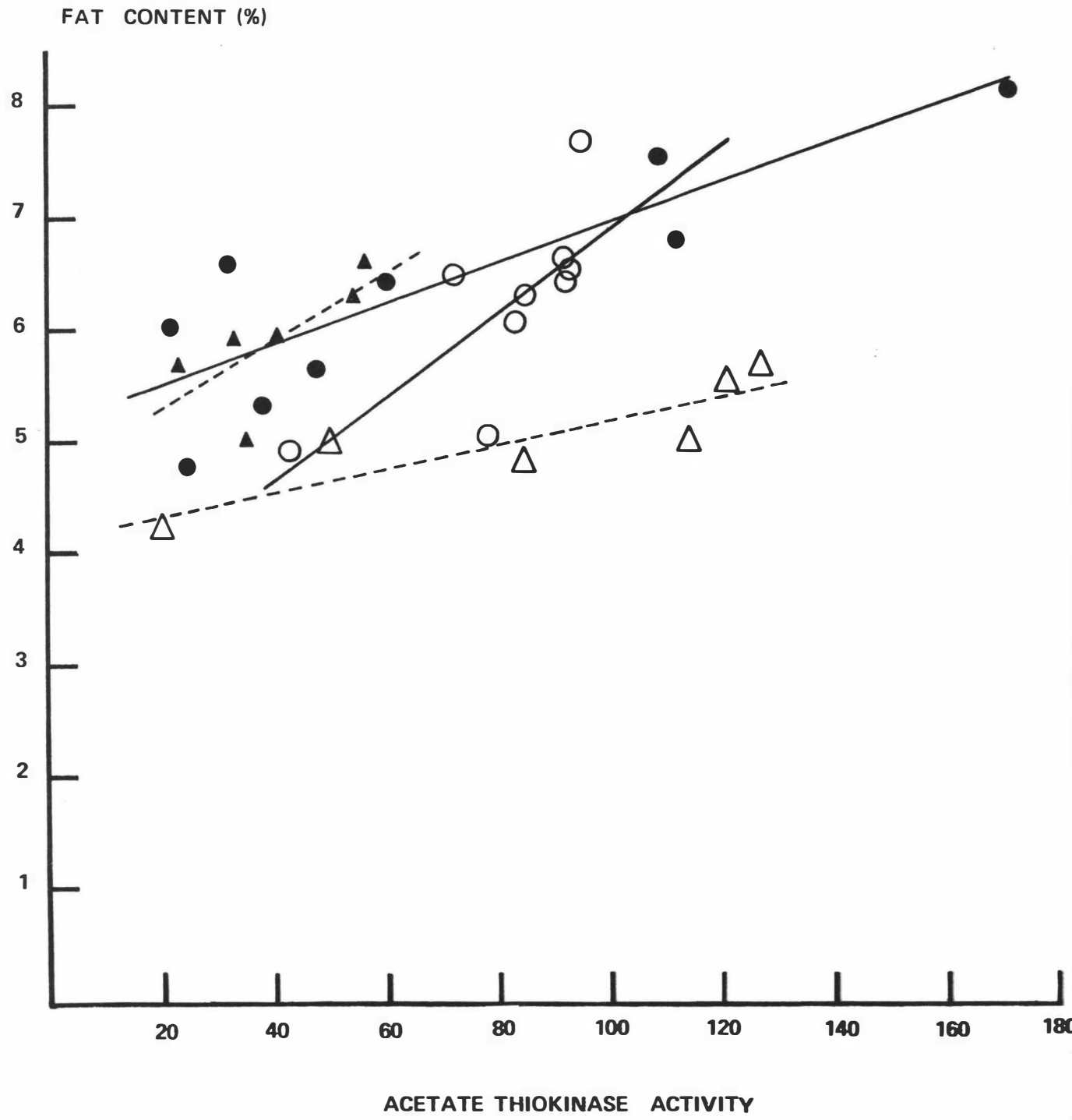


Fig. 3.9 Simple linear regressions of milk fat content on Acetate thiokinase activity.
(Symbols as in Fig. 3.1)

Deviation of intercepts from zero suggested that the fitted regressions explained variation in only part of the milk fat. Because the fat contains fatty acids which reached the gland preformed and should bear no relationship with mammary acetate thiokinase, further analyses were made on milk fat content adjusted for fatty acid composition as described below.

3.5.2 On the basis of the studies reviewed earlier in 1.7 and 1.17, the milk fat fatty acids can be considered as being of three groups: (a) those synthesised almost entirely within the gland (C4 - C14), (b) those reaching the gland preformed from the blood (18:0, 18:1) and (c) those, predominantly 16:0, associated with both (a) and (b) above. The proportion of 18:1 and 18:0 in milk fat followed a similar pattern of changes during the lactation (Fig. 3.7 and Table 3.8), so it was argued that the variation in that fraction of 16:0 arising from the same blood lipid source would be related to the variation in the 18C acids (see perfusion studies reviewed in 1.3). Individual components in (a) were positively correlated by pairs and it appeared likely that the fraction of 16:0 synthesised de novo would be similarly correlated with other acids in group (a). That fraction of 16:0 could then be estimated by:

$$\text{(I)} \qquad \frac{\%\,16{:}0}{100} \cdot \Sigma\left(\%4{:}0 + \%6{:}0 + \cdots + \%15{:}0\right)\%$$

and the total fatty acids synthesised de novo by:

$$\text{(II)} \qquad \left(1 + \frac{\%\,16{:}0}{100}\right) \cdot \Sigma\left(\%4{:}0 + \%6{:}0 + \cdots + \%15{:}0\right)\%$$

Since the components are interdependent and sum to 100% for given samples there is no appropriate test to evaluate the adjustment used above. Ignoring the minor weight contribution of glycerol, milk fat contents were adjusted according to (II) and used as the dependent variable in the following regression analyses (Table 3.10).

Table 3.10 Simple linear regressions of fat content (adjusted for fatty acid composition) on acetate thiokinase activity (DNA).

Cow	n	$\hat{r}$	$\hat{b}$	$\hat{a}$	F	P
19	9	0.8203	0.0073	2.914	14.39	<0.01**
118	9	0.5464	0.0204	1.819	2.98	>0.10 ns
5	6	0.4949	0.0163	2.835	1.30	>0.25 ns
26	6	0.8541	0.0081	1.941	10.79	<0.05*
Common	29	0.3221	0.0049	2.9497	3.24	<0.10
	Between Slopes				$F_{3,22}$ 0.80	>0.25 ns
	Homogeneity of Variance				χ^2 = 4.6 (d.f. = 3)	>0.10 ns
	Between Intercepts				$F_{3,25}$ 11.90	<0.01**

(For analyses of variance of regression - see Appendices A-12, A-13)

Comparison of the two sets of analyses (Tables 3.9, 3.10) shows that for all cows there was a fall in the extent to which regressions on acetate thiokinase activities explained variation in fat content when the latter was adjusted to those fatty acids synthesised within the gland. Significant differences exist between the intercepts for the four cows, but overall comparison of the lines show that elevations were reduced approximately two fat percentage units by the adjustment. The fraction of fat content still not accounted for possibly arose from non-acetate substrates such as the 4C acids. These substrates would be expected to contribute to short and medium chain length acids (e.g. Sum [%4:0 + %6:0 + ... + %15:0]) but make a negligible contribution to any of the medium to long chain acids considered alone. In an attempt to determine this possibility, two further regressions were fitted using as the dependent variable (a) the content in milk of 14:0 and (b) the content in milk of all acids of chain length less than 16C (Table 3.11).

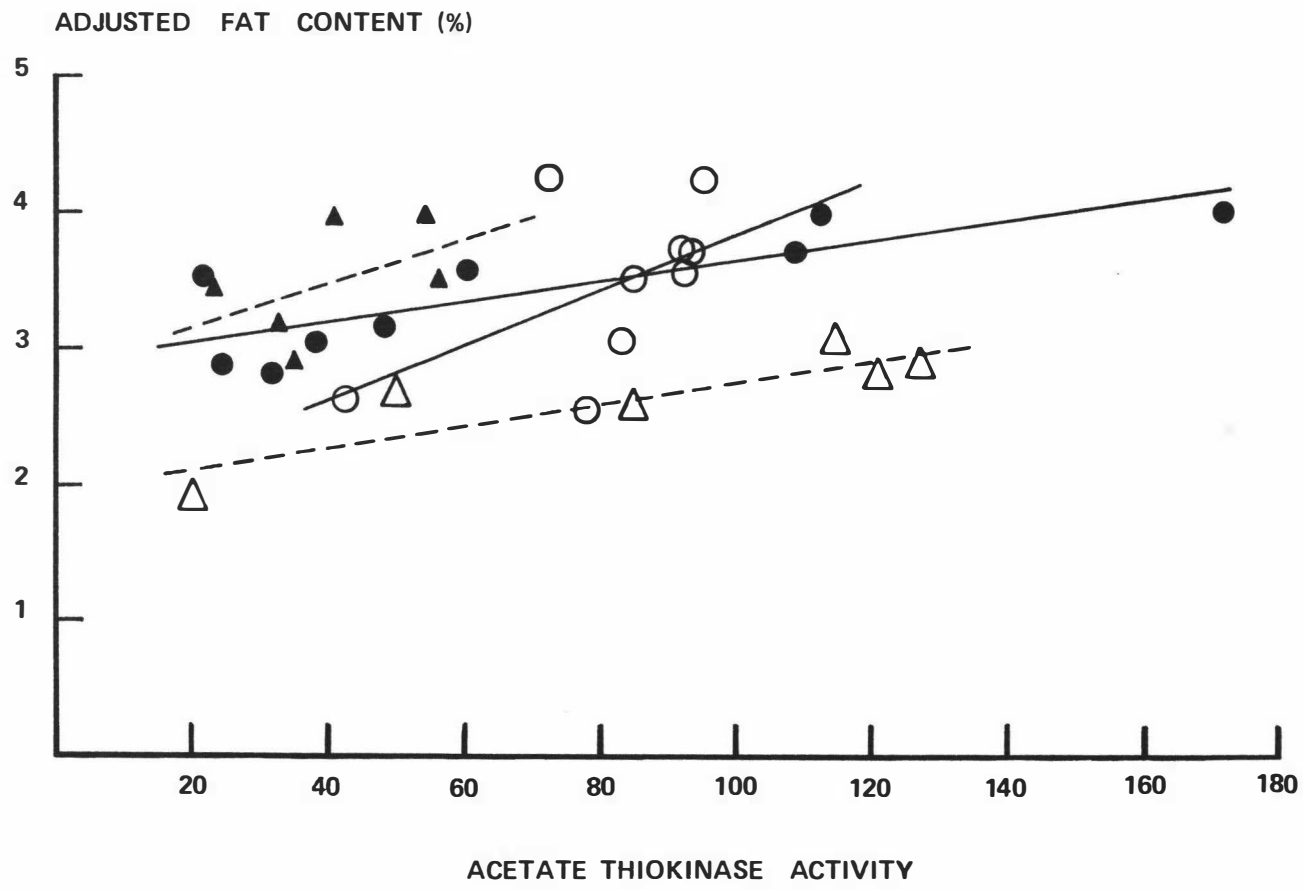


Fig. 3.10 Simple linear regressions of milk fat content (adjusted to fatty acids synthesised de novo) on Acetate thiokinase activity.
(Symbols as in Fig. 3.1)

Table 3.11 (a) Simple linear regressions of the content of 14:0 in milk on acetate thiokinase activity (DMA).

Cow	n	$\hat{r}$	$\hat{b}$	$\hat{a}$	F	P
19	9	0.8285	0.0025	0.815	15.33	<0.01**
118	9	0.6913	0.0079	0.324	6.41	<0.05*
5	6	0.4194	0.0032	0.825	0.85	>0.25 ns
26	6	0.7317	0.0021	0.562	4.61	0.10

(b) Simple linear regressions of the content of acids chain length less than 16C in milk on acetate thiokinase activity.

Cow	n	$\hat{r}$	$\hat{b}$	$\hat{a}$	F	P
19	9	0.7541	0.0048	2.311	9.23	<0.05*
118	9	0.4331	0.0017	1.703	1.62	>0.10 ns
5	6	0.4513	0.0115	2.213	1.02	>0.25 ns
26	6	0.4876	0.0032	1.585	1.25	>0.25 ns

(For analyses of variance of regression - see Appendices A-14, A-15)

The correlations in Table 3.11 indicated that variations in the dependent variable in (a) were somewhat better explained by the regressions than was the dependent variable in (b).

3.5.3 Summary:

Part of the variation in the content of fat in milk could be explained by linear regressions on the activity of mammary acetate thiokinase.

Adjustment of milk fat content for the fraction of fatty acids derived from the blood lipids did not improve the relationship between the two variables but did account for a portion of estimated fat content at zero enzyme activity.

Another part of the fat content remaining unexplained may reflact fatty acids synthesised from substrates other than acetate.

Chapter Four DISCUSSION

4.1 Discussion of Methods Used

4.1.1 Design

The major factor influencing the design of the experiment was the time and facilities available to the author (see 2.1). Some six months were spent in preliminary work and even then, the assay of one enzyme - acetyl CoA carboxylase - was not developed sufficiently to be used during the experiment. Only two cows could be sampled each day and, by operating on two consecutive days, enzymes from four cows could be assayed on the two days of sampling and the DNA extracts prepared for analysis on the third.

Variations in milk composition arising from changing nutrition were expected, so cows calving at two dates were chosen in an attempt to separate effects due to differences in stages of lactation (physiological) from those due to season (mainly nutrition). Despite this attempt, the calving interval between the two groups was not sufficient for the serious underfeeding in the late summer to occur at effectively different stages of lactation. An alternative approach, to house the cows and feed a diet changing only in quantity according to production, would have ignored an important source of variation.

4.1.2 Surgical Biopsy Method

The method described herein appeared suitable for repeatedly obtaining small (0.5 - 1.0 g) samples of tissue from cows' udders without seriously influencing milk production. Previously published methods (Hibbit, 1964; Marx & Caruolo, 1963) enabled much larger samples to be obtained but the severe depression in milk production which followed surgery prevented their use in the present study where a reliable measure of milk production was required. The method described was not evaluated with respect to obtaining a wholly representative sample of the total secretory mass. This would have involved multiple samplings of all quarters preferably in conjunction with excision of the gland, its dissection and random sampling of the secretory parenchyma. The author could not justify the sacrifice

of one or more lactating cows to meet this requirement. The symmetry of changes in enzyme activities over the early stages of lactation suggested that the samples taken might well have been representative of the gland as a whole, but caution was required with all inferences drawn from the biochemical data (see below).

In general, the extent of difficulties encountered during individual operations was reflected in the degree by which milk production in the operated quarter was depressed in the following few days. The main requirement from a surgical standpoint was to avoid regions of heavy vascularisation as complete haemostasis was difficult to effect. The forequarters in the region described were sampled for this reason but another suitable site would be the mid- and lower-caudal region of the udder as used by Hibbit. Regeneration of fibrous tissue into biopsy sites is rapid (Marx & Caruolo, 1963) hence previously operated sites need to be avoided (c.f. 3.1.1).

4.1.3 Enzyme Assays

Optical methods, modified from the originals by increased levels of substrates and cofactors, were suitable for product accumulation assays in the present study. The major difficulty in the use of enzyme determinations performed *in vitro* lies in interpretation to the overall pathway or *in vivo* situation. The mammary gland comprises several different cell types - secretory epithelium, adipose, vascular and connective elements - and information is required solely on the first of these. All tissues contribute to the total enzyme 'pool' according to the tissue composition and the enzyme complements of each. As a result, prediction of ratios of two or more enzyme activities in the tissue of interest would be quite unreliable unless it is known *a priori* (a) that the enzymes are confined to the given tissue or (b) that changes in the activities occur only in that tissue. The first condition is met in lactating mammary tissue at present only for selected enzymes of lactose synthesis, but the second condition can probably be assumed for the enzymes of the major synthetic pathways.

Mammary enzymes are subject to both activation and induction controls (Emery & Baldwin, 1966) so the interpretations of changes and

their causes is made more difficult. Emery & Baldwin attempted to measure turnover rates of mammary enzymes in rats previously injected with C^{14}-labelled amino acids, using immunological separation of enzyme proteins. The purity of the enzymes used to raise the antisera and hence the specificity of the latter were not firmly established so their results may not be reliable but the technique could be developed as a useful adjunct to enzyme assays, performed *post mortem* as in the rodent species. Another supplementary approach involves the measurement of tissue metabolite levels (Baldwin *et al*, 1969) but when considered alone, these methods have interpretive difficulties similar to those discussed above for the enzyme assays.

4.1.4 DNA Determinations

The modified Burton (1956) procedure was quite satifactory for the measurement of deoxyribonucleic acid in bovine mammary tissue. Munford (1963) found no interference with the determination from a range of sugars which could be present either intracellularly or in retained milk. DNA is the most suitable base for the expression of enzyme activities in this type of study as the ratio obtained approximates a per cell 'content'. Other bases such as the wet or dry weight of tissue, extractable protein or total nitrogen are less satisfactory because of variable amounts of retained milk present in the tissue sample. Although corrections, based on lactose content, have been used to account for retained milk, they are not entirely satisfactory for analytical reasons (Munford, 1966) and there remains a problem in partitioning the lactose in retained milk from that present intracellularly in non-secreted form.

Similar comments to those discussed in 4.1.3 apply when using DNA as a metabolic base. If cells not contributing to the enzyme 'pool' increase in number, DNA content rises and enzyme activities, as ratios, fall. Between sample errors can then arise through changing tissue composition. Other criticisms of the use of DNA as an index of mammogenic and metabolic status, more fully discussed by Munford (1964) and Jones (1969), are not relevant to the use made of DNA levels in the present study.

4.2 Discussion of Experimental Results

4.2.1 Changes in Biochemical Parameters

The fall in the content of DNA in biopsy samples following calving probably reflects hypertrophy of existing cells rather than hypoplastic changes in the gland as a whole. The hypertrophy could have accompanied post-partum development of mammary cells and an increase in cytoplasm with the establishment of maximum secretory effort. The increases in DNA content which followed from above indicates either (a) decreases in cell size associated possibly with diminishing secretory activity or (b) possible increases in cell numbers with advancing lactation.

Changes in cell dynamics have not been characterised in the lactating mammary glands of ruminants despite a large research effort in the rodent species (Munford, 1964; Traurig, 1967; Tucker, 1969). Pulse labelling of mammary DNA by injecting H^3-thymidine as used by Traurig, with a surgical biopsy method such as that described here could be combined to obtain some estimates of mitotic activity in lactating udders of farm animals.

The changes in enzyme activities reported in 3.2.2 are the first determinations made through complete lactations in cows. Baldwin (1966) measured DNA content and the activities of hexokinase, glucose-6-phosphate dehydrogenase, UDPglucose-4'-epimerase and glycerol phosphate dehydrogenase inter alia in bovine mammary samples taken early in lactation. Heifers were sampled using Hibbit's (1964) method 14 days pre-partum, at parturition and at 3, 10 and 35 days post-partum. Results were presented as means for 2 - 11 determinations at each stage and Baldwin found no significant differences (methods of testing not stated) in enzyme activities over the first 35 days of lactation. He compared the ratios of the activities of UDPglucose-4'-epimerase: fatty acid synthetase in mammary extracts from rats, guinea pigs and cows with ratios of lactose:fat contents in milk (values taken from Biochemists' Handbook - Long, 1961) and claimed a correlation among the pairs of ratios. For the reasons advanced in 4.1.3, the present author considers that this type of comparison is unlikely to yield useful information on in vivo relationships. Baldwin's results

agree in part with those of the present study insomuch that there were no significant changes in enzyme activities over the first two stages, corresponding roughly to Baldwin's last two samples.

Longer term changes did occur in the activities of all enzymes (see 3.2.2) although differences among stages of lactation failed to reach significance except in the case of hexokinase. Differences between cows were statistically significant for three enzymes indicating the dangers in using mean values obtained from several animals. Despite the lack of statistical significance, the symmetry of changes illustrated in Fig. 3.5 a suggests changes in the capacity of mammary tissue to metabolise glucose at different stages of lactation. There were no obvious relationships between changes in any of the three enzymes' activities and milk composition with stages of lactation. When data were considered together within cows, the differences among cow means for the activity of UDPglucose-4'-epimerase corresponded to the differences among cow means for lactose content in milk (see 3.2.2 and 3.3.1). The enzymes whose activities are shown in Fig. 3.5 b followed less regular changes but despite the lack of significant differences among stages of lactation, positive relationships were established between acetate thiokinase activities and changes in milk fat content for all cows. Glucose-6-phosphate dehydrogenase, the enzyme concerned generally in synthetic processes (see 2.1) was related on a cow basis with whole lactation means for fat and protein contents. There were no relationships between the activity of the dehydrogenase and any of the milk constituents when compared on a stage of lactation basis.

Since it is not generally believed that UDPglucose-4'-epimerase or glucose-6-phosphate dehydrogenase activities are rate limiting for the synthesis of any of the milk constituents, it was surprising to find even the weak relationships indicated above. General agreement that acetate is the major lipogenic substrate in the ruminant mammary gland (Folley & McNaught, 1961) and is of great importance in the energy metabolism of these species (Blaxter, 1962) highlights the possible role of acetate thiokinase as a regulator. There are no known physiological controls operating on any of the thiokinases which is surprising in light of their importance in lipid

metabolism in all mammalian species (Fritz, 1961). If the nutritional concept (Rook, 1961) that acetate availability is limiting for milk fat synthesis has any substance, it might be expected that the activity of acetate thiokinase in mammary tissue could be rate limiting for the synthetic pathway. It must be stressed however, that all available evidence, albeit from rodents (Jones, 1969), indicates that acetyl CoA carboxylase is the limiting enzyme and failure to measure its activity in the present experiment severely restricts further discussion of this interesting question

4.2.2 Changes in Milk Production

The aim in selecting cows likely to have had divergent milk yields and compositions was obviously met (see 3.3.1). Variations in milk production were analysed on a cow and stage of lactation basis for comparisons with the biochemical data and on a seasonal basis in an attempt to adjust for the marked effect of changing nutrition on the contents of fat and protein in milk. Variations arising from differences among stages of lactation and seasonal periods were however confounded to an indeterminate degree as discussed in 4.1.1. The changes in milk yield and composition with stage of lactation, ignoring seasonal effects, were fairly typical of cattle grazing pasture (Johansson & Claesson, 1957) except that the content of fat and protein rose sharply from shortly after calving. Overriding the pattern of changes with advancing lactation was the sharp elevation in fat contents over the last three seasonal periods. Milk fat compositions (see below - 4.2.3) and general observation indicated that the changes in fat content were the result of a combination of chronic underfeeding and drying off since the content of protein in milk rose rather than fell over those periods.

4.2.3 Changes in Milk Fat Fatty Acid Compositions

Since variations in the composition of milk fat from different cows were expected, analyses were made on samples from individuals and not on pooled fats as in the studies of Decaen & Adda (1966) and Stull *et al* (1966). Differences in compositions for individual cows were identified (see 3.4.1) and the major components involved

were the 18C acids derived from the blood lipids. Since there was no reason to believe that the cows received different nutritional treatment during the experiment, the results indicate either (a) cow to cow variation in the contribution made by depot acids to milk fat or (b) variations in the mammary utilisation of these acids. Wide variations in milk fat content among cattle according to breeds, ages and individuals are well recognised and accordingly it is reasonable to expect genetically based differences in lipid metabolism amongst individuals. The glucose - insulin - lipolysis inhibition theory of McClymont & Vallance (1962) discussed in 1.14, is an attractive explanation for the effects of 'fat depressing diets' and the physiological mechanism actively involved in the response could vary from animal to animal. Variations in the content in milk fat of the 18C acids in the present experiment could be associated with differences in the extent of depot reserves, their exposure to and/or responsiveness to lipolytic influences.

Changes in fat composition following calving did not follow the smooth patterns provided by the data of Decaen & Adda (Fig. 1.2), but the cows in the French study were calved in high condition (620 Kg) and fed under very controlled conditions during lactation. The somewhat higher proportions of 18:1 and 18:0 in milk fat after the initial stages in the present experiment are consistent with the generally lower plane of nutrition and presumably greater contribution, over a longer time, of body fat fatty acids to milk fat. The increased content of this fraction in the late summer and early autumn periods were used to confirm that the changes in milk composition at that time were partly due to underfeeding. The most consistent change in the milk fat componenets with advancing lactation was the increase in the content of palmitic acid (16:0). This was similar to the results of Decaen & Adda (Fig. 1.2) but quite different from those of Stull *et al*. The marked seasonal influence on the content of practically all components arose from the abnormal compositions recorded in 3.4.3 and Fig. 3.8. The compositions, with low proportions of long chain length acids, were similar to those seen on fat depressing diets (McCarthy *et al*, 1966) but elevated fat contents discount this type of nutritive cause. Since 18:1, 18:0 and 16:0 were the acids most obviously affected, reciprocal changes in the content of shorter chain length acids were considered secondary (see 1.16). The infra-

red spectrophotometric data indicated that the cis-unsaturated rather than the trans-unsaturated fraction was responsible for the fall in total octadecenoic acids. Since there are no known factors affecting mammary desaturation (see 1.3) other than the availability of substrate (mainly 18:0), the results indicate either a profound change in the diet or an altered lipid metabolism of the cows. No record was made of day to day feeding conditions but the direct relationship between the extent of changes in composition and the condition of the cows (3.4.3) favours the second possible explanation. Changes in lipid metabolism involving lipolysis in adipose tissue without drastic dietary changes could still explain the less marked fall in the content of the trans-unsaturated acids since the depot lipids contain the trans isomer of octadecenoic acid (Hilditch, 1956).

Weather records collected by D.S.I.R. Grasslands Division showed strong to gale force NW winds on the day before and day of sampling the milk with abnormal compositions. The similarity of changes in fat composition with the changes in fat properties which followed the injection of ACTH to grazing cattle (Campbell, Davey, McDowall, Wilson & Munford, 1964) suggested that changes in fat composition may have been the result of some type of 'stress' - possibly associated with the strong wind. Further study is required to examine the mode of action of 'stress' factors in changing the composition of milk as the lipolytic action of ACTH in ruminants (Radloff & Schultz, 1966) would be expected to elevate the content of the depot derived acids in milk fat leading to high iodine values, and not the reverse as was the case in the study of Campbell et al and probably in the present study.

Results from the present study clearly illustrate the marked sample to sample variation in the composition of milk fat from grazing cattle. Whilst variations in pasture composition could be expected to affect the composition of fat through (a) the provision of long chain length acids derived from the pasture lipids (Hawke, 1963), (b) the availability of lipogenic substrates at the mammary gland (Rook, 1961) and (c) adjusting, through indirect mechanisms (McClymont & Vallance, 1962), the equilibrium of fatty acids into and out of the adipose tissue, an overriding effect of quantity of pasture and hence

the extent of underfeeding may well interact with the three causes above. This study highlights the need for suitable statistical techniques to aid the detection of changes in fat composition and more detailed information on the mechanisms underlying the changes.

4.2.4 Relationships between changes in Enzyme Activities and the Composition of Milk

The relative infrequency of biopsy samplings and the wide range of errors of interpretation (discussed in detail in 4.1.3) were factors influencing the enzyme - milk composition comparions. However the data collected over whole lactations indicated some differences among cows in the ranking of means of enzyme activities and the milk components. It was tempting to interpret the relationship between UDPglucose-4'-epimerase and lactose content (4.2.1) in a causal sense although all studies conducted on the rodent mammary gland (Kuhn 1967; 1968; Jones, 1969) suggest that lactose synthetase and not the epimerase is rate limiting for the synthetic pathway. The lack of change in lactose content at various stages of lactation despite large, though not significant, differences in the activity of the enzyme is further support against a causal relationship between the two variables. Similar comments to those above apply to the weak relationship between cows, but not stages of lactation, in the activity of glucose-6-phosphate dehydrogenase and the contents of fat and protein in milk.

The clearest relationship was between the activity of acetate thiokinase and the content of fat in milk (see 3.5) where significant fractions of the variation in fat content were accounted for by linear regressions on the activity of the enzyme. The sizeable intercepts were reduced by adjusting milk fat content for the fraction of fatty acids calculated to have reached the gland preformed from the blood lipids. The adjustment used (3.5.2) appears similar to the method employed by Decaen & Adda (1966) and in both cases fat contents were reduced approximately 50%. Because of the large sample to sample variation in milk fat composition (4.2.3), the efficacy of the adjustment was limited and may underlie the loss in degree of

correlation in Table 3.10 rather than the increase which was expected. Similarly, the attempts to relate fractions of the acids synthesised within the gland with the enzyme (c.f. Bartley et al, 1967) were of little value. There was however a suggestion that a fraction of the fatty acids synthesised de novo may have resulted from the use of substrates other than acetate.

The similarity of regressions on a within cow basis did suggest a causal relationship between the activity of acetate thiokinase and milk fat content. Since acetate is considered to be of major importance in the metabolic economy of ruminants (4.2.1), this part of the study merits repeating especially if assays for acetyl CoA carboxylase and enzymes of the pathways incorporating 4C precursors (see 1.1) could be performed.

4.3 Conclusions

This thesis describes a new biopsy technique suitable for repeated samplings of mammary tissue from lactating cows. During the experiment, some sampling precision was sacrificed so that deleterious effects on milk production were minimised. The content of deoxyribonucleic acid and the activities of five enzymes, concerned in major synthetic pathways for milk solids, were measured in tissue samples collected at 28 day intervals throughout lactations of four cows. These parameters were examined for relationships with estimates of milk production obtained at 14 day intervals throughout the experiment.

Differences between cow lactation means for enzyme activities bore weak relationships with differences between cow's milk compositions. One enzyme - acetate thiokinase - was related, on a stage of lactation basis, to changes in milk fat content. The possibility of this being a causal relationship has been discussed in light of published results. Two main factors influenced the enzyme - composition comparisons: (a) the validity of the interpretation of enzyme activity estimates in an *in vivo* sense and (b) the marked effect on milk production of changing nutrition especially towards the end of the experiment. The first difficulty might have been partly overcome by a more rigorous sampling routine at the expense of reliable milk production (see above), but problems involved in arguing from the determinations made *in vitro* to the corresponding biochemical pathways *in vivo* present the greatest difficulties to this type of experimental approach (see 4.1.3 and 4.1.4). Attempts were made to accomodate changing nutrition in the design and statistical analyses but the confounding of seasonal periods and stages of lactation made a clear separation impossible. As a result, statistical probabilities came largely from analyses based on sources of variation considered one at a time, ignoring others until inferences were drawn using the partly confounded results of separate and combined analyses. Errors in interpretation, which resulted from this compromise, must have influenced the precision of the comparisons.

Detailed analyses of milk fat were undertaken to assist in the interpretation of changes in milk composition and to give estimates of

the fraction of milk fat fatty acids synthesised de novo by the mammary gland. Nutritional changes throughout the experimental period led to quite variable milk fat compositions, probably reflecting the contribution of depot reserves to milk fat. Statistical analyses of changes in composition are not readily interpreted and the extent of sample to sample variation in all milk parameters measured, restricted the use of more straightforward analyses of fatty acid yields.

Controlled indoor feeding would best satisfy part of the requirements of this type of study by removing nutritional fluctuations. For reasons advanced in 4.1.1, controlled feeding conditions would have ignored an important source of variation and animals would have to be studied much more intensively to justify the extra resources employed. The areas encountered in the present experiment where additional information would have aided interpretation, viz cell dynamics in the lactating udder and the identity of rate limiting enzymes in the bovine gland, would still hinder the more controlled experiment above.

Provided information on these two points was made available, the most promising field for a causal relationship approach to the lactating mammary gland and its secreted products would appear to be in further study of the enzymes of lipogenesis and the nature of the milk fat so produced.

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Appendix A-1 Determination of Isolated *trans* - Unsaturation in 'Trielaidin' Reference Material

A sample of 'Trielaidin' was methylated and analysed by gas-liquid chromatography as described in 2.5.3 except that the column was operated isothermally at 185°C. The resulting chromatograph is presented in Fig. A-1 (i). In view of the contamination by saturated and other unsaturated acids, the mixture of esters was partitioned into saturated, *trans* - unsaturated and *cis* - unsaturated components on thin layers of $AgNO_3$ impregnated silica gel (Morris, 1962). Plates were layered with $AgNO_3$: silica gel G (Merck) : water (6.6 : 30 : 60), dried and activated at 110°C for 1 hour. Esters were applied along with methyl stearate, methyl oleate and methyl elaidate standards and the plates developed in hexane : diethyl ether (90 : 10). Plates were sprayed with 0.2% (w/v) 2'7' dichlorofluoroscein in ethanol and fractions marked under UV light.

Three portions of the plates containing the saturated, *trans* and *cis* esters were scraped and eluted three times with diethyl ether. After evaporating the ether, the esters were taken up in heptane, dried over anhydrous Na_2SO_4 and analysed by GLC as above. The resulting chromatographs are presented in Fig. A-1 (ii), (iii) and (iv). Data from the four graphs were combined as in Table A-1 and an estimate of 79.4% isolated *trans* was obtained.

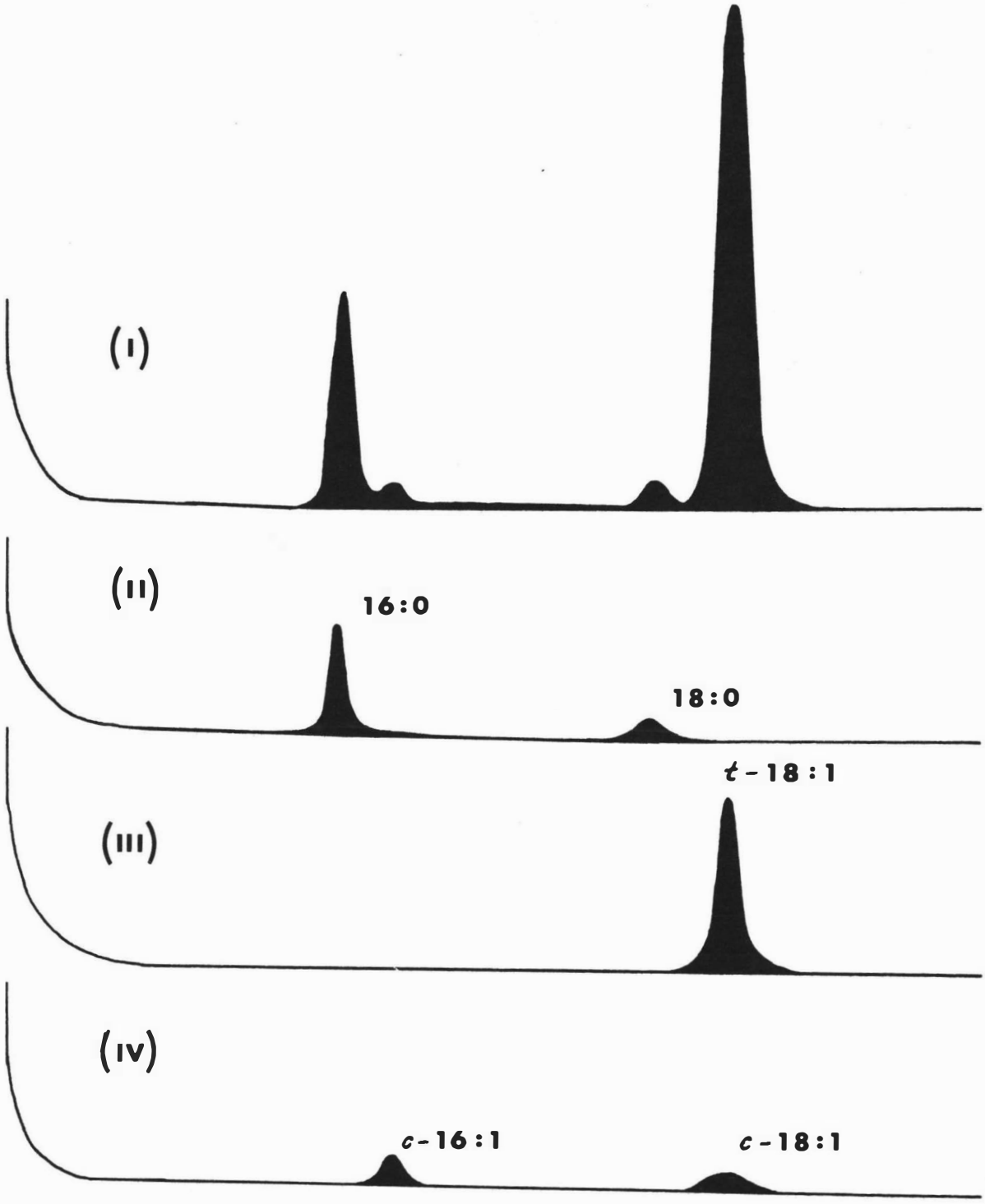

Fig. A-1 Chromatograms of fatty acid methyl esters in 'Trielaidin' Infra-Red reference standard.

(i) Total methyl esters.

(ii) Saturated methyl esters.

(iii) trans - unsaturated methyl esters.

(iv) cis - unsaturated methyl esters.

Table A-1 Estimation of Weight Percentage trans-18:1 in Trielaidin Reference Material

From Fig. A-1 (i) Total Fatty Acid Methyl Esters

16:0	16.63%		
16:1	0.45%	$\frac{16:0}{18:0}$	= 6.38
18:0	2.61%		
18:1	80.31%		

(ii) Saturated Fatty Acid Methyl Esters

16:0	85.45%	$\frac{16:0}{18:0}$	= 6.35
18:0	13.61%		

(iii) trans-Unsaturated Fatty Acid Methyl Esters

18:1	100%

(iv) cis- Unsaturated Fatty Acid Methyl Esters

16:1	33%	$\frac{18:1}{16:1}$	= 2
18:1	67%		

Since all 16:1 is of the cis configuration, the product of the ratio from (iv) and the weight percentage of 16:1 in (i) gives the weight percentage of cis 18:1 in (i).

$2 \times 0.45 = 0.9$ cis 18:1

and $80.31 - 0.9 = 79.4$ trans 18:1

Estimated Weight Percentage Composition of Reference Material	
16:0	16.63 %
c 16:1	0.45 %
18:0	2.61 %
c 18:1	0.90 %
t 18:1	79.4 %

Appendix A-2 Cow Means for Enzyme Activities analysed on ENZ 1

$$Y_{il} = \mu_{il} + IA_i + e_{il}$$

Cow (index)	n	Acetate Thiokinase	UDPG Epimerase	Glycerol P Dehydrog.	Hexokinase	Gluc. 6 P Dehydrog.
19 (1)	9	68.82	457.10	487.22	97.98	195.76
118 (2)	9	80.39	752.56	966.93	244.29	204.51
5 (3)	6	40.68	135.82	188.70	75.48	59.13
26 (4)	6	86.47	340.68	324.77	106.53	51.87

Appendix A-3 Stage of Lactation Means for Enzyme Activities analysed on ENZ 2

$$Y_{jl} = \mu_{jl} + K_j + e_{jl}$$

Stage of Lactation	n	Acetate Thiokinase	UDPG Epimerase	Glycerol P Dehydrog.	Hexokinase	Gluc. 6 P Dehydrog.
1	2	33.90	357.75	395.50	89.45	174.85
2	4	43.23	308.85	327.15	119.28	189.53
3	4	71.88	783.75	1123.28	546.60	134.75
4	4	63.50	683.00	767.70	61.90	235.58
5	4	67.05	240.93	252.50	51.78	65.45
6	4	73.05	318.68	379.18	48.13	89.95
7	3	71.33	208.53	277.90	30.50	63.93
8	2	101.10	594.85	595.35	46.85	244.75
9	2	133.30	560.90	493.30	126.70	85.95

Appendix A-4 Cow Means for Milk Production Variables analysed on MYC 1

$$\bar{Y}_{il} = \mu_{il} + IA_i + e_{il}$$

Cow (index)	n	Milk Yield lbs	Fat Content %	Protein Content %	Lactose Content %	Fat Yield lbs	Protein Yield lbs	Lactose Yield lbs
19 (1)	18	21.455	6.592	4.881	5.289	1.359	1.017	1.139
118 (2)	18	20.828	6.409	4.902	5.127	1.308	1.011	1.071
5 (3)	15	32.113	5.663	4.317	4.776	1.771	1.371	1.543
26 (4)	14	41.329	5.146	3.936	5.224	2.065	1.599	2.171

Appendix A-5 Stage of Lactation Means for Milk Production Variables analysed on MYC 2

$$Y_{jl} = \mu_{jl} + K_j + e_{jl}$$

Stage of Lactation	n	Milk Yield lbs	Fat Content %	Protein Content %	Lactose Content %	Fat Yield lbs	Protein Yield lbs	Lactose Yield lbs
1	2	44.550	3.990	4.330	4.985	1.805	1.915	2.235
2	8	36.638	4.663	4.009	5.118	1.678	1.443	1.893
3	8	36.900	5.535	4.234	5.190	2.019	1.539	1.923
4	8	33.288	5.586	4.321	5.208	1.831	1.413	1.734
5	8	30.188	6.013	4.455	5.123	1.771	1.310	1.543
6	8	28.163	6.331	4.508	5.098	1.759	1.241	1.433
7	8	22.275	5.998	4.584	5.093	1.323	1.005	1.126
8	7	18.086	6.787	4.893	4.920	1.229	0.886	0.890
9	4	14.975	7.325	5.270	5.180	1.105	0.790	0.775
10	4	11.550	8.303	5.770	5.005	0.955	0.668	0.580

Appendix A-6 Seasonal Means for Milk Production Variables analysed on MYC 3

$$Y_{kl} = \mu_{kl} + L_k + e_{kl}$$

Seasonal Period	n	Milk Yield lbs	Fat Content %	Protein Content %	Lactose Content %	Fat Yield lbs	Protein Yield lbs	Lactose Yield lbs
1	4	26.300	4.968	4.228	5.275	1.308	1.110	1.388
2	5	30.920	5.282	4.482	5.124	1.596	1.388	1.582
3	8	36.413	5.101	4.235	5.139	1.780	1.504	1.864
4	8	35.438	6.005	4.523	5.207	2.048	1.543	1.848
5	8	32.000	5.889	4.489	5.224	1.814	1.386	1.674
6	8	27.013	5.791	4.350	5.165	1.509	1.121	1.388
7	8	26.188	6.418	4.475	5.036	1.620	1.131	1.319
8	8	21.975	6.494	4.743	5.044	1.369	1.000	1.104
9	8	16.175	7.393	5.265	4.878	1.146	0.825	0.788

Appendix A-7 Cow Means for Milk Fat Fatty Acid Molar Components analysed on FA 1

$$Y_{il} = \mu_{il} + IA_i + e_{il}$$

Cow (index)	n	18:1 %	18:0 %	16:0 %	14:0 %	12:0 %	10:0 %	8:0 %	6:0 %	4:0 %
19 (1)	18	17.787	10.321	29.341	15.482	6.161	5.740	2.231	3.205	4.734
118 (2)	18	15.219	9.294	31.932	15.583	5.793	5.841	2.298	3.577	5.014
5 (3)	14	14.955	7.361	30.420	16.414	6.403	6.114	2.490	3.829	5.161
26 (4)	14	16.106	11.134	31.486	14.950	5.439	4.949	2.151	3.328	5.150

Appendix A-8 Stage of Lactation Means for Milk Fat Fatty Acid Molar Components analysed on FA 2

$$Y_{jl} = \mu_{jl} + K_j + e_{jl}$$

Stage of Lactation	n	18:1 %	18:0 %	16:0 %	14:0 %	12:0 %	10:0 %	8:0 %	6:0 %	4:0 %
2	9	17.247	12.197	27.512	14.871	5.631	5.820	2.380	3.474	5.683
3	8	14.175	9.491	27.993	17.000	7.123	6.875	2.838	3.975	5.138
4	8	15.895	9.993	30.738	16.104	6.189	5.795	2.228	3.330	4.638
5	8	14.579	8.539	30.144	16.450	6.598	6.436	2.650	3.918	5.543
6	8	15.770	8.710	32.763	16.036	5.748	5.310	2.193	3.369	4.473
7	8	16.959	9.376	33.493	14.568	5.533	4.626	1.874	3.044	4.506
8	7	16.043	8.083	32.334	14.767	5.349	5.394	2.126	3.650	5.574
9	4	17.965	9.323	30.750	15.563	5.603	5.440	2.253	3.203	4.255
10	4	17.640	9.873	32.908	14.385	5.230	4.775	1.735	2.890	4.565

Appendix A-9 Seasonal Period Means for Milk Fat Fatty Acid Molar Components analysed on FA 3

$$Y_{kl} = \mu_{kl} + L_k + e_{kl}$$

Seasonal Period	n	18:1 %	18:0 %	16:0 %	14:0 %	12:0 %	10:0 %	8:0 %	6:0 %	4:0 %
1	4	16.595	11.015	27.145	14.903	5.655	6.143	2.493	3.735	7.045
2	4	17.278	11.438	27.775	16.343	6.088	6.068	2.245	3.360	4.420
3	8	17.070	11.339	28.071	15.845	6.376	6.063	2.349	3.310	4.483
4	8	12.289	7.953	28.608	16.625	7.825	7.530	3.226	4.549	5.838
5	8	14.543	9.306	32.635	16.876	6.028	5.635	2.195	3.368	4.616
6	8	17.426	10.479	30.648	14.976	5.498	5.300	2.206	3.305	4.861
7	8	17.349	9.001	34.040	14.800	4.751	4.688	1.966	3.058	4.735
8	8	16.968	8.670	32.738	15.339	5.554	5.093	2.129	3.270	4.603
9	8	16.040	8.526	32.003	14.699	5.718	5.005	1.871	3.336	5.110

Appendix A-10 Analyses of Variance of Regressions of Milk Fat Content on Acetate Thiokinase Activity

Cow 19

Source	d.f.	SS	MS	F	P
Total	8	8.9398			
Regression	1	6.4955	6.4955	18.60	< 0.01**
Deviations	7	2.4442	0.3492		

Cow 118

Source	d.f.	SS	MS	F	P
Total	8	5.6486			
Regression	1	3.1047	3.1047	8.54	< 0.05*
Deviations	7	2.5438	0.3634		

Cow 5

Source	d.f.	SS	MS	F	P
Total	5	1.4737			
Regression	1	0.7435	0.7435	4.07	> 0.10 ns
Deviations	4	0.7304	0.1826		

Cow 26

Source	d.f.	SS	MS	F	P
Total	5	1.3555			
Regression	1	1.0207	1.0207	12.20	< 0.05*
Deviations	4	0.3348	0.0837		

Common

Source	d.f.	SS	MS	F	P
Total	29	24.8018			
Regression	1	6.4850	6.4850	9.91	< 0.01**
Deviations	28	18.3168	0.6542		

Test of Homogeneity of Variances:

$$\chi^2 = \frac{2.7809}{1.0617} = 2.62 \text{ (d.f. = 3)} \qquad P > 0.25 \text{ ns}$$

Appendix A-11 Comparisons of Regressions of Milk Fat Content on Acetate Thiokinase Activity

Source	d.f.	Σx^2	Σxy	Σy^2	$\hat{b}$	Dev SS	d.f.	Dev MS
19	8	20893.0156	368.3934	8.9398	0.0176	2.4442	7	0.3492
118	8	2169.5822	82.0731	5.6486	0.0378	2.5438	7	0.3634
5	5	830.1083	24.8637	1.4737	0.0299	0.7302	4	0.1826
26	5	9365.3533	97.7703	1.3555	0.0104	0.3348	4	0.0837
				Pooled Deviations		6.0530	22	0.2751
Pooled Cow	26	33258.0594	573.0806	17.4175	0.0172	7.5427	25	0.3017
				Between Slopes		1.4897	3	0.4966
Common	29	41287.0697	517.4353	24.8018	0.0125	18.3168	28	0.6542
				Between Intercepts		10.7740	3	3.5913

$$\text{Between Slopes } F_{3,22} = \frac{\text{Between Slopes Dev MS}}{\text{Pooled Deviations MS}} = 1.80 \qquad P > 0.10 \text{ ns}$$

$$\text{Between Intercepts } F_{3,25} = \frac{\text{Between Intercepts MS}}{\text{Pooled Cow Dev MS}} = 11.90 \qquad P < 0.01^{**}$$

Appendix A-12 Analyses of Variance of Regressions of Adjusted Fat Content on Acetate Thiokinase Activity

Cow 19

Source	d.f.	SS	MS	F	P
Total	8	1.6528			
Regression	1	1.1119	1.1119	14.39	< 0.01 **
Deviations	7	0.5408	0.0773		

Cow 118

Source	d.f.	SS	MS	F	P
Total	8	3.0366			
Regression	1	0.9067	0.9067	2.98	> 0.10 ns
Deviations	7	2.1299	0.3042		

Cow 5

Source	d.f.	SS	MS	F	P
Total	5	0.9012			
Regression	1	0.2207	0.2207	1.30	> 0.25 ns
Deviations	4	0.6805	0.1701		

Cow 26

Source	d.f.	SS	MS	F	P
Total	5	0.8338			
Regression	1	0.6082	0.6082	10.79	< 0.05 *
Deviations	4	0.2256	0.0564		

Common

Source	d.f.	SS	MS	F	P
Total	29	9.7433			
Regression	1	1.0112	1.0112	3.24	< 0.10
Deviations	28	8.7321	0.3119		

Test of Homogeneity of Variances:

$$\chi^2 = \frac{4.8783}{1.0617} = 4.61 \text{ (d.f. = 3)} \qquad P > 0.10 \text{ ns}$$

Appendix A-13 Comparisons of Regressions of Adjusted Milk Fat Content on Acetate Thiokinase Activity

Source	d.f.	Σx^2	Σxy	Σy^2	$\hat{b}$	Dev SS	d.f.	Dev MS
19	8	20893.0156	152.4246	1.6528	0.0073	0.5408	7	0.0773
118	8	2169.5822	44.3522	3.0366	0.0204	2.1299	7	0.3043
5	5	830.1083	13.5349	0.9012	0.0164	0.0805	4	0.1701
26	5	9365.3533	75.4722	0.8338	0.0081	0.2256	4	0.0564
				Pooled Deviations		3.5768	22	0.1626
Pooled Cow	26	33258.0594	285.7839	6.4243	0.0086	3.9685	25	0.1587
				Between Slopes		0.3918	3	0.1306
Common	29	41287.0697	204.3168	9.7433	0.0049	8.7321	28	0.3119
				Between Intercepts		4.7635	3	1.5879

Between Slopes $F_{3,22} = \frac{\text{Between Slopes Dev MS}}{\text{Pooled Deviations MS}} = 0.8$ $P > 0.25$ ns

Between Intercepts $F_{3,25} = \frac{\text{Between Intercepts MS}}{\text{Pooled Cow Dev MS}} = 10.0$ $P < 0.01$**

Appendix A-14 Analyses of Variance of Regressions of Content of 14:0 in Milk on Acetate Thiokinase Activity

Cow 19

Source	d.f.	SS	MS	F	P
Total	8	0.1904			
Regression	1	0.1307	0.1307	15.33	< 0.01 **
Deviations	7	0.0597	0.0085		

Cow 118

Source	d.f.	SS	MS	F	P
Total	8	0.2858			
Regression	1	0.1366	0.1366	6.41	< 0.05 *
Deviations	7	0.1492	0.0213		

Cow 5

Source	d.f.	SS	MS	F	P
Total	5	0.0495			
Regression	1	0.0087	0.0087	0.85	> 0.25 ns
Deviations	4	0.0408	0.0102		

Cow 26

Source	d.f.	SS	MS	F	P
Total	5	0.0768			
Regression	1	0.0411	0.0411	4.61	< 0.10
Deviations	4	0.0357	0.0089		

Appendix A-15 Analyses of Variance of Regressions of Content of Total Acids - C 16 in Milk on Acetate Thiokinase Activity

Cow 19

Source	d.f.	SS	MS	F	P
Total	8	0.8592			
Regression	1	0.4886	0.4886	9.23	< 0.05 *
Deviations	7	0.3706	0.0529		

Cow 118

Source	d.f.	SS	MS	F	P
Total	8	1.5949			
Regression	1	0.2992	0.2992	1.62	> 0.10 ns
Deviations	7	1.2957	0.1851		

Cow 26

Source	d.f.	SS	MS	F	P
Total	5	0.5384			
Regression	1	0.1097	0.1097	1.02	> 0.25 ns
Deviations	4	0.4287	0.1072		

Cow 5

Source	d.f.	SS	MS	F	P
Total	5	0.4132			
Regression	1	0.0983	0.0983	1.25	> 0.25 ns
Deviations	4	0.3150	0.0787		