STUDIES ON THE FATTY CONSTITUENTS OF MILK.

A thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Science.

By

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STUDIES ON THE FATTY CONSTITUENTS OF MILK.

C O N T E N T S.

S Y N O P S I S.

STUDIES ON THE FATTY CONSTITUENTS OF MILK.

Part I. : The Effect of Inanition on the Yield and Composition of Milk Fat.

The effect on the secretion and composition of butterfat from lactating cows during 12 day period of inanition has been investigated with the object of studying the mechanism of milk fat secretion. It was found that the daily secretion of milk fat fell by about 40 per cent. during the latter period of the fast and that on re-alimentation it returned to some 60 per cent. of the pre-fast value at the end of about 6 weeks. The chemical composition showed very marked changes, even on the first day of the fast.

A detailed analysis of the milk fat component acids of the milk fat from cows on a normal diet and after fasting have been made by a modified process for the analysis of butterfat, using a fractional distillation method from which prolonged steam distillation of the saponified fat is omitted. The chief change in the nature of the butterfat due to inanition was a decrease of about 80 per cent. in the original content of lower acids up to and including C_{14}, a deficiency which was almost entirely made up by an increase in the content of oleic acid. The palmitic acid content of the fat was unaltered by inanition, while the higher unsaturated components, represented as C_{20}, showed a considerable increase.

This significant inverse relationship between the

oleic acid and the lower fatty acids has been discussed in connexion with the different theories put forward to explain the occurrence of the lower glycerides in milk fat. The bearing of these results in fixing the chemical constants for normal butterfat has also been indicated.

Part II. : The effect of Thyroxine Administration on the blood Lipoids and on the Nature of the Milk Fat.

The object of this section of the work was to find whether the administration of thyroxine, which is known to cause large increases in the daily yield of milk fat, would bring about any changes in the level and nature of the blood lipoids and in the quality of the milk fat.

In agreement with previous work of others, thyroxine administration caused an increase of 50 per cent. in the daily yields of both milk and butterfat, but only extremely small changes were found in the actual composition of the fat. There was also no significant change in the percentage of non-fatty solids of the milk.

During hyperthyroidism there was no measurable alteration in the lipoids of the corpuscles, but in the plasma the level of the phosphatide fatty acids was reduced on an average by 17%, the non-phosphatide fatty acids by 15%, and the ester cholesterol by 14%. This hypolipaemia was not accompanied by any detectable change in the actual nature of the plasma fatty acids, and in spite of the general decreases in the lipoid

constituents of the blood, the relative proportions of the various components were very little altered.

ACKNOWLEDGEMENT.

I wish to express my grateful appreciation of the facilities accorded to me by the Council of the Hannah Dairy Research Institute, Kirkhill, Ayr. I am particularly indebted to Dr. N.C. Wright, Dr. J.A.B. Smith and other members of the Institute staff for their constant interest and valuable advice which was readily granted at all times.

GENERAL INTRODUCTION

to

Parts I. and II.

The biochemical processes involved in the secretion of milk fat and the factors which affect the amount and the nature of the secreted fat are still little understood. In 1911, Foa (7) put forward evidence which led him to conclude that milk fat was derived from the neutral triglyceride fraction of the blood lipoids of the lactating animal. But in 1919, Meigs et al. (17) suggested from phosphorus estimations in blood samples taken simultaneously from the mammary and jugular veins that blood phosphatides were also precursors of milk fat. This view was generally held by many workers for some years until Blackwood (6) demonstrated that blood from the jugular vein could not in fact be assumed to represent mammary-arterial blood. Blackwood also found that when samples were obtained simultaneously from the radial artery and mammary vein no difference was found in the lipoid phosphorus content of the two samples, thus contradicting the suggestion that blood phosphatides are removed from the blood as precursors of milk fat. Lintzel (14) in his studies with goats found no reduction in the fatty acids of either the phosphatide or the cholesteryl esters in the blood as it passed through the gland. Since, however, there was a lowering of the total fatty acids, calculation indicated that neutral fat was taken up. Graham et al. (11), using a new and perhaps more reliable technique, found that when the mean differences between arterial and venous blood were expressed as percentages of the

average arterial concentration, the gland apparently withdrew 2 - 4 per cent. of the fatty acids, and these authors also showed that neither phosphatides nor cholesteryl esters took part in the production of the milk fat. These findings were confirmed in general by Maynard et al. (16). Aten and Hevesy (4), in experiments with goats in which they applied radioactive phosphorus to the study of milk secretion, have confirmed the fact that plasma phosphatides do not give rise to the milk fat and inorganic phosphorus in the gland.

At first sight the evidence just outlined might seem to suggest that for the secretion of milk fat the gland simply selects from the blood the particular type of glycerides which it requires, and that these are then allowed to diffuse into the milk. But it is clear from the peculiar composition of the fat itself, and also from the fact that the respiratory quotient of the actively lactating mammary gland has now been found by Graham et al. (10) to be considerably greater than unity, that more complex processes such as the synthesis of fat from carbohydrates may also possibly be involved. There may therefore be two main precursors of milk fat in the blood, the neutral glycerides on the one hand and some form of carbohydrate material on the other. If this is true it seems reasonable to suppose that the composition of the milk fat produced by an individual animal at any particular time will depend on the extent to which each of these

precursors contributes towards its production.

As the fatty acids of blood are undoubtedly the chief precursors of milk fat, the question naturally arises as to whether the level of the fatty acids in blood is related to the amount of milk and butterfat produced. Maynard et al. (15) have carried out investigations on the influence of the lactation cycle on the total fatty acids, phosphatide fatty acids and cholesterol of the blood. They found that following parturition there is a rapid and approximately parallel rise in all these constituents, succeeded by a gradual fall to normal levels as the dry period is reached. In experiments with animals which were held at a constant level of food and fat intake during the dry period and earlier weeks of lactation, the same rise in the blood lipoids occurred during the onset of milk secretion, thus demonstrating that lactation is accompanied by a change in the blood lipoid level which is independent of changes in the fat intake. Porcher and Maynard (19) and Schaible (20) have also found an increase in fatty acids and unsaponifiable matter after parturition. Despite marked changes in the level of these constituents from the dry to the lactating state there is no change in the distribution of the fatty acids or in their nature as determined by the iodine number. The requirements for lactation so far as blood is concerned seem therefore to be an increase in the quantity without any measurable change in the quality.

Some authors have made interesting investigations into the possibility of blood lipoid being used as a valuable indicator in problems connected with the breeding of dairy cattle. Porcher (18) seems to have been the first to call attention to the possible relationship of the fat of the blood to the ability of cows to produce milk fat. In 1931 Leroy et al. (15) from their studies on five different breeds of cattle, concluded that there exists a high co-efficient of correlation between the total fatty acids in the blood and the butterfat content of milk. They suggested that the determination of fatty acids and total lipoids in the blood would furnish a value by which the future capacity of young cattle to produce butterfat might be established. Schoorl (21) reached the same conclusion from his studies. As opposed to these workers, however, Allen (3) in his recent bulletin gives a very extensive review of the literature on the subject and arrives at some valuable conclusions from his own results. He finds that there is no statistically significant relationship between the plasma lipoid level and the capacity of the animal for milk fat production, but he rightly points out that, even if plasma lipoid level is a factor of importance, the problem would be complicated by other factors such as the rate of blood flow through the mammary gland, the total plasma volume of the animal and the volumetric relationship between the cells and the plasma of the blood. He found no marked relation-

ship between the butterfat level in the plasma and the breed of cow and confirmed previous workers in showing that during lactation there is a rise in blood lipoid level.

With regard to the effect of the nature and the amount of fat in the diet on milk fat production much work has been carried out. That increases in the fat intake may have some slight temporary beneficial effect has been shown by several workers, e.g. Golding et al. (9), Allen (1,2), and Garner et al. (8). But it is exceedingly doubtful if any lasting effect can be obtained in this way. Small changes in the nature of the milk fat can readily be brought about by feeding different fats. In general it may be said that, within certain limits, the more unsaturated the fat of the food, the more unsaturated is the fat of the milk. This has been shown in recent years, for example, by Hilditch and Thompson (12) who obtained small changes in the composition of milk fat by feeding linseed and rape oils. With linseed oil there was an increase in oleic acid of 7%, while the inclusion of rape oil in the diet resulted in the presence of 4% of erucic acid in the butterfat. That there is a close relationship between the amount of a particular dietary fat in the blood and its amount in the milk was shown by Aylward et al. (5), using "labelled" fatty acids.

From this brief review of the literature the following conclusions may be drawn: (i) The chief

though not perhaps the only, precursor of milk fat is the triglyceride fraction of the plasma lipoids; (ii) Although lactation is accompanied by an increase in the total lipoid level of the plasma, it is still doubtful whether this level is an important factor in controlling the actual amount of milk fat secreted; (iii) Some slight temporary changes in the amount and nature of the milk fat can be brought about by changes in the amount and nature of the fat fed.

In the work so far described changes in the blood fat have undoubtedly always been very slight and, where they took place, have always been brought about slowly over a long period. It was therefore felt that further evidence regarding the biochemical and physiological processes involved in milk fat secretion might be obtained by a detailed study of both the blood and milk fats of animals kept under such experimental conditions as would be expected to alter markedly and quickly the amount of the milk fat precursors in the blood and therefore probably the milk fat itself. Two series of experiments were therefore planned, one involving inanition and the other the administration of thyroxine, the main object being to study the effect of these two procedures on the blood and milk fats. In the past total fatty acids and phosphatides have been studied in some detail but seldom has much attention been paid to the triglyceride fraction itself. In the scheme of which the present work forms a part, it was decided to make a special study of this part-

lecular Fraction so far as existing analytical procedure would allow.

The inanition experiments are described in Part I. of this thesis (pp.8-56) and the thyroxine investigations in Part II. (pp.57-95).

PART I.

The Effect of Inanition on the Yield and Composition of Milk Fat.

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INTRODUCTION

Up till now there have been very few references in the literature with regard to the nature of milk secreted by animals during a period of inanition, and no data at all has been published for the composition of butterfat under such conditions. Overman and Wright (49), in experiments with lactating cows which had been starved for 5 - 6 days, found that inanition caused a marked decrease in the yield of milk and lactose. On the other hand, the percentages of fat, total solids and ash were found to increase, although as a result of the decrease in yield the total daily output of fat was lowered. In the work of Gowen and Tobey (28) several animals were starved for three days. They found a decrease in the milk production but a rapid increase in all the solids of the milk except lactose, the butterfat reaching 2.5 times its normal percentage. In neither of these experiments was the change in the nature of the milk fat considered. Further, the periods during which food was withheld were somewhat short to allow the attainment of a true state of inanition with ruminants.

A suitable opportunity was provided to study this problem more thoroughly during an experiment carried out by Dr. B. Morris of this Institute to determine the effect of inanition on the general metabolism of lactating and non-lactating ruminants. In these experiments the effect of inanition on the lactating

cow was studied from various different points of view. A section of the work dealing with the general nitrogen metabolism of the animals has been published by Morris and Ray (48). Another section was concerned with changes in the yield and chemical composition of the milk and has been described by Smith et al. (53), and a third section on the changes in blood lipoids during this interval has been carried out by Smith (51). The particular aspect of the work described here deals only with the changes in the yield and composition of the butterfat during inanition (cf. Smith and Dastur, 52) and in this connexion a modified form of the fractional distillation method for analysing butterfat has been evolved and described. It must be borne in mind that a starvation period of 12 days as employed in these experiments can only be looked upon as a special case of undernutrition when the animal is on the "minimum energy" intake that is possible.

EXPERIMENTAL.

The animals used for the inanition experiment were three non-pregnant Ayrshire cows in the sixth to seventh months of their lactation period, and were denoted in this work as Nos. 1, 2 and 3. At the end of a few weeks, during which the animals had been receiving a normal well-balanced diet, food was withheld from them for a period of 12 days, after which free access to food was again permitted. Water was at all times freely given.

With the third cow the experiment was stopped after the seventh day owing to the unsatisfactory condition of the animal. It may be noted that this animal was in a somewhat poor condition at the commencement of the fast, and on subsequent post-mortem examination was found to be almost completely devoid of depot fat. But even with this animal the fast may be looked upon as a case of simple inanition, up to the sixth or seventh day.

The animals were milked twice daily and a composite sample of evening and morning milk taken for analysis. The total yield of fat was calculated each day from the yield of milk and its fat content. The results for the total fat yields are recorded in Fig. 1, and will be discussed later.

In order to detect any change which might take place in the actual nature of the milk fat as a result of inanition, a sample of the fat was prepared each day by the following method. Approximately 500 ml. of milk were centrifuged for 30 minutes at 2,000 r.p.m. and then chilled in the refrigerator and the cake of fat so formed carefully removed. The fat was purified by dispersing it in lukewarm water (35°) and re-centrifuging. By this means most of the adherent proteins were removed. The cake of fat after the second centrifuging was then extracted with ether and the solution filtered. The residue was extracted three times with the same solvent to make sure all the fat was removed. After this the ether was distilled off

and the fat, which still contained some water, was dehydrated by adding a small quantity of benzene and alcohol. This was then evaporated on a water-bath under vacuum and the residual fat dissolved in light petroleum. When required for analysis the solvent was distilled off and completely removed under vacuum.

The Reichert Meissl and iodine values of the fat were then determined, and the results are shown diagrammatically in Fig. 2, where very marked changes can be observed. For the determination of the R.M. value the method used was that described in the Methods of Analysis of the Association of Official Agricultural Chemists (47), while for the iodine values the process adopted was that of Rosenmund and Kuhnhenn, using the bromine-pyridine-sulphate reagent (cf. Vere Jones, 55).

As it was so obvious from Fig. 2 that there were such marked changes in the chemical composition of the fat, it was decided to analyse in considerable detail samples secreted before and during inanition. For this purpose large quantities of fat of the order of 500 g. were required. These were prepared by separating the milk, and churning the cream into butter, from which the pure fat was obtained by warm filtration. To ensure that the sample obtained was truly representative of the whole, tests were always made to ascertain that the skimmed milk and buttermilk contained no more than the merest traces of

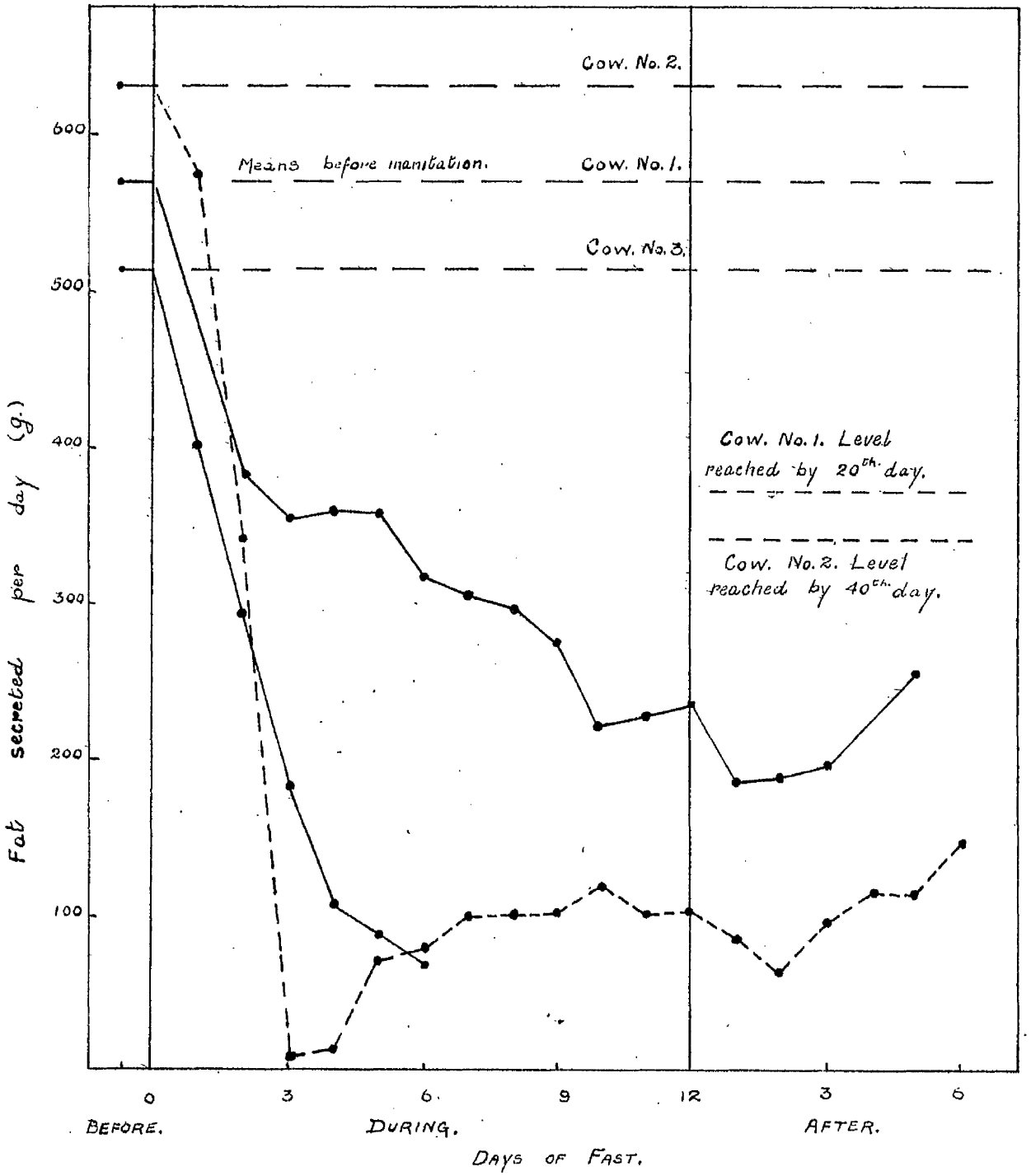


Fig. 1. The effect of inanitation on the total daily yields of milk fat.

The extremely low yield from Cow No. 2. on the 3rd and 4th days was due mainly to the incidence of milk fever.

fat. The fats were preserved over light petroleum in order to prevent any oxidation. If, on testing, the buttermilk was found to contain more than very small amounts of fat, the latter were removed by centrifuging and were then added to the bulk. A sample of the fat from cow No.1 before and at the end of the fasting period and one from cow No.2 obtained by mixing the fats secreted by the animal on the last six days of inanition were analysed by a method which will shortly be described (p.15) and which depends mainly on the fractional distillation of methyl esters.

The details of the three fats analysed in this way are recorded in Table I. while the analytical data are shown in Tables II. to IV.

TABLE I.

Details of fats used for general analysis.

Description	Amount of fat used for analysis in g.	Reichert Meissl value	Iodine value
Fat secreted by cow No.1 before starvation	287	26.0	36.6
Fat secreted by cow No.1 on the 11th & 12th days of starvation	285	9.8	32.5
Fat secreted by cow No.2 during the last 6 days of starvation	254	13.9	33.6

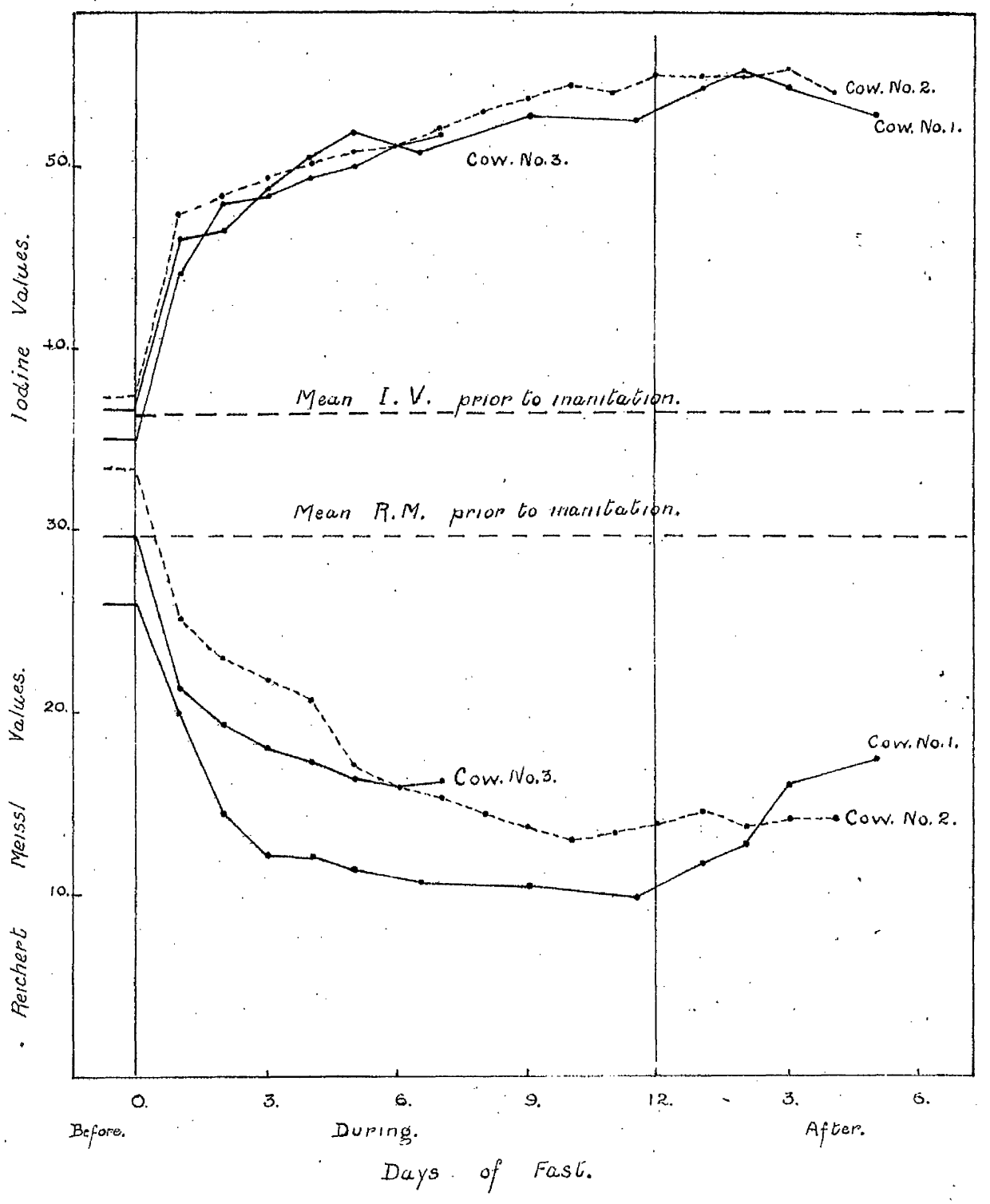


FIG. 2. The effect of inanitation on the iodine values and Reichert Meissl values of the milk fat.

THE ANALYSIS OF BUTTERFAT.

In the past few years two main procedures have been used for the analysis of butterfat. Hilditch and his colleagues (54, 56, 57, 59) first saponify the fat and then distil the acidified products for some hours in a current of steam. This is followed by fractional distillation of the steam-volatile compounds, while the non-volatile residue is separated by the Twitchell process into solid and liquid acids. These are then converted into methyl esters and fractionated. Owing to other sources of error which seem to be unavoidable in any process of this type which can at present be devised, this procedure probably gives results which are as accurate as possible, but there are some slight disadvantages in the actual technique. One of these lies in the fact that, during steam distillation, the non-volatile fatty acids have to be heated to about 100° in the presence of steam for several hours, a process which ought to be avoided if possible where unsaturated acids are present, and that unless very large quantities of the original butterfat are available, the amount of volatile compounds resulting from the steam distillation must be exceedingly small for their accurate fractionation. In the modified procedure now to be described, distillation in steam is dispensed with, and the lower acids can be estimated more readily on small amounts of initial material. Bosworth and Brown (22) also avoided the use of steam distillation in making a detailed invest-

igation of the lower components of butterfat and were thus able to establish the presence in butter of very small amounts of decenoic and tetradecenoic acids, but they did not adapt their method for the complete analysis of the total fatty acids, as that presumably was not the main purpose which they had in view. To do this the Twitchell separation of liquid and solid acids must be introduced at some stage in the process. The scheme which was therefore adopted in the present work may be summarised very briefly by saying that the fat was converted directly into methyl esters, the lower components fractionally distilled from the whole bulk, the higher members separated into liquid and solid acids, methylated and also fractionally distilled. To illustrate the procedure more fully the analysis of the butterfat secreted by cow No.1 before inanition will now be described in detail.

237 g. of the butterfat were converted into methyl esters by refluxing for 24 hours with 2,000 ml. of re-distilled methyl alcohol containing 5 per cent. (by weight) of sulphuric acid. Hydrochloric acid cannot be used owing to the disadvantage of its volatile nature at a later stage of the analysis when butyric acid is being estimated. Most of the alcohol (1,000 - 1,500 ml.) was then distilled off, the distillate made up to a known volume and preserved for subsequent analysis (solution A). Care must always be taken not to increase the concentration of the sulphuric acid too much by reducing the volume too far.

The residue was quickly cooled, transferred to a two litres separating funnel and diluted with water. It was extracted exhaustively with ether to remove all the water insoluble esters and the ether extracts collected in a separating funnel. They were then washed about four times with water to remove sulphuric acid, a process which also inevitably removes traces of lower water-soluble esters if present. These washings were then collected in a round-bottomed flask, made distinctly alkaline with KOH and heated on the open flame to reduce the volume to about 500 ml. The residue was acidified and steam distilled, the distillate being made up to a known volume (solution B).

The washed ether extracts were dehydrated over sodium sulphate and filtered. Most of the solvent was distilled off (solution C), and then the flask containing the esters was connected to the fractionating apparatus described on page 22 and very gentle suction applied. Small residual traces of solvent were thus removed and added to the solution C. The mixture was then made up to a known volume (solution D).

Aliquots were taken from solutions A and D and saponified with standard alcoholic potash. The amount of acid found in the usual way was expressed a butyric acid. As all the methyl esters higher than the butyrate are insoluble in water, it was reasonable to assume that the acidity present in the various solvents was due almost entirely to butyric acid. Known amounts of solution B were titrated against N/10 alkali

and the results again calculated as butyric acid. The sum total of these results gives the amount of C_4 acid originally present in the fat. A blank determination was carried out with a similar quantity of lard for which the R.M. value was negligible. The amounts of "butyric acid" estimated to be present for the milk fat (cow No.1) and the lard were 0.9 and 0.03% in the aqueous phase (solution B), 2.73 and 0.03% in the methyl alcohol (solution A) and 0.68 and 0.25% in the ether (solution D), making totals of 3.5 and 0.31% of the butter and lard respectively. Since lard contains no butyric acid, it is evident from these results that the estimated figure for butyric acid by this method tends to be a little high.

(i) Fractionation of the Lower Esters: The methyl esters were first gently heated at 15 mm. pressure until the lower fractions had passed over (cf. fractions 2 and 3 in Table II.A). The pressure was then decreased to 2 mm. and a large primary fraction containing all the esters distilling below 153 - 160° collected. (In preliminary trials it had been found that practically all the lower compounds up to and including most of the myristate pass over below this temperature, while the distillation of oleate was still relatively slight). This large primary fraction containing the lower esters was then redistilled and about 20 small fractions collected, the details of which are recorded in Table II.A. When the iodine values of the various fractions were estimated, the value for the last fraction (not

shown in the table) was found to be greater than 20 and as this indicated that it contained a large proportion of oleate, this particular fraction was returned to the residue which was then separated into solid and liquid acids.

(ii) Separation into Solid and Liquid Acids: The residue from this first distillation was saponified by heating it on a water-bath for one hour with 1.5 litres of ethyl alcohol and an excess of KOH (about 70 g.) dissolved in 100 ml. water. Most of the alcohol was then distilled off and the residue transferred to a separating funnel. The fatty acids were recovered by acidifying the mixture with HCl, followed by exhaustive extraction with ether. The united extracts were washed free from mineral acid, dehydrated with anhydrous Na_2SO_4, filtered and the ether distilled off.

The fatty acids so obtained were separated into 'solid' and 'liquid' fractions by the process originated by Twitchell (54) and modified by Hilditch and Priestman (59). The acids were dissolved in 5 volumes of ethyl alcohol containing 90 g. of lead acetate. Sufficient lead acetate must be used to combine with all the solid acids present but at the same time an unnecessarily large excess must be avoided. The alcoholic solutions were mixed while hot and the mixture was then boiled for a few minutes after which it was put in an insulated box and the lead soaps allowed to precipitate very slowly as the solution

cooled over-night. The final temperature must be between 15° and 20°. The precipitate of solid lead soaps was filtered and washed several times with cold alcohol. It was then dissolved again in the same volume of alcohol as before and 20 ml. of acetic acid added. The whole mixture was refluxed for about 1 hour till the soaps were dissolved, when it was again allowed to cool slowly over-night. Next day the precipitate was again filtered, washed, drained well and dissolved in dry benzene. The two alcoholic filtrates and washings were united and after distilling off all the alcohol the liquid soaps were dissolved in benzene. (It is particularly important to remove all the excess of alcohol at this stage if emulsions of lead soaps are to be avoided in the next process). The lead present was removed from both fractions by a current of H_2S, the lead sulphide filtered off and washed several times with hot benzene. Finally the benzene containing excess of H_2S was distilled both from the solid and from the liquid fatty acids.

(a) Solid Esters (predominantly saturated): The solid acids were dissolved in 1 litre of methyl alcohol and esterified by passing a rapid stream of dry hydrochloric acid gas for 20 minutes and refluxing the mixture for 1 hour. Most of the alcohol was then distilled off and the residue transferred to a separating funnel, diluted with water and extracted with ether. The combined ether extracts were washed free from HCl, dehydrated, the esters transferred to a 500 ml. round-

bottomed flask, and the solvent almost completely distilled off. They were then distilled in vacuo through the electrically heated fractionating column described overleaf.

(b) Liquid Esters (predominantly unsaturated): Before esterifying the liquid acids it is advisable to remove any small amounts of unsaponifiable matter which they may contain. In butter there are always small amounts of cholesterol and other unsaponifiable matter, but owing to the solubility of these substances in alcohol they will always pass into the liquid fraction in the Twitchell separation. They might then distil with some of the higher liquid esters and give slightly erroneous iodine and saponification values towards the end of the analysis. The effect of the presence of the unsaponifiable matter would at any time be exceedingly slight, but as a precautionary measure it is well to remove it.

The liquid acids were saponified by adding excess of KOH dissolved in water and the aqueous solution of the soaps extracted thoroughly with ether to remove the unsaponifiable matter. The ether was then washed with water, and the washings added to the solution of soap which was now acidified with HCl and the fatty acids extracted with ether. After dehydrating the ether solution with $MgSO_4$, the solvent was distilled off and the residue dissolved in methyl alcohol and esterified by the same method as that used for the solid esters. The esters were now ready for fraction-

al distillation.

(iii) The Fractionating Apparatus: The general arrangement of the apparatus is shown in Plates I and II. The fractionating column is similar to that described by Longenecker (46) and consists of a Pyrex glass tube (A) about 90 cm. in height and 17 mm. internal diameter. About 3.5 cm. from the bottom of the tube a perforated glass disc is fused in. A 360° thermometer is tied round this tube at a distance of about 10 cm. from the lower end. The column is packed to two-thirds its height with glass helices prepared according to the method described by Wilson et al. (56). These authors have shown that this packing material is far superior to the usual 5 x 5 mm. glass tubes. The flooding tendency is less, enabling more efficient distillation. The column is surrounded by another Pyrex tube (B) of about 95 cm. long and 30 mm. internal diameter on which is wound about 15 ft. of No.22 nichrome wire for heating the column. The heating jacket is protected by a further Pyrex tube with an internal diameter of about 37 mm. The open ends of the two outer tubes are plugged with cotton wool to prevent the loss of heat.

At the top of the column is a still head carrying a thermometer and distilling arm of about 16 cm. When lower esters are being distilled, the condenser is surrounded by flowing water but when the distillation temperature rises to 110° the water is cut off. The distillate is collected by means of the receiver for

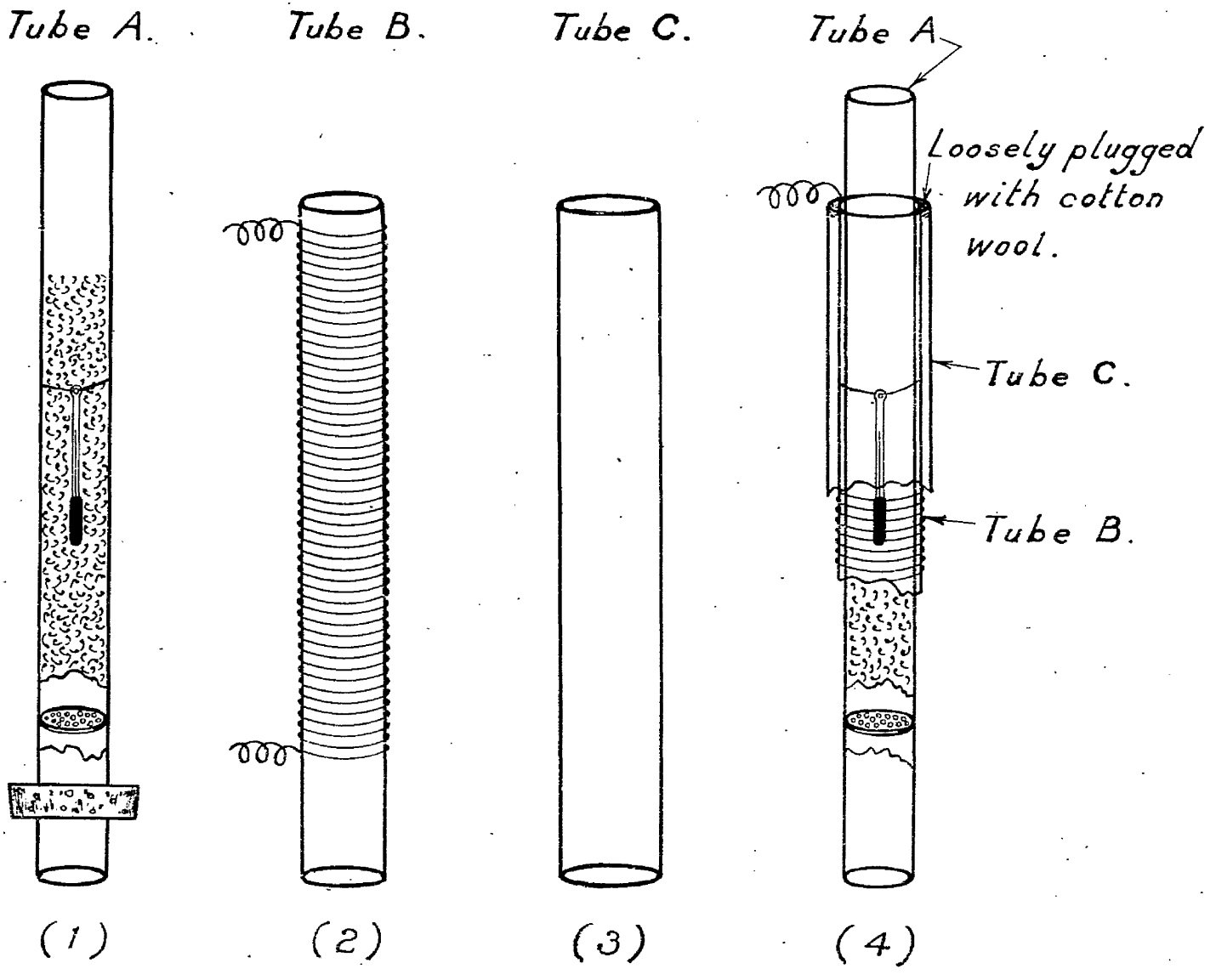
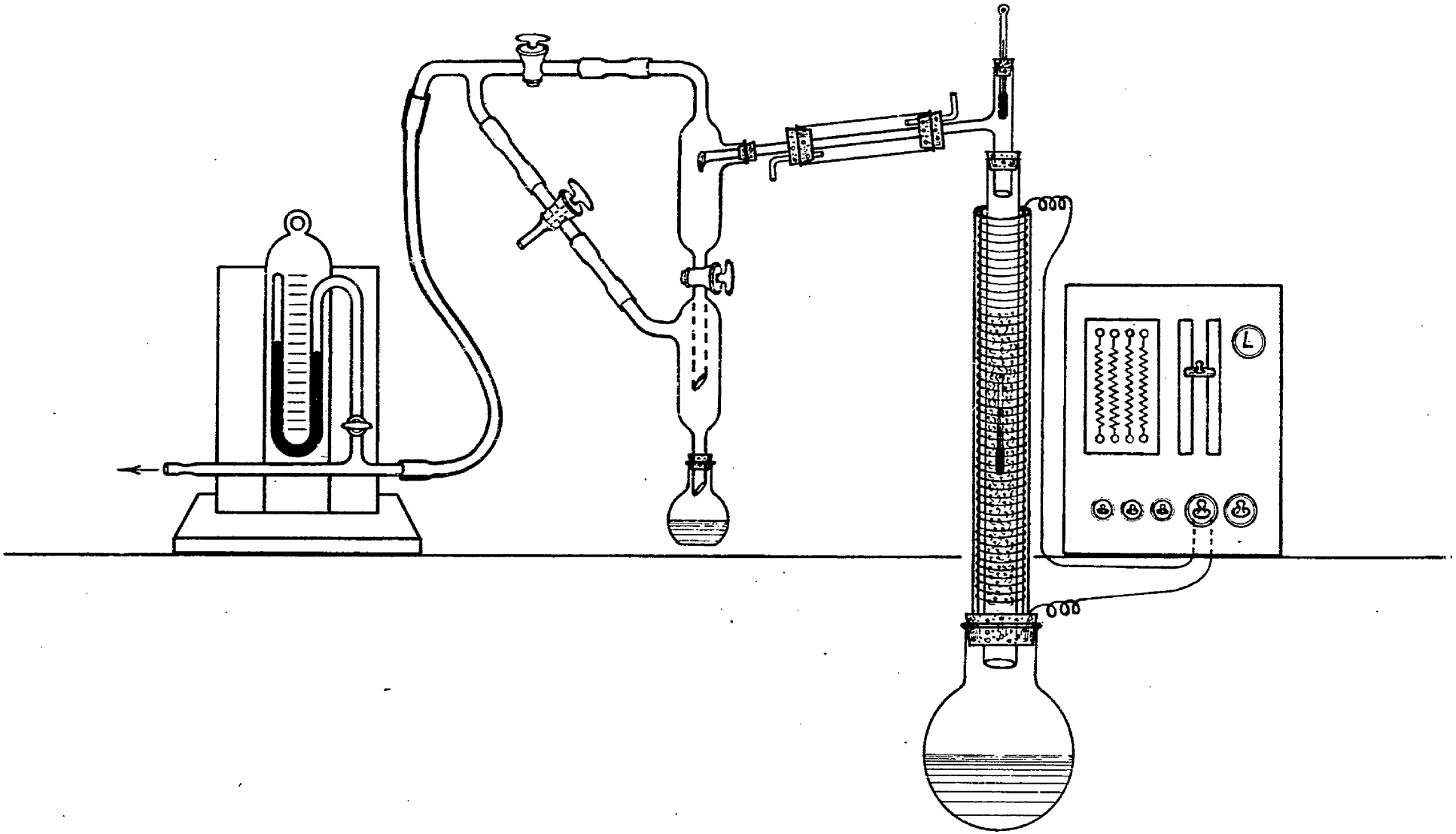

Tube A.
Tube B.
Tube C.
Tube A
Loosely plugged with cotton wool.
Tube C.
Tube B.
(1)
(2)
(3)
(4)

Plate I.



Apparatus for Fractionation.

Plate II.

fractional distillation as improved by Geissler. The various fractions are collected in weighed 100 ml. flat bottomed flasks. All connections throughout must be as air tight as possible.

By a system of resistance the temperature of the heating column as recorded by the thermometer on the tube (A) can be raised from room temperature to 250° or even higher. It has been found in practice that when the distillate is passing over say at 100°, the temperature of the column as registered by this termometer is usually 30° higher. The magnitude of this difference will naturally depend on the position of the lower thermometer, and as it is moved higher the difference will become smaller.

The flask containing the methyl esters is heated directly by a small flame which is kept continually moving to prevent bumping, and the conditions are so adjusted that there is no visible accumulation of liquid in the column. If the temperature of the distillate as it passes over is rising, fractions are taken at every few degrees, but if it is remaining constant for a period, fractions are removed at regular but less frequent intervals until it again begins to rise. The iodine and saponification values of the various fractions are then determined.

(iv) Determination of Iodine Values and Molecular Weights:

The iodine values of all the fractions were estimated in the present work by the method of Rosenmund and Kuhnhenn using the pyridine-sulphate reagent

(55). The mean molecular weights were determined by saponifying a known amount of each fraction for 60 minutes with excess of alcoholic potash (approximately 0.5N) and back titrating with standard HCl. Preliminary trials indicated that in order to obtain consistent results the weight of esters saponified should be as large as possible (0.6 - 1.0 g.). Also to avoid the presence of a large excess of water the concentration of the acid used for back titration should be about 0.5N. When only small quantities of the fractions were available the determination had to be carried out with more dilute reagents (0.25N). From time to time the molecular weight determined by this method was checked by extracting the acids and directly titrating them against 0.1N alkali. For estimating the iodine value and molecular weight of the esters present in the residues left after fractionation, the small amounts of unsaponifiable matter formed due to slight decomposition during the prolonged distillation process were always first removed in the usual way and the fatty acids isolated. The mean molecular weight of these acids was then found by direct titration, and, by adding 14 units to the value so determined, the molecular weight of the methyl esters was obtained. The iodine value was also calculated from that of the acids.

(v) <u>The Method Calculation</u>: The details of the method used for deducing the composition of the fat from these data is somewhat complex but the main

TABLE II.A

TABLE II.A.

Fractionation of the methyl esters prepared from 287 g. of the normal butterfat secreted by cow No.1 prior to starvation

Lower Esters.

Fraction No.	B.P. at (o) 2 mm. up to	% of total esters	Molecular weight	Iodine value	M.W. of the saturated esters only (by calculation)	Saturated esters of the acid							Unsaturated esters of the acids			
						C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{10}	C_{12}	C_{14}	C_{16}
1	Methyl butyrate	3.88	-	-	-	3.88	-	-	-	-	-	-	-	-	-	-
2	45*	0.35	132.1	0.6	132.1	-	0.32	0.03	-	-	-	-	-	-	-	-
3	45*	0.32	131.8	0.6	131.8	-	0.30	0.02	-	-	-	-	-	-	-	-
4	56	0.22	158.5	2.6	158.5	-	-	0.22	-	-	-	-	-	-	-	-
5	56	0.42	157.4	2.7	156.7	-	0.02	0.39	-	-	-	-	0.01	-	-	-
6	56	0.07	159.2	-	159.2	-	-	0.07	-	-	-	-	-	-	-	-
7	70-80	0.56	177.8	12.8	177.2	-	-	0.16	0.35	-	-	-	0.05	-	-	-
8	82	0.67	184.8	16.5	184.9	-	-	0.03	0.56	-	-	-	0.08	-	-	-
9	84	0.61	185.1	16.4	185.0	-	-	0.06	0.48	-	-	-	0.07	-	-	-
10	102	0.51	195.5	12.9	195.2	-	-	-	0.31	0.15	-	-	0.03	0.02	-	-
11	106	0.94	209.5	8.0	209.5	-	-	-	0.15	0.73	-	-	-	0.06	-	-
12	118	0.76	216.4	9.1	216.7	-	-	-	-	0.64	0.06	-	-	0.06	-	-
13	124	1.05	232.4	11.6	234.5	-	-	-	-	0.26	0.69	-	-	0.10	-	-
14	127	1.12	235.4	10.8	232.7	-	-	-	-	0.34	0.67	-	-	-	0.11	-
15	127	1.61	238.3	10.7	238.1	-	-	-	-	0.21	1.24	-	-	-	0.16	-
16	128	2.38	239.8	10.5	239.8	-	-	-	-	0.18	1.96	-	-	-	0.24	-
17	131	1.66	242.3	9.2	242.3	-	-	-	-	-	1.52	-	-	-	0.14	-
18	141	1.31	248.4	9.0	249.2	-	-	-	-	-	0.92	0.28	-	-	0.11	-
19	145	1.56	260.9	8.9	260.2	-	-	-	-	-	0.51	0.90	-	-	-	0.15
20	148	5.44	266.2	9.5	266.0	-	-	-	-	-	0.47	2.62	-	-	-	0.35
21	149	1.76	269.5	11.3	269.8	-	-	-	-	-	0.03	1.52	-	-	-	0.21
	Total	25.20				3.88	0.64	0.98	1.85	2.51	8.07	5.32	0.24	0.24	0.76	0.71

* At 15 mm. pressure.

TABLE II.B

Fractionation of solid esters from cow No.1 prior to starvation.

Fraction No.	B.P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	M.W. corrected for methyl oleate	Saturated esters of the acids				Methyl oleate
						C_{14}	C_{16}	C_{18}	C_{20}	
22	140	0.45	245.0	0.7	245.0	0.41	0.04	-	-	-
23	149	0.67	252.3	0.4	252.3	0.19	0.48	-	-	-
24	153	1.61	268.4	1.3	267.8	0.14	1.44	-	-	0.03
25	153	2.38	274.4	4.0	273.3	-	2.03	0.24	-	0.11
26	159	2.93	274.5	4.1	273.5	-	2.47	0.32	-	0.14
27	157	3.47	277.8	5.0	276.7	-	2.52	0.75	-	0.20
28	161	3.20	278.0	5.6	276.7	-	2.31	0.68	-	0.21
29	162	5.16	281.0	8.2	279.4	-	1.93	0.93	-	0.50
30	158	3.25	283.2	9.0	281.7	-	1.72	1.19	-	0.34
31	161	2.73	284.0	9.3	282.4	-	1.37	1.05	-	0.31
32	165	2.92	289.2	11.2	288.1	-	0.93	1.61	-	0.38
33	165	1.93	290.2	15.7	289.1	-	0.53	1.09	-	0.31
34	170	2.69	297.3	13.9	297.5	-	0.06	2.19	-	0.44
35	Residue	3.05	307.7	17.1	310.8	-	-	1.33	1.08	0.64
		34.44				0.74	17.83	11.38	1.08	3.41

TABLE II.C

TABLE II.C

Fractionation of liquid unsaturated esters from cow No.1 prior to starvation

Fraction No.	B.P. at (o)3 mm. up to	% of total esters	Molecular weight	Iodine value	M.W. of saturated esters only (by calculation)	H atoms required for full saturation	Calculated M.W. of fully saturated fraction	I.V. of the C_{18} and C_{20} esters	Saturated methyl esters of the acids C_{12}	C_{14}	C_{16}	C_{18}	Unsaturated esters. C_{14}	C_{16}	C_{18}'	C_{18}''	C_{20}*
36	132	0.30	237.2	17.1	236.6				0.05	0.20	-	-	0.05	-	-	-	-
37	132	0.46	244.2	17.4	245.2				-	0.34	0.04	-	0.08	-	-	-	-
38	145	0.37	254.9	27.4	255.1				-	0.15	0.12	-	0.05	0.05	-	-	-
39	155	0.60	268.9	46.3	269.7				-	0.15	0.15	-	-	0.15	0.15	-	-
40	160	1.20	281.5	67.9		1.5	283.0	79.3	-	0.22	-	0.05	-	0.22	0.71	-	-
41	155	2.40	282.2	72.2		1.6	283.8	85.0	-	0.41	-	0.02	-	0.41	1.56	-	-
42	165	2.18	284.7	72.4		1.6	286.3	82.4	-	0.31	-	0.06	-	0.31	1.50	-	-
43	161	2.40	287.1	76.6		1.7	288.8	85.2	-	0.27	-	0.01	-	0.27	1.85	-	-
44	165	3.27	287.8	77.6		1.8	289.6	85.5	-	0.34	-	0.01	-	0.34	2.58	-	-
45	164	2.76	288.7	79.9		1.8	290.5	87.6	-	0.26	-	-	-	0.25	2.20	0.05	-
46	165	2.78	290.5	82.2		1.9	292.4	87.3	-	0.19	-	-	-	0.20	2.34	0.05	-
47	166	4.72	295.1	85.9		2.0	297.1	87.1	-	0.07	-	-	-	0.07	4.50	0.08	-
48	163	4.91	295.3	87.5		2.0	297.3	88.4	-	0.05	-	-	-	0.06	4.64	0.15	-
49	168	4.26	295.5	88.7		2.1	297.6	89.5	-	0.04	-	-	-	0.03	4.00	0.19	-
50	169-175	4.47	294.8	90.3		2.1	296.9	91.7	-	0.07	-	-	-	0.08	4.02	0.30	-
51	Residue	3.28	305.1	95.1		2.2	305.3	98.1	-	-	-	-	-	-	2.11	0.35	0.82
	Total	40.56							0.05	3.08	0.31	0.15	0.18	2.44	32.16	1.17	0.82

(') Methyl oleate; (") Methyl linoleate; (*) Calculated as the esters of C_{20} acid with one double bond only.

TABLE II.D

Summary of the results for the butterfat obtained from cow No.1 prior to starvation.

	Acid	Per cent. as methyl esters.			
		Lower	Solid	Liquid	Total
Saturated.	C_4	3.88	-	-	3.88
	C_6	0.64	-	-	0.64
	C_8	0.98	-	-	0.98
	C_{10}	1.85	-	-	1.85
	C_{12}	2.51	-	0.05	2.56
	C_{14}	3.07	0.74	8.08	11.89
	C_{16}	5.52	17.83	0.31	23.46
	C_{18}	-	11.38	0.16	11.53
	C_{20}	-	1.08	-	1.08
	Total	23.25	31.03	8.59	57.87
Unsaturated.	C_{10}	0.24	-	-	0.24
	C_{12}	0.24	-	-	0.24
	C_{14}	0.76	-	0.18	0.94
	C_{16}	0.71	-	2.44	3.15
	Oleic	-	3.41	32.16	35.57
	Linoleic	-	-	1.17	1.17
	C_{20}	-	-	0.82	0.82
	Total	1.95	3.41	36.77	42.13
	Sum of the saturated & unsaturated esters.	25.20	34.44	40.36	100.0

principles underlying the method are best explained by reference to the "saturated" ester fractions for which the details are recorded in Table II.B. Reference to that table will show that the iodine value of these fractions rose from 0.7 to 17.1 as the distillation proceeded. As oleic acid is by far the predominating unsaturated acid in the original butterfat it is most probable that these iodine values are due almost entirely to methyl oleate, since small amounts of oleic acid tend to be carried down with the solid lead soaps in the Twitchell separation. The amount of methyl oleate present in each fraction is therefore calculated as a percentage of the original mixed esters from the formula P x I.V./85.7, where I.V. is the iodine value of the fraction, P its percentage of the whole and 85.7 the theoretical iodine value of methyl oleate. The mean molecular weight of the esters other than oleate present in each fraction can now be calculated by simple proportion and the figures recorded as shown in the sixth column of Table II.B. It is now assumed that the saturated esters in each fraction consist of only two adjacent even homologues, and therefore, from the corrected molecular weight just mentioned the amount of each of these two homologues is readily obtained. Although this assumption will not in all cases be strictly true, a study of Table II.B will suggest that any error arising from its adoption in the composition finally obtained for the original mixture

is likely to be exceedingly small. The small amounts of acids higher than C_{18} have been grouped together as "C_{20}".

Turning now to the calculations necessary for the lower acids, this can best be outlined by reference to Table IIA. Here it will be observed that the iodine values of the fractions do not increase regularly as with the higher saturated esters. In this case it first rises to 16.5 at the 8th fraction and then falls to 8.0 at the 11th and rises again to 11.6 at the 13th. After a further slight fall it rises once more to 11.3 at the 21st fraction. These variations are now known to be due to the presence of small amounts of lower unsaturated acids in butterfat. As far back as 1912, Smedley (50) inferred the presence of a C_{10} unsaturated acid, a finding which was confirmed by Grun & Wirth in 1922 (31). The presence of C_{12}, C_{14} and C_{16} unsaturated acids was indicated in later studies by Grun & Winkler (30). It was not however until 1933 that Bosworth & Brown (22) presented indisputable evidence for the existence of decenoic and tetradecenoic acids in cow butterfat. Hilditch & Paul (38), Longenecker (46), and Hilditch & Longenecker (37) have since confirmed the work of Bosworth & Brown and have established the position of the double bond in these lower unsaturated acids as being in the 9:10 position as in oleic acid itself. Since the presence of all these acids has been so adequately proved no

attempt has been made in the present studies to identify them. In Table IIA it was therefore assumed that the iodine value of the fractions having a mean molecular weight less than or roughly equivalent to that of methyl decanoate was due to the C_{10} unsaturated acid, while the iodine value of those with a molecular weight roughly corresponding to methyl dodecanoate was assumed to be due to the C_{12} unsaturated compound, and so on for the C_{14} and C_{16} unsaturated acids. The mean molecular weight of the remaining saturated acids is then obtained by simple proportion. And, again by assuming that only two saturated homologues are present in any one fraction, the amount of each homologue can be calculated.

In calculating the composition of the liquid fractions (Table IIC), it must first be realised that owing to the high boiling point of even the lowest fraction (No.36) and to the very small percentage of methyl laurate in the original mixture, extremely little, if any, of this particular ester can be present in these fractions. On the other hand due to its higher boiling point and greater concentration in the original mixture the ester of myristic acid would be expected to be present in these liquid ester fractions to a marked extent, particularly as a considerable proportion of lead myristate is known to pass into the liquid fraction in the Twitchell process (Hilditch & Priestman, 39). Again the

amounts of methyl palmitate and stearate must be exceedingly small as the Twitchell process is known to be very efficient in separating the C_{16} and C_{18} saturated acids from oleic and other unsaturated acids.

The composition of fractions 36 to 39 in Table IIC, which are small in themselves and possess relatively small iodine values, are calculated by a method very similar to that already described for the lower esters (Table IIA). From fraction 40 onwards the method is somewhat different. From the iodine value of each fraction it is very simple to calculate what its mean molecular weight would have been had it been fully saturated. Thus in fraction 40 for example an iodine value of 67.9 represents a mean unsaturation of 1.5 atoms of hydrogen (the 6th column in the table). The molecular weight of the fraction had it been saturated would thus have been 281.5 + 1.5 = 283.0. From this saturated molecular weight, the amount of C_{14}, C_{16} and C_{18} methyl esters present in each fraction is calculated, assuming that the C_{14} and C_{16} acids are present in equal amounts. Some such assumption as this is necessary because, when three homologues exist together in the same mixture, it is impossible to calculate the amount of each present unless the relative proportions of any two of them is assumed. By taking the C_{14} and C_{16} as being equal throughout in fractions 40 - 50 in Table IIC, very small errors in the calculated composition of some of the fractions will tend to be

neutralised by small opposite errors in some of the others. In any event such errors will have no more than an extremely small effect on the composition of the fat as finally deduced.

Having thus obtained the approximate amount of methyl myristate and palmitoleate likely to be present, the iodine value of the remainder of each fraction, consisting of methyl esters of C_{18} acids, is then calculated. From this iodine value, which is recorded in the 8th column of Table IIC, the amount of stearate, oleate and linoleate is readily obtained.

For the residue from the distillation of the liquid esters (fraction 51, Table IIC) the mean molecular weight was found to be higher than that of C_{18} esters. According to previous work on rat fat analysis by Channon et al. (25), the higher acid component was assumed for calculation purposes to be a C_{20} acid with one double bond. Actually, as pointed out by these authors, the mean unsaturation of these higher acids is probably considerably greater than this would suggest, because it is certain that they contain an appreciable amount of arachidonic acid which is a C_{20} acid with four double bonds. The existence of this acid was in fact proved as recorded on page 46. The advantage of assuming that the mean unsaturation of the C_{20} acids is equivalent to only one double bond is that it gives the maximum possible content for the linoleic acid in that particular fraction. If a higher degree of unsaturation for

the C_{20} acids were assumed, it would reduce the linoleic figure in fraction 51, Table IIC, from 0.35 to a slightly small amount, but the difference would come well within the limits of the analysis as a whole. It should be observed that the proportions of C_{18} and C_{20} acids in the fraction now under discussion are determined before any assumption is made as to the degree of unsaturation of the C_{20} acids. The assumption therefore only affects the division of the C_{18} acids into oleic and linoleic.

It will be observed that in all the tables just cited the results of the intermediate calculations are recorded to two decimal places, while the final figures shown in Table IID for the composition of the original mixture are quoted only to one place. In giving the intermediate calculations to two decimal places, no claim is made that the method as a whole possesses such a high degree of accuracy. It is simply a matter of following the commonly accepted practice of quoting the decimals to one place further than has any real significance. The general accuracy of this type of analysis has been discussed by Hilditch (35) and by Irving and Smith (44). The latter authors state that owing to the various assumptions which have to be made, the final results for the composition of the mixed fatty acids will approximate only to within a few units of the true value in the large figures recorded, with corresponding errors in

TABLE III.A.

TABLE III.A.

Fractionation of the methyl esters prepared from 285 g. of the butterfat secreted by cow No.1 on the 11th and 12th days of inanition.

Lower Esters.

Fraction No.	B.P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	M.W. of the saturated esters only (by calculation)	Saturated esters of the acids							Unsaturated esters of the acids		
						C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{14}	C_{16}	Oleate
1	Methyl butyrate	1.27	-	-	-	1.27	-	-	-	-	-	-	-	-	-
2	30-40	0.05	164.7	-	164.7	-	-	0.04	0.01	-	-	-	-	-	-
3	70-90	0.15	178.0	14.4	171.1	-	-	0.06	0.05	-	-	-	0.02	-	-
4	120	0.15	195.9	15.0	185.9	-	-	0.01	0.12	-	-	-	0.02	-	-
5	130	0.20	222.3	20.8	217.7	-	-	-	-	0.13	0.03	-	0.04	-	-
6	140	0.47	242.8	24.9	241.6	-	-	-	-	0.01	0.35	-	0.11	-	-
7	145	0.45	258.8	22.2	257.9	-	-	-	-	-	0.15	0.19	-	0.11	-
8	150	0.74	267.0	21.3	267.0	-	-	-	-	-	0.07	0.50	-	0.17	-
9	153	1.25	270.6	21.1	262.8	-	-	-	-	-	0.25	0.69	-	-	0.31
10	157	1.11	275.4	29.0	264.4	-	-	-	-	-	0.15	0.58	-	-	0.38
	Total	5.82				1.27	-	0.11	0.18	0.14	1.00	1.96	0.19	0.28	0.69

TABLE IIIB.

Fractionation of solid esters from cow No.1 during inanition.

Fraction No.	B. P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	M.W. corrected for methyl oleate	Saturated esters of the acids				Methyl oleate
						C_{14}	C_{16}	C_{18}	C_{20}	
11	147	0.26	250.9	2.5	248.8	0.19	0.06	-	-	0.01
12	150	0.74	262.4	0.8	262.4	0.21	0.52	-	-	0.01
13	152	1.79	269.0	1.0	268.3	0.13	1.64	-	-	0.02
14	152	3.50	271.7	2.0	270.8	-	3.36	0.06	-	0.08
15	153	3.15	275.6	3.0	274.6	-	2.57	0.47	-	0.11
16	153	4.25	275.2	2.1	274.6	-	3.51	0.64	-	0.10
17	154	3.86	277.3	3.5	277.3	-	2.75	0.95	-	0.16
18	154	2.86	279.8	4.4	279.7	-	1.80	0.91	-	0.15
19	160	1.81	282.0	6.4	279.7	-	1.12	0.56	-	0.13
20	165	3.32	294.2	9.9	293.5	-	0.51	2.45	-	0.38
21	167	4.06	298.0	11.1	298.5	-	-	3.50	0.03	0.53
22	168	3.55	300.4	11.9	300.7	-	-	2.80	0.26	0.49
23	Residue	3.08	305.7	14.9	304.9	-	-	1.95	0.60	0.53
	Total	36.23				0.53	17.84	14.27	0.89	2.70

TABLE III.C.

TABLE III.C.

Fractionation of liquid unsaturated esters from cow No.1 during inanition.

Fraction No.	B.P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	Calculated M.W. of fully saturated fraction	I.V. of the C_{18} and C_{20} esters.	Saturated methyl esters of the acids C_{14}	C_{16}	C_{18}	Unsaturated esters C_{14}	C_{16}	C'_{18}	C''_{18}	C^{*}_{20}
					M.W. of unsaturated esters only (by calculation)									
24	130-140	0.33	243.6	40.9	248.5		0.16	0.04	-	0.13	-	-	-	-
25	150	0.46	260.0	49.2	265.7		0.04	0.19	-	0.12	0.11	-	-	-
26	160	0.96	274.6	61.6	275.9	78.9	0.26	-	0.04	-	0.25	0.41	-	-
27	164	1.49	288.3	79.3	290.1	86.6	0.14	-	-	-	0.15	0.19	0.01	-
28	166	1.59	291.6	85.0	293.5	87.6	0.09	-	-	-	0.09	1.38	0.03	-
29	170	2.08	291.1	85.2	293.0	88.3	0.13	-	-	-	0.13	1.76	0.06	-
30	185	1.46	294.2	84.0	296.2	85.6	0.03	-	-	-	0.04	1.39	-	-
31	182	2.47	294.7	85.5	296.7	86.9	0.05	-	-	-	0.04	2.35	0.03	-
32	170	4.11	294.4	85.9	296.4	87.6	0.09	-	-	-	0.10	3.83	0.09	-
33	170	5.17	294.0	86.1	296.0	88.3	0.14	-	-	-	0.14	4.74	0.15	-
34	175	1.30	295.0	87.5	295.0	90.8	0.05	-	-	-	0.05	1.13	0.07	-
35	185	2.47	294.5	86.9	296.5	88.9	0.06	-	-	-	0.06	2.26	0.09	-
36	180	7.16	296.2	87.4	298.5	87.5	0.01	-	-	-	0.01	6.99	0.15	-
37	180	8.12	296.8	89.2	298.9	89.5	-	-	-	-	-	7.60	0.35	0.17
38	182	7.65	297.8	90.1	299.9	90.8	-	-	-	-	-	6.76	0.45	0.44
39	182	4.95	299.0	91.5	301.2	95.0	-	-	-	-	-	4.06	0.38	0.51
40	Residue	6.20	306.0	91.9	308.2	99.4	-	-	-	-	-	3.38	0.63	2.19
	Total	57.95					1.25	0.23	0.04	0.25	1.17	49.23	2.47	3.31

(') Methyl oleate; (") Methyl linoleate; (*) Calculated as the esters of C_{20} acid with one double bond only.

T A B L E XIID.

Summary of results obtained for butter-fat from cow No.1 on the 11th and 12th days of inanition.

	Acid	Per cent as methyl esters. Lower	Solid	Liquid	Total
Saturated.	C_4	1.27	-	-	1.27
	C_6	-	-	-	-
	C_8	0.11	-	-	0.11
	C_{10}	0.18	-	-	0.18
	C_{12}	0.14	-	-	0.14
	C_{14}	1.00	0.53	1.25	2.78
	C_{16}	1.96	17.84	0.23	20.03
	C_{18}	-	14.27	0.04	14.31
	C_{20}	-	0.89	-	0.89
	Total	4.66	33.53	1.52	39.71
Unsaturated.	C_{10}	-	-	-	-
	C_{12}	-	-	-	-
	C_{14}	0.19	-	0.25	0.44
	C_{16}	0.28	-	1.17	1.45
	Oleic	0.69	2.70	49.23	52.62
	Linoleic	-	-	2.47	2.47
	C_{20}	-	-	3.31	3.31
	Total	1.16	2.70	56.43	60.29
	Sum of the saturated and unsaturated esters.	5.82	36.23	57.95	100.0

TABLE IV.A.

TABLE IV.A.

Fractionation of the methyl esters prepared from 254 g. of butterfat secreted by cow No.2 on the 7th, 8th, 9th, 10th, 11th and 12th days of inanition.

Lower esters.

Fraction No.	B.P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	M.W. of the saturated esters only (by calculation)	Saturated esters of the acids							Unsaturated esters of the acids				
						C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{10}	C_{12}	C_{14}	C_{16}	Ole
1	Methyl butyrate	2.98	–	–	–	2.98	–	–	–	–	–	–	–	–	–	–	–
2	50	0.15	144.7	10.5	142.0	–	0.08	0.06	–	–	–	–	0.01	–	–	–	–
3	60	0.18	174.6	15.5	175.5	–	–	0.07	0.09	–	–	–	0.02	–	–	–	–
4	88	0.45	185.8	14.4	186.0	–	–	–	0.40	–	–	–	0.05	–	–	–	–
5	104	0.35	188.3	17.9	188.9	–	–	–	0.28	0.05	–	–	0.04	–	–	–	–
6	120	0.32	200.7	25.0	198.0	–	–	–	0.15	0.11	–	–	–	0.06	–	–	–
7	130	0.59	227.5	25.0	228.0	–	–	–	–	0.23	0.23	–	–	0.07	0.06	–	–
8	145	0.77	243.4	22.3	245.9	–	–	–	–	–	0.53	0.08	–	–	0.16	–	–
9	150	2.06	262.4	18.8	257.1	–	–	–	–	–	0.77	0.86	–	–	–	0.22	0.
10	157	1.01	273.4	24.2	269.5	–	–	–	–	–	0.03	0.71	–	–	–	0.13	0.
	Total	8.86				2.98	0.08	0.13	0.92	0.37	1.56	1.65	0.12	0.13	0.22	0.35	0.

TABLE IVB.

Fractionation of solid esters from cow No.2 during inanition.

Fraction No.	B. P. at 2 mm. (°) up to	% of Total esters	Molecular weight	Iodine value	M.W. corrected for methyl oleate	Saturated esters of the acids				Methyl oleate
						C_{14}	C_{16}	C_{18}	C_{20}	
11	140-150	0.33	252.0	1.6	250.7	0.22	0.10	-	-	0.01
12	152	0.82	264.5	0.7	264.0	0.18	0.63	-	-	0.01
13	153	1.73	267.6	0.9	267.3	0.18	1.53	-	-	0.02
14	155	4.54	270.7	2.5	270.1	0.01	4.40	-	-	0.13
15	155	4.29	273.0	2.7	272.0	-	3.90	0.25	-	0.14
16	158	3.63	276.1	4.0	275.1	-	2.87	0.59	-	0.17
17	161	3.89	278.6	4.8	277.3	-	2.75	0.92	-	0.22
18	165	3.91	282.4	6.5	281.3	-	2.14	1.38	-	0.29
19	165	2.98	287.5	8.3	286.6	-	1.12	1.55	-	0.31
20	167	4.04	291.7	10.0	291.3	-	0.89	2.68	-	0.47
21	Residue	4.16	304.0	18.5	306.2	-	-	2.34	0.92	0.90
	Total	34.22				0.59	20.33	9.71	0.92	2.67

TABLE IV.C.

T A B L E IV.C.

Fractionation of liquid unsaturated esters from cow No.2 during inanition.

Fraction No.	B.P. at 2 mm. (°) up to	% of total esters	Molecular weight	Iodine value	M.W. of unsaturated esters only (by Cal.)	Calculated M.W. of fully saturated fraction	I.V. of the C_{18} and C_{20} esters	Saturated methyl esters of the acids C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	Unsaturated esters C_{10}	C_{12}	C_{14}	C_{16}	C^{*}_{18}	C^{+}_{18}	C^{ϕ}_{20}
22	130	0.38	202.7	31.9	204.1			0.10	0.19	-	-	-	0.04	0.05	-	-	-	-	-
23	150	0.26	256.6	47.5	244.3			-	-	0.11	0.04	-	-	0.05	0.06	-	-	-	-
24	160	0.44	256.9	58.8	259.0			-	-	0.07	0.11	-	-	-	0.13	0.13	-	-	-
25	164	1.16	279.1	71.9		280.7	89.2	-	-	0.24	-	-	-	-	-	0.25	0.64	0.03	-
26	165	2.92	289.5	76.9		291.3	85.1	-	-	0.25	-	0.07	-	-	-	0.24	2.36	-	-
27	173	4.80	289.7	79.4		291.5	85.7	-	-	0.39	-	-	-	-	-	0.39	4.02	-	-
28	173	5.72	291.5	82.8		293.2	87.7	-	-	0.35	-	-	-	-	-	0.35	4.90	0.12	-
29	173	9.84	295.0	83.9		297.0	85.0	-	-	0.15	-	0.07	-	-	-	0.16	9.46	-	-
30	175	8.87	295.2	85.0		297.2	86.1	-	-	0.12	-	-	-	-	-	0.11	8.60	0.04	-
31	177	5.80	296.7	86.3		298.7	86.3	-	-	-	-	-	-	-	-	-	5.68	0.04	0.
32	180	4.30	300.9	87.1		303.0	88.8	-	-	-	-	-	-	-	-	-	3.45	0.13	0.
33	185	5.63	297.1	87.0		299.1	87.3	-	-	-	-	-	-	-	-	-	5.37	0.10	0.
34	187	2.63	301.4	87.1		303.5	89.2	-	-	-	-	-	-	-	-	-	2.05	0.09	0.
35	Residue	4.17	310.1	87.1		312.2	95.8	-	-	-	-	-	-	-	-	-	1.86	0.24	2.
	Total	56.92						0.10	0.19	1.68	0.15	0.14	0.04	0.10	0.19	1.63	48.39	0.79	3.

* Methyl oleate; + Methyl linoleate; ϕ Calculated as the esters of C_{20} acid with one double bond only.

TABLE IVD. Summary of the results for the butterfat obtained from cow No.2 during inanition.

	Acid	Per cent. as methyl esters			
		Lower	Solid	Liquid	Total
Saturated.	C_4	2.98	-	-	2.98
	C_6	0.03	-	-	0.03
	C_8	0.13	-	-	0.13
	C_{10}	0.92	-	0.10	1.02
	C_{12}	0.37	-	0.19	0.56
	C_{14}	1.56	0.59	1.68	3.83
	C_{16}	1.65	20.35	0.15	22.13
	C_{18}	-	9.71	0.14	9.85
	C_{20}	-	0.92	-	0.92
	Total	7.69	31.55	2.26	41.50
Unsaturated.	C_{10}	0.12	-	0.04	0.16
	C_{12}	0.13	-	0.10	0.23
	C_{14}	0.22	-	0.19	0.41
	C_{16}	0.35	-	1.63	1.98
	Oleic	0.35	2.67	48.39	51.41
	Linoleic	-	-	0.79	0.79
	C_{20}	-	-	3.52	3.52
	Total	1.17	2.67	54.66	58.50
	Sum of the saturated & unsaturated esters	8.86	34.22	56.92	100.0

the smaller figures. Unfortunately no method giving a more accurate analysis for fats has so far been devised.

For the three fats analysed and for which details are given in Table I, the total loss of material throughout the whole analytical process was 5 - 7%. Considering the various processes involved in the complete analysis this loss is very small. In calculating the results the total weight of all the fractions has been used for estimating the composition rather than the weight of fat initially taken. This is in accordance with the general practice of others (cf. Hilditch et al. and Channon et al.).

Experiments to Identify Linoleic, Lower Unsaturated and Arachidonic Acids:

(A) Linoleic Acids: Reports in the literature regarding the linoleic acid content of butterfat are conflicting. Hilditch and Sleightholme (40) concluded that some butterfats may contain as much as 4.5% of this acid, whereas on the other hand Holland et al. (43) and Bosworth and Brown (22) failed to verify its presence. Eckstein (26) reported that several butterfats examined by him contained approximately 0.2 - 0.5% of linoleic acid, and demonstrated that the amount in butterfat was related to the total quantity ingested by the lactating animal.

That linoleic acid does generally exist in milk fat and to the extent of a few units per cent. has been

adequately proved by Hilditch et al. (39), (42) when, by oxidation experiments, they isolated appreciable amounts of tetra-hydroxystearic acid which could only have arisen from linoleic acid. But as the matter has been disputed by some American workers and as inanition might cause appreciable difference in the linoleic acid content of the butterfat, its presence was sought by oxidation in various fractions in the present work.

The oxidation was carried out by the method of Lapworth and Mottram (45) as recommended by Channon et al. (24). The fatty acids are first isolated from the particular fraction to be analysed. It is dissolved in a dilute aqueous solution of alkali (0.73 g. of KOH for 1 g. acid) so as to make the concentration of organic acid 0.1%. The whole mixture is kept in the refrigerator till the temperature is reduced to 0°. An aqueous solution of potassium permanganate is also chilled at the same time (0.76 g. of $KMnO_4$ for 1 g. of acid). When calculating the amount of permanganate a correction is always made for the approximate amount of saturated acids present, since an excess of the reagent is harmful. The permanganate is then added with continuous shaking to the solution containing the acids. The oxidation is allowed to proceed for 5 minutes and is then stopped by adding a small quantity of concentrated sodium metabisulphite solution and 150 ml. of concentrated HCl. After allowing the mixture to stand for some time it is filtered and the

precipitate washed several times with water. The residue is dried in a vacuum desiccator and extracted with light petroleum to take out any unsaturated acids present. The residual powder is refluxed with ether (one litre for 1 g.) for 2 - 3 hours, which dissolves the dihydroxystearic acid formed from oleic. If there is undissolved material left, the mixture is filtered through a warm funnel (30°) and the residue extracted with ethyl acetate. The hot ethyl acetate solution is filtered under suction and washed. The residue is purified by dissolving it in alcohol, filtering and precipitating it from the filtrate by adding ether. Its molecular weight and melting point are then determined. If linoleic acid is originally present in sufficient quantity the M.P. of this tetrahydroxy derivative will be 173°. Fractions 40, 41 and 50 in Table IIC and 25, 26, 39 and 40 in Table IIIC were treated in this way, but in no case could any tetrahydroxystearic acid be isolated. This was doubtless due to the fact suggested by the final results of the fractionation that the linoleic acid content of the milk fat from these particular cows was as low as 1 - 2% and that the amount of fat available was limited. That linoleic acid was almost certainly present in small amounts is shown by the iodine values recorded in Tables IIC, IIIC and IVC for the higher liquid fractions.

(B) Lower Unsaturated Acids: As already explained, owing to the excellent proof which has recently been published for the existence of lower unsaturated fatty

acids in normal milk fat, their presence in many of the fractions from the distillation process in the present work has been assumed. In order to ensure that this assumption was justified for the fat secreted during inanition, fractions 8 and 9 (Table IIIA) and 25 and 26 (Table IIIC) were subjected to oxidation as already described for linoleic acid. When the dihydroxy derivatives were isolated from these two pairs of fractions, the mean molecular weight of the product was 288 for the first pair and 297 for the second, the theoretical molecular weights for the dihydroxy derivatives from C_{14}, C_{16} and C_{18} unsaturated acids being 260, 288 and 316 respectively. When one or two higher fractions were analysed in the same way the molecular weight of the dihydroxy derivatives was 317 - 318 and the M.P. 130°. This indicated that whereas in the higher fractions the main unsaturated acid was oleic, in the lower fractions there were considerable proportions of lower unsaturated acids.

(c) Arachidonic Acid: This highly unsaturated acid has been shown to occur in normal milk fat by Bosworth and Sisson (23) and it was of interest to determine whether the acid was also secreted by the mammary gland during inanition. Acids of this particular type are identified by the following method. The fatty acids obtained by saponifying fractions are dissolved in absolute ether and then cooled to 0°. To the cooled solution, bromine is

is added in small quantities with continuous shaking, the temperature being maintained below 2°, till the solution acquires a permanent orange tint. It is then allowed to stand for 24 hours in the refrigerator, after which the precipitated bromo derivative is filtered off and washed with ether. Its weight, M.P. and bromine content are then determined. For all the fractions treated in this way, a very small quantity of a bromo-derivative was obtained, M.P. about 228° with decomposition, and bromine content of 65.5%. $C_{20}H_{32}O_2Br_8$ contains 67.8% of bromine and has a similar indefinite melting point. It is therefore safe to conclude that both before and during inanition a small amount of some such highly unsaturated C_{20} acid was being secreted. The amount of crude octabromo-derivative obtained from each fraction suggested that the arachidonic acid present in the total mixture was at least 0.1%.

DISCUSSION.

The Yields of Fat: For all three animals the period of inanition was characterised by a very considerable decrease in the daily secretion of milk fat. Thus it can readily be seen from Fig.1 that for the first cow in which inanition was not complicated by other factors, the yield fell quickly from 568 g. before the fast to 353 g. on the third day, and then more slowly to a mean of 227 g. or 40% of the original during the last three days of the fast. On realimentation a

further slight drop was noticeable, but five days later the daily yield began to increase and at the end of about six weeks it had returned to some 60% of the prefast value. Allowing, therefore, for the usual drop in milk yield as lactation advances, it may be assumed that six weeks after the end of the fast, the yield had returned almost to normal. With cow No.2 in which milk fever symptoms developed on the third day, the milk secretion almost ceased while this complication lasted, but by the fifth day the fat production had risen to 12%, and before the end of the fast to 20% of the original. On realimentation the yield followed a course almost parallel to that of cow No.1 during the same period.

The Composition of the Fats : Fig.2 shows clearly that inanition caused a marked progressive change in the nature of the fats secreted by each of the three cows as judged by their iodine and Reichert Meissl values, and that these changes were very similar for all the three animals. It will be observed that the initial iodine values of 36.6 and 37.1 for cows 1 and 2 rose quickly to 46.1 and 47.3 by the first day of the fast, and then slowly to 52.5 and 54.9 by the 12th day, the figures for cow No.3 following a similar course till the 7th day when this particular animal was withdrawn from the experiment. Then again the R.M. values for cows 1 and 2 fell rapidly in the first two days from 26.0 and 33.3 to 14.5 and 22.9 and then still further to 9.8 and 13.8 by the end of

the fast. On realimentation a slight tendency to return to normal was observed in both values after the first few days and this tendency continued somewhat spasmodically for three or four weeks until figures similar to the pre-fast values were attained. These results at once suggest that inanition causes a considerable increase in the content of the unsaturated acids and a marked fall in that of the lower fatty acids normally present as glycerides in butterfat. This was confirmed by more detailed analyses based on fractional distillation methods. Thus reference to the totals recorded in Tables IID and IIID shows that, while the proportion of higher solid esters remained almost unaffected as a result of inanition, the lower esters were reduced from a pre-fast value of 25.2 to 5.8%, a difference of 19.4 units, and that the corresponding figures for the liquid esters showed an almost equal increase from 40.4 to 58.0%, a difference of 17.6 units. Again it will be observed from the weight percentages given in Table V that the sum of all the acids up to and including C_{14} amounted to 22.6% before the fast and to no more than 4.8% during inanition, a decrease of 17.8 units, while the oleic acid content rose by 16.9 units, i.e. from 35.9 to 52.8%. Calculated also on a molar basis, as shown in the same table, the fall of 24.2 molar per cent. in the lower acids was mainly accounted for by a rise of almost 20 molar per cent. in the oleic acid. It seemed evident therefore that the chief effect of

inanition was the replacement of about 80% of the lower constituents of the fat mainly by oleic acids, and that the whole of this decrease was distributed over all the lower members from C_4 to C_{14} but the first and last of these appeared to suffer least. Thus the butyric acid content only fell from 3.5 to 1.2%, a trifle more than a third of the original, the myristic by a somewhat similar amount and the others to less than an eighth of their pre-fast values. Of the remaining saturated acids the amount of palmitic fell slightly from 23.5 to 20.0% while the stearic increased from 11.6 to 14.3%. That the latter rise has probably some significance was shown best by reference to the molar percentages where it will be observed that stearic acid rose from 9.8 to 13.5%, almost a 40% increase, whereas the fall in palmitic acid amounted only to some 5% of the pre-fast value. In fact the constancy of the palmitic acid in all three sets of results is worthy of particular notice. The saturated acids represented as C_{20} also remained virtually unchanged. In the unsaturated acids other than oleic and higher than C_{14}, the chief change appeared to be a decrease in palmitoleic acid from 3.2 to 1.4% and a most marked rise from 0.8 to 3.3% in the higher unsaturated components calculated as "C_{20}".

The results for the fat secreted by cow No.2 during inanition are also recorded in Table V and if these are considered in conjunction with the curves in Fig.2, it will readily be seen that with the possible

TABLE V : The component fatty acids of the different butterfats expressed in weight & molar percentages.

Acid	Weight percentages.			Molar percentages.		
	Cow No. 1 Before inanition	Cow No. 1 During* inanition	Cow No. 2 During+ inanition	Cow No. 1 Before inanition	Cow No. 1 During* inanition	Cow No. 2 During+ inanition.
Saturated.						
C_4	3.5	1.2	2.7	9.7	3.5	7.9
C_6	0.6	-	0.1	1.2	-	0.1
C_8	1.0	0.1	0.1	1.6	0.2	0.2
C_{10}	1.8	0.2	1.0	2.5	0.3	1.5
C_{12}	2.5	0.1	0.6	3.0	0.2	0.7
C_{14}	11.9	2.8	3.8	12.5	3.2	4.3
C_{16}	25.5	20.0	22.1	22.1	20.9	22.1
C_{18}	11.6	14.5	9.9	9.8	13.5	8.9
C_{20}	1.1	0.9	0.9	0.8	0.8	0.8
Total	57.6	39.8	41.2	63.2	42.6	46.5

* A sample obtained by mixing the fat secreted on the 11th and 12th days of inanition.

\+ A pooled sample from the fat secreted on the last six days of inanition.

TABLE V : The component fatty acids of the different butterfats expressed in weight & molar percentages.

Acid	Weight percentages. Cow No. 1 Before inanition	Weight percentages. Cow No. 1 During* inanition	Weight percentages. Cow No. 2 During+ inanition	Molar percentages. Cow No. 1 Before inanition	Molar percentages. Cow No. 1 During* inanition	Molar percentages. Cow No. 2 During+ inanition
C_{10}	0.2	-	0.2ϕ	0.3	-	0.2
C_{12}	0.2	-	0.2ϕ	0.3	-	0.3
C_{14}	0.9	0.4ϕ	0.4ϕ	1.0	0.5	0.5
C_{16}	3.2	1.4	2.0	3.0	1.5	2.0
Oleic	35.9	52.8	51.7	30.5	50.1	46.9
Linoleic	1.2	2.5	0.8	1.0	2.4	0.7
C_{20}	0.8	3.3	3.5	0.6	2.9	2.9
Total Unsaturated.	42.4	60.4	58.8	36.7	57.4	53.5
Total of all the acids up to C_{14}	22.6	4.8	9.1	32.1	7.9	15.7

* A sample obtained by mixing the fat secreted on the 11th and 12th days of inanition.
+ A pooled sample from the fat secreted on the last six days of inanition.
ϕ Where the amount of lower acids present is very low and oleic acid predominates as in the fats of inanition, it is probable that the figures marked ϕ are somewhat higher than the true values.

exception of stearic and linoleic acids, the general effect of the fast on the composition of the milk fat was the same for both animals.

The great change obtained in the amount of lower fatty acids during inanition is worthy of special consideration. With regard to their source in normal butterfat there are probably four main possibilities. In the first place they may pre-exist in the blood and arise from it directly. Secondly, they may be formed in the mammary gland itself by breakdown of oleo-glycerides as suggested by Hilditch and Thompson (42) and by Hilditch and Paul (38). Thirdly, they may arise, again in the gland itself, as by-products in the synthesis of the fat from some form of carbohydrate material, or fourthly, by some combination of the three foregoing processes. The theory that they may arise directly from the blood gains support from the suggestion that the lower fatty acids may be formed by bacterial action in the rumen of the cow and that these are then transported by the blood to the mammary gland, whereas in the human species, in which the formation of lower acids in this particular way is not possible, the content of the lower acids in the milk fat is exceedingly small (Espe, 27). From the present work this theory might also appear to gain confirmation from the fact that as rumination ceased as a result of the enforced fast, the lower fatty acid content of the milk also fell, but in this connexion there are two points of interest. In the first place

it would be very natural for these lower compounds, if they exist in blood, to be present in the non-phosphatide fraction of the plasma. But from the results of Smith (51) it was suggested that if any of these lower acids were present at all as esters in this fraction, their amount must have been very small and that they certainly did not appear to decrease during inanition. It seemed always possible, however, that during the analysis of the blood lipoids free water-soluble acids were lost, for such an occurrence would be exceedingly difficult either to detect or avoid. Moreover, only exceedingly small concentrations of these lower fatty acids in blood plasma would be required to substantiate this theory, for it has been shown by Graham et al. (33) that on an average only about 5 mg. of fatty acids per 100 ml. of blood are removed by the mammary gland to produce the total daily yield of milk fat which may amount to as much as 1 kg. or more. Consequently if only 5 mg. of fatty acids are removed for the production of the total milk fat, the amount necessary for producing the lower fatty acids which amount to no more than about 20% of the whole must be very small indeed and certainly much too small to be measured by any method so far devised. On these grounds, therefore, it is quite possible that the lower fatty acids of milk do arise directly from the blood in which they may be present in small amounts. In the second place, however, if the production of these lower constituents

of milk fat was due to a deficiency of the same acids in the blood, it would be natural to expect that their decrease in the milk fat during inanition would be counter-balanced by increases fairly evenly distributed over all the other constituents, whereas it has already been shown that almost the whole loss is made good by a corresponding increase in oleic acid alone. Indeed this last observation might lend considerable support to Hilditch's suggestion that the lower acids arise by the oxidation of oleo-glycerides in the gland itself, for if this were so, the catabolism of these glycerides might not be so essential during inanition when the general activity of the gland was considerably reduced, with the consequence that the content of the lower constituents would fall and that of oleic acid rise. Recent work, however, by Graham et al. (32), based on respiratory quotient measurments, has suggested that fat metabolism in the mammary gland probably includes some form of synthesis rather than of breakdown. Too much stress must nevertheless not be laid on the possibility of fat synthesis within the mammary gland until the work of Graham et al. has been adequately confirmed. Assuming that it is correct, the explanation of the present results may be that the lower compounds are formed as by-products in the synthesis of oleic acid, but that during inanition, when the gland is producing much less than its usual amount of fat, the smaller amount of synthesis taking place is able to proceed to its final stages, so that the lower acids are almost completely converted to

oleic. This suggestion might possibly gain support from the well-known fact that diets rich in carbohydrates and poor in fat cause an increase in the lower fatty acids of the milk fat, while those poor in carbohydrates and rich in fat have the reverse effect, indicating that on diets with which fat synthesis would probably be most necessary the lower fatty acids increase. Here again the explanation may be that when fat is deficient in the diet, the mammary gland tends to take up from the blood a higher proportion of the lower fatty acids which may be formed in the rumen. All that can definitely be established at this stage is that there is a marked inverse relationship between the lower fatty acids and oleic, but whether the lower acids arise by breakdown of oleic or as by-products in the synthesis of that acid or directly from blood is a problem which must await further work.

Apart from the theoretical aspects of the problem, the present investigations show that when the animals were not well nourished, the fat secreted by them greatly resembled a sample of butterfat adulterated with fat from another source. Usually it is the practice in most countries to fix the standard Reichert Meissl value for normal butterfat to be not less than 24 and the iodine value not greater than 40. If a sample of fat falls outside these limits it is looked upon with suspicion. When reference is made to the analytical constants of the fats analysed here

(Table I), it is seen that they closely resemble what might be regarded as adulterated samples. Fig.2 shows that even two days of inanition produces a considerable change in the composition of butterfat. When it is remembered that in many parts of the world it is not uncommon to find considerable degrees of under-nutrition among cattle, greater consideration should be given to this particular factor in fixing the standards for chemical purity of butterfat than has perhaps hitherto been the custom. Further reference will be made to this point in Part II of the present thesis (p. 66).

SUMMARY.

1. The effect of inanition on the butterfat secreted by three lactating cows has been investigated in detail with the object of studying the mechanism of milk fat secretion.

2. The chief change in the composition of the milk fat was a decrease of about 80% in the original content of lower acids up to and including C_{14}, a deficiency which was almost entirely made good by an increase in the content of oleic acid.

3. The fact that oleic acid tended so completely to replace the lower constituents has been discussed in connexion with different theories previously put forward to explain the occurrence of these lower homologues in milk fat.

4. A description is given of a modified procedure for the analysis of butterfat by the fractional distillation method from which prolonged distillation of the saponified fat in steam is omitted.

5. The various findings are briefly discussed in relation to the subject of the adulteration of butterfat.

PART II.

The Effect of Thyroxine Administration on the blood Lipoids and on the Nature of the Milk Fat.

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INTRODUCTION.

That the thyroid gland plays an important part in the control of mammary activity has been known since 1918 (70), but it is only recently that a number of systematic experiments have been carried out to study the effect of this hormone on milk secretion. Graham (66, 67) found that when a cow in declining lactation was treated with either desiccated thyroid tissue or synthetic thyroxine, there was a rise both in the yields of milk and of milk fat. This author suggested that the action of thyroxine on milk secretion is connected with its effect on the basal metabolic rate. Jack and Bechdel (73) also found an increase in milk and fat secretion on injecting an alkaline thyroxine solution, but there was no rise in the concentration of the other milk constituents. Folley and White (64), by injecting 10 mg. of thyroxine solution daily for 15 days, caused four Shorthorn cows in the declining phase of lactation to increase their milk production on an average by 26%, the fat production by 50%, and the concentration of non-fatty solids of the milk by 4% above the basal levels. The rise in the milk yield took place almost at once but the concentration of fat in the milk was not enhanced till after the first 6 days of the hormone treatment. It was also observed that the pulse rate of the animals increased

by 22 beats per minute.

Herman et al. (71, 77), who have carried out similar studies, agree with other workers in finding that the cows at the peak of production and those nearing the end of the lactation period fail to increase in milk yield, either with desiccated gland or with thyroxine itself. They also found that subcutaneous injection of thyroxine produced a much greater effect than the same amount of thyroid fed orally, suggesting that in the ruminant some of the activity of the hormone may be destroyed, probably by bacterial action in the rumen.

In recent years Reece and Turner (78) have been carrying out experiments to find whether, for normal cows, there is any connexion between the thyroid activity and the capacity of animals to produce milk. They report that pituitry glands from lactating cows contain more of the thyrotropic hormone than those from dry cows and that, on the basis of pituitry size, the glands of dairy cows contain potentially more of this hormone than those of beef cows. They make the interesting suggestion that a study of the endocrinology of the dairy cow may ultimately be of great value to geneticists in the breeding of high milk producers.

In none of the experiments so far carried out with ruminants has any attempt been made to study the changes in the various lipoid fractions of the blood, although a large number of investigations have been made on human subjects and on other animals. Boyd and Connell

(61) have published an extensive bibliography in which most of the studies described are confined to the estimation of blood cholesterol. In man hyperthyroidism is accompanied by a decrease in the concentration of blood cholesterol and probably also of other lipoids, the decrease being less marked and frequently insignificant when whole blood is analysed instead of plasma. The lipopenic action of thyroxine, (i.e. its action in decreasing the amount of blood lipoids), does not appear to be common to all species of animals. It has been observed in cats and dogs, but not in rabbits and birds. The only reference in the literature to the effect of thyroxine on the blood lipoids of cows is that of Graham et al. (69), who refer to some unpublished work of Graham and Jones in 1935 which has not yet appeared in print. They report that an increase in the blood sugar and a decrease in the total blood fatty acids was found during thyroxine treatment. In this brief account by Graham et al. only a few values are given and evidently no attempt was made to study the different individual fractions of the blood lipoids. This latter point is extremely important because it is very possible for the total fatty acids of the blood to fall and yet the tri-glycerides of the plasma, which are probably the main precursors of the milk fat, to rise, since tri-glyceride fatty acids form a very small proportion of the whole. It was therefore decided in the present work to analyse as extensively as possible

the various fractions of the blood lipoids.

If the suggestion of Reece and Turner already cited ever became possible in practice and greater milk production were to be secured by breeding cows possessing greater thyroid activity, the question naturally arises as to whether thyroxine causes changes in the actual nature of the milk fat when it brings about a 50% increase in the yield. Only one publication has so far appeared in which milk fat from cows receiving thyroxine has been analysed. In this paper by Hilditch and Paul (72), the object of the experiment was to study the minor fatty acid components of milk fat and not the effect of thyroxine on the general composition of the fat. Consequently the nature of the fat secreted before, during and after thyroxine treatment was not studied. The fat used by these authors appeared to be normal, but the limits within which milk fat can vary and yet can still be regarded as normal are extremely wide, and the fat might well have been within these limits, although it had changed appreciably on administering the hormone. It was therefore decided to examine this point in detail.

The object of the present section of the work was therefore two-fold; First, to examine as far as possible the changes brought about by thyroxine in the amount and nature of the blood lipoids; and second, to study the quality of the milk fat before,

during and after hormone administration.

EXPERIMENTAL.

The particulars of the four animals and controls used in the present experiment are given in Table VI. Cows 1, 2 and 3 were treated with thyroxine in the autumn of 1938 and cow No.4 during the summer of 1939. All the experimental animals were allowed free access to pasture except when brought in for milking, when they were given a little crushed oats and maize. They were milked twice daily and composite samples of milk and fat were collected as described for the inanition experiments (Part I, p.10).

TABLE VI.

Particulars of the animals used for the Experiment.

	Age in years.	Number of Calvings.	Weeks in milk.	Time when experiment was made.
Cow No.1	4	2	27	
Cow No.2	7	5	34	September
Cow No.3	6½	4	37	to beginning
Control I	6	3	28	of October.
Control II	10	7	34	
Cow No.4	11	8	15	End of May
Control	7½	5	14	to middle of July.

The thyroxine solution was prepared from B.D.H. thyroxine sodium B.P. by dissolving it in dilute NaOH solution and neutralising the excess of alkali with dilute HCl until the first trace of precipitate was

formed. The mixture was then made up to a known volume and filtered before use. 10 ml. of this solution, containing 10 mg. of thyroxine, were injected intramuscularly in the thigh each day. The treatment was continued for ten days with cows 1, 2 and 3 and for 15 days with cow No.4.

MILK ANALYSIS: Milk samples were analysed for fat (Gerber), lactose (polarimetrically), chloride (Davies, 62), protein (total nitrogen by Kjeldhal method multiplied by 6.38) and total solids. Large quantities of milk fat were also collected at intervals during the experiment and a sample of these studied in detail by the fractional distillation method. The results for the milk and milk fat before, during and after the hormone treatment are shown in Figs. 5 to 8 and are discussed in detail later. The Reichert Meissl values recorded in Fig.8 represent an average of two determinations carried out with only 2.5 g. of fat instead of the 5 g. which it is customary to use for this process. A series of tests carried out to find if there was any difference in the results obtained by the two methods showed that when only 2.5 g. of butterfat were used the values were about two units higher than those obtained by using 5 g. For example, a mixed sample of butterfat from cow No.3 gave a R.M. value of 18.9 when 2.5 g. were used and 16.9 when 5 g. of fat were employed. The method used here, involving 2.5 g., must therefore

have caused the R.M. values to be slightly higher than they would otherwise have been, but this will not in any way effect the interpretation of the results.

A study of Fig. 8 will show that the iodine and Reichert Meissl values for the butterfats from cows 1, 2 and 3 were very abnormal (i.e. I.V. 44-54; R.M. 17 - 25). It was therefore decided to study in more detail two samples of the fat from cow No.3, one taken at the peak of fat production during thyroxine treatment and the other taken later when the yield of fat had returned almost to normal. The results are shown in Table VII, where it will be seen that low saponification and Krischner values confirmed the low Reichert Meissl numbers already determined. The values as a whole suggest that the two fats were similar to each other but were abnormal. One of them was therefore examined in detail by the method of fractional distillation already described in Part I (p. 15). Its composition is recorded in Table VIII.

These results show that the content of oleic and higher unsaturated acids was greater, while the content of acids up to and including C_{12} was somewhat less than usual. For example, the percentage of oleic, linoleic and C_{20} unsaturated acids was 46.5 as compared with only 37.9 in the sample of normal butterfat obtained before inanition and analysed in Part I (p. 50). Similarly, the lower acids up to and including C_{12} amounted in the present mixture to only 6.4 as against

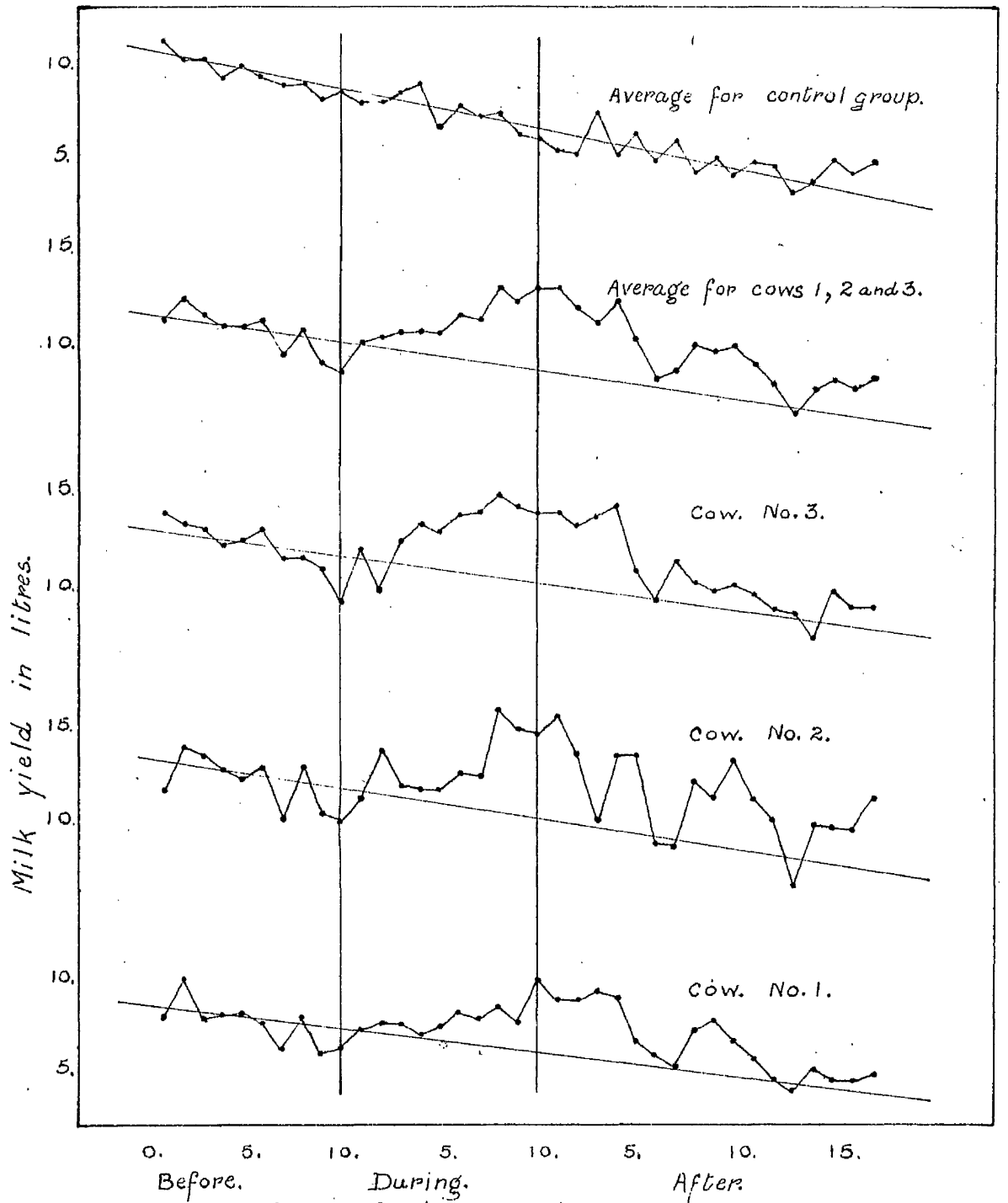

FIG. 3. The effect of thyroxine on the daily milk yields of cows 1, 2 and 3.

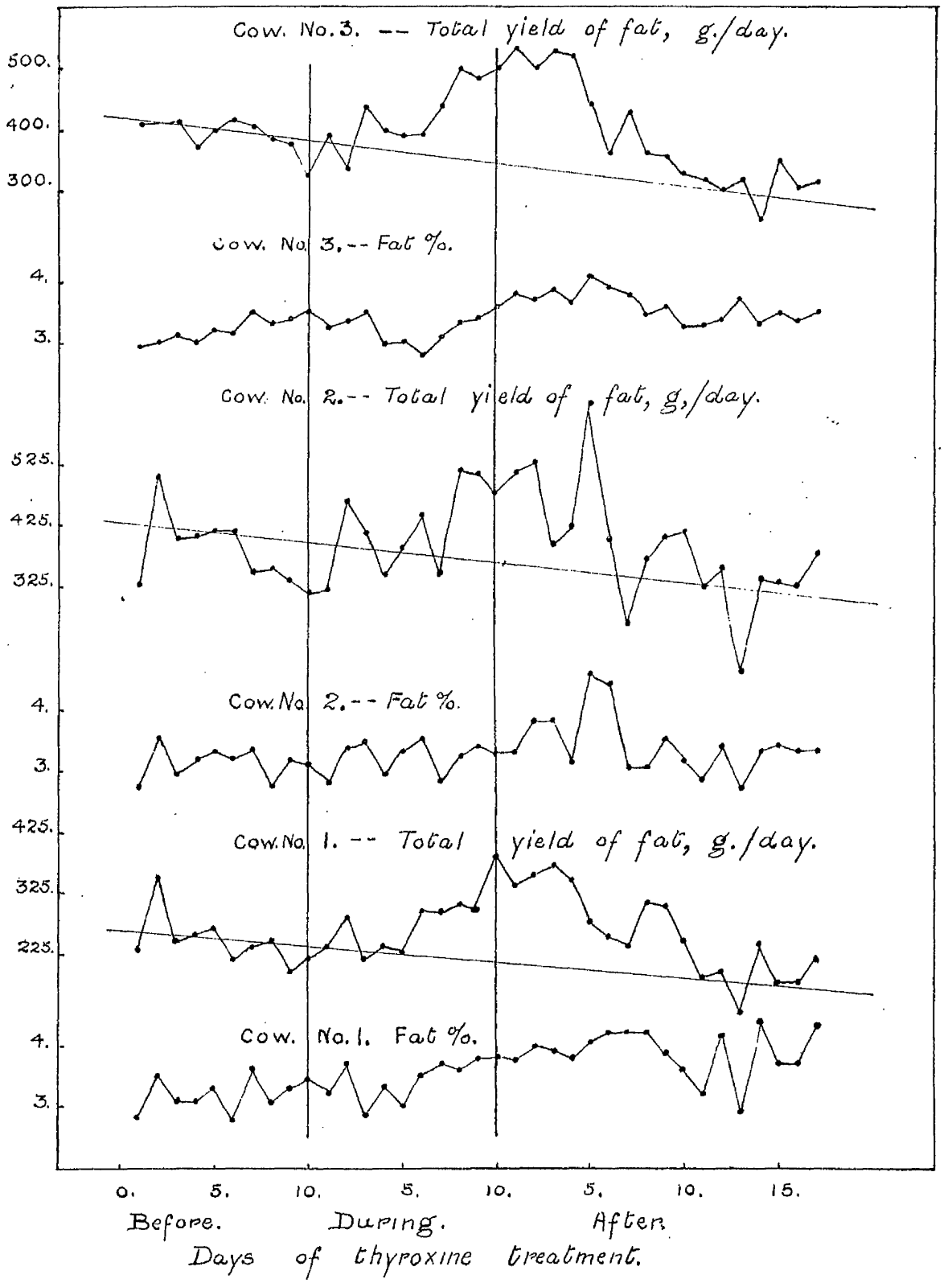

FIG. 4. The effect of thyroxine on the fat percentage and total daily fat yields from cows 1, 2 and 3.

TABLE VII.

Analytical constants of samples of butterfat from cow No.3 obtained during thyroxine experiment.

Description	R. M. value	Polenske value	Kirschner value	Iodine value	Sapon. value
Sample of fat obtained on the 9th and 10th days of thyroxine experiment	17.2	1.0	14.6	48.4	213.4
Sample of fat obtained on the 9th, 14th and 15th days after thyroxine treatment	16.8	1.0	14.1	46.5	213.8

9.8 for the normal sample, a decrease of about 35%. The molar percentages recorded in the same table also exhibit similar differences. These results, taken in conjunction with those already described for the inanition experiment, show how difficult it is to fix definite limits for the chemical constants by which the purity of butterfat is tested, since so many factors, - such as the system of management, the quality and quantity of the food, the season of the year and the stage of lactation, - may combine to have very marked effects.

BLOOD ANALYSIS: The work of Maynard and McCay (75) and of Porcher and Maynard (76) has shown that the blood lipoid level of ruminants under normal

TABLE VIII : The component fatty acids of a pooled sample of fat secreted by cow No.3 on the 9th, 14th and 15th days after the final thyroxine injection.

	Acid	Weight percentages	Molar percentages
Saturated.	C_4	2.5	7.1
	C_6	0.5	1.1
	C_8	0.5	0.9
	C_{10}	1.1	1.5
	C_{12}	1.5	1.9
	C_{14}	9.5	10.4
	C_{16}	25.3	24.6
	C_{18}	8.1	7.1
	C_{20}	0.6	0.5
	Total	49.5	55.0
Unsaturated.	C_{10}	0.1	0.2
	C_{12}	0.2	0.2
	C_{14}	0.8	0.9
	C_{16}	2.9	2.8
	Oleic	41.5	36.6
	Linoleic	2.9	2.6
	C_{20}	2.1	1.7
	Total	49.5	45.0

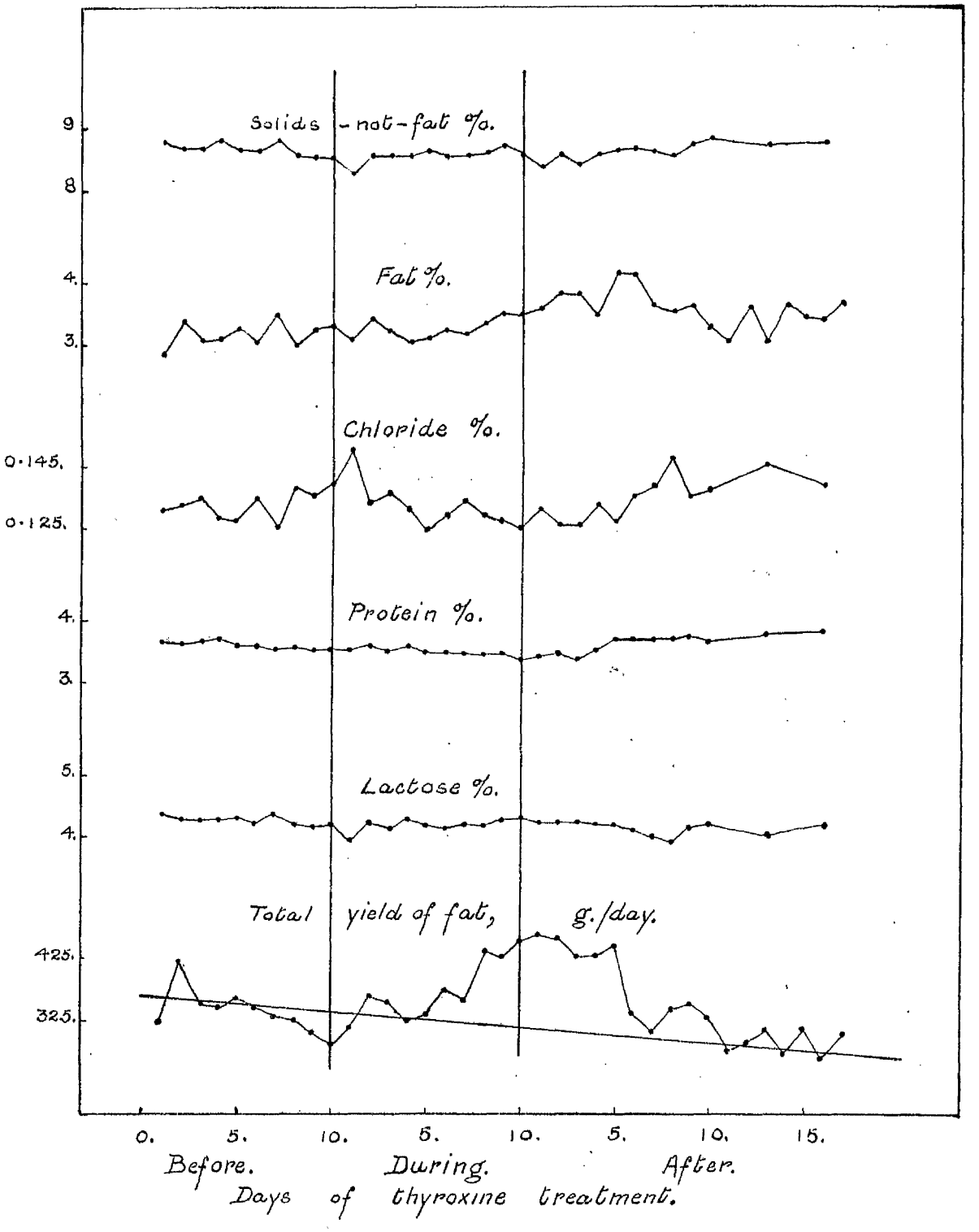

FIG. 5. The effect of thyroxine on the constituents of milk. The curves show the average values for cows 1, 2, and 3.

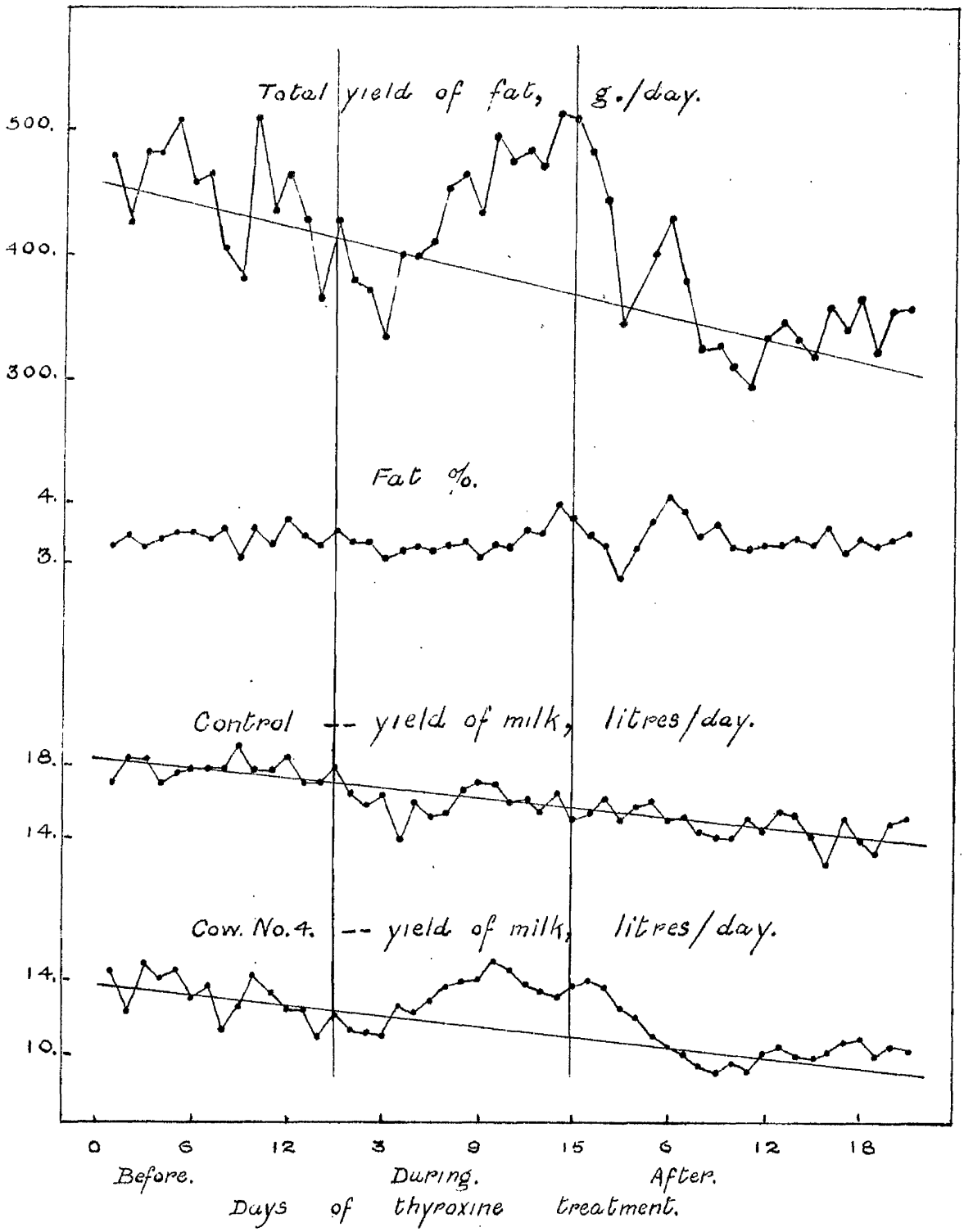

FIG. 6. The effect of thyroxine on the milk yield, fat % and total daily fat yield for cow No. 4.

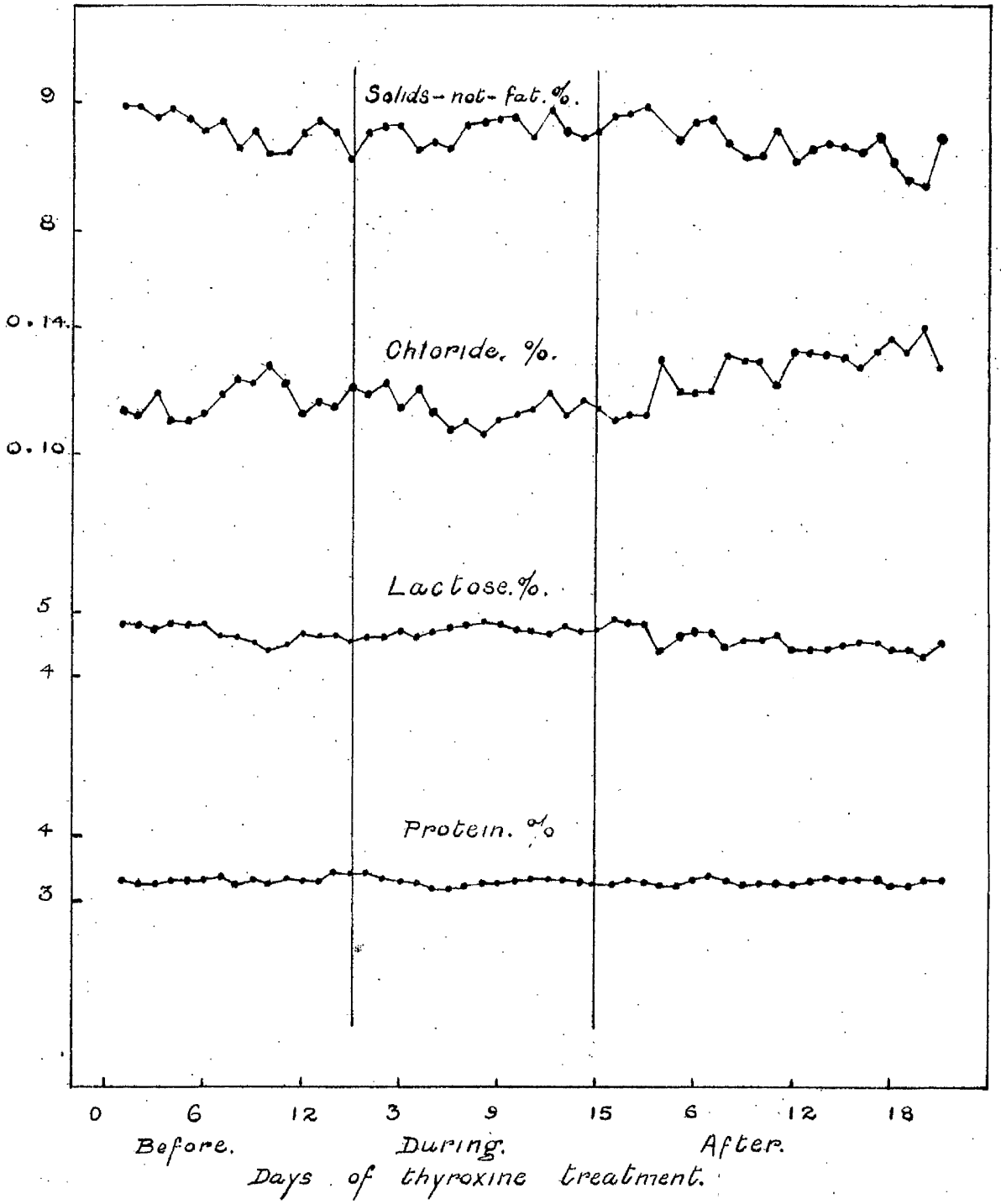

FIG. 7. The effect of thyroxine on the constituents of milk from cow No. 4.

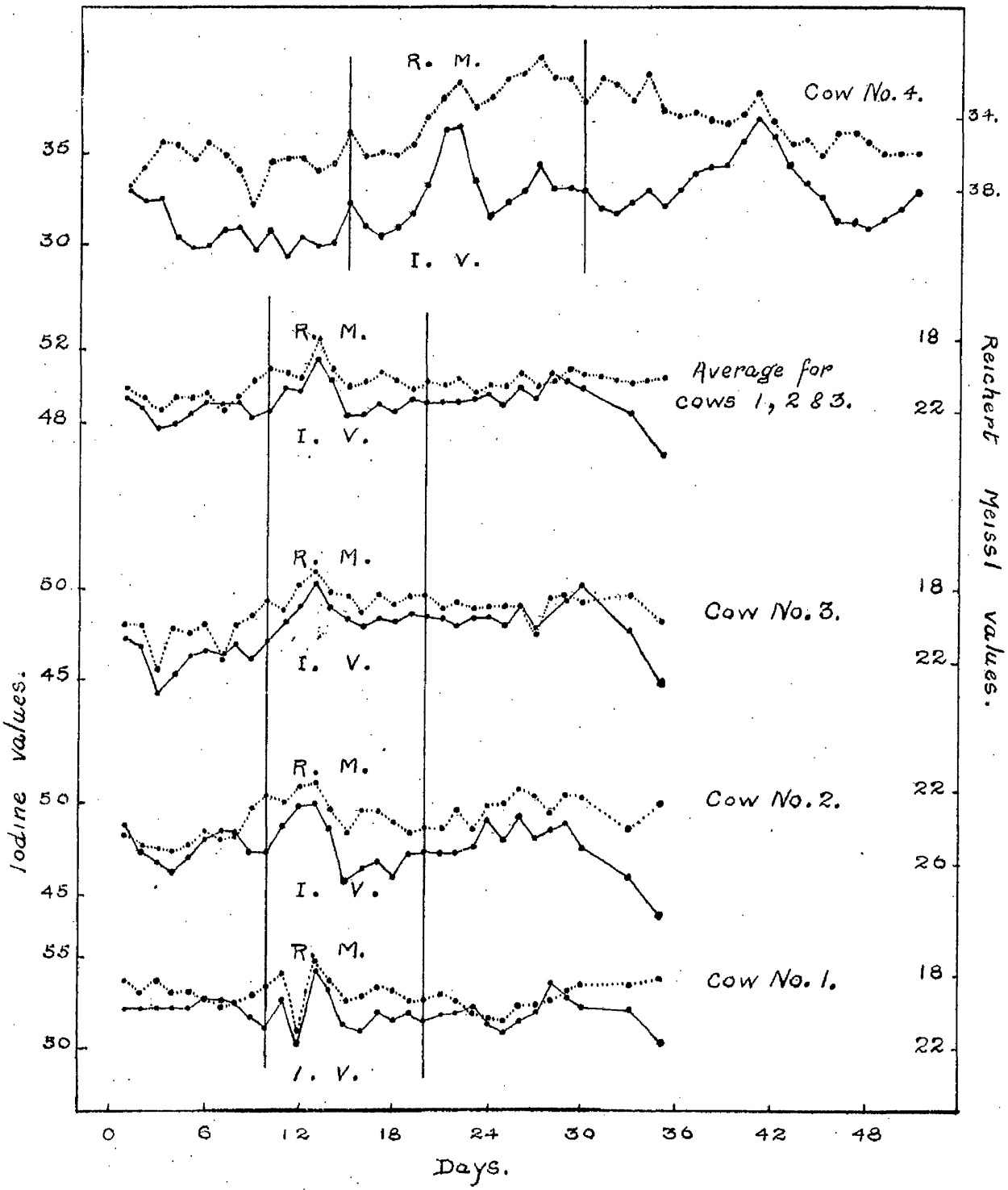

Fig. 8. The effect of thyroxine on the Reichert Meissl and iodine values of the milk fat.

circumstances changes extremely slowly and that variations in it tend to be but slight. In fact with non-lactating cows Aylward and Blackwood (57) found no definite or consistent alteration in the total fatty acid content of the blood even after a fast of 10½ days, while later results of Aylward et al. (58) showed that, although for two days after a drench of "labelled" fat the blood of the cow became gradually enriched with the "labelled" constituents of the drench, the actual lipoid level itself was not measurably affected. It was therefore obvious that if the small changes in composition or in amount which may be expected in the blood fat of ruminants were to be detected, the methods adopted for analysis would have to be as searching and as accurate as possible. For this reason it was felt that the micro procedures now commonly in use, such as that of Bloor (59, 60), were not applicable to the present experiments as they would not involve sufficient material for the thorough examination which was in view. Nor was another type of method, such as that published by Stoddart and Drury (61), of much value in the present work. This particular method depends on the assumption that the mean molecular weight of the fatty acids is always a given value and from this value the amount of the fatty acid is calculated. But in the thyroxine experiments the constancy or variability of these particular molecular weights before and during hormone

treatment was one of the properties which it was desired to investigate. It was therefore decided that the macro procedure, suitably adapted, would be of much greater value than more numerous but less reliable analyses carried out on very small amounts. The modified macro method described by Smith (79) was therefore adopted. For the analyses of the blood lipoids, blood samples were taken from one of the two abdominal subcutaneous mammary veins of cows 1, 2 and 4 at suitable times before, during and after the thyroxine treatment. About 200 ml. of blood were drawn into a bottle containing approximately 0.2 g. of potassium oxalate to prevent coagulation. The sample was then centrifuged for 45 minutes at 2000 r.p.m. and the plasma separated from the corpuscles. Sugar was determined by a macro form of Hagedorn-Jensen's method described by Fujita and Iwatake (65) using 5 ml. of plasma. The results are given in Table IX.

The remainder of the plasma and corpuscles were extracted with alcohol-ether and aliquots taken for phosphorus estimation by the method of Fiske and Subbarow (63) as modified by King (74). Known volumes of the extracts were then evaporated to dryness on a water-bath under reduced pressure in a gentle stream of nitrogen and the products so obtained extracted exhaustively with light petroleum and filtered from the relatively large amounts of blood

TABLE IX.

Blood plasma sugar from cows 1, 2 and 4.

Days of thyroxine treatment for cows 1 and 2.	mg./100 ml. of plasma			Days of thyroxine treatment for cow No.4.
	Cow No.1	Cow No.2	Cow No.4	
Before:				Before:
9	75	65	59	11
7	68	63	52	8
5	70	66	56	6
3	66	68	59	4
Average (A)	70	65	57	
During:				During:
4	75	75	64	4
6	75	76	75	8
8	76	73	76	11
10	81	75	72	14
Average (B)	77	75	72	
After:				After:
7	67	67	71	8
9	67	63	69	14
			64	17
Average	67	65	68	
% increase (B-A)x100/A	10.0	15.4	26.3	

inorganic salts which accumulate at this stage. The petroleum was then allowed to boil off, the residue dissolved in about 10 ml. of absolute ether, transferred to a 50 ml. centrifuge tube and the phosphatides precipitated with 30 ml. of dry acetone. After standing at room temperature for 10 minutes, the mixture was centrifuged, the clear supernatant liquid poured

off and the residue washed with acetone. It was then dissolved in ether and reprecipitated. The phosphatide residue was dispersed in benzene with gentle heating and centrifuged. The clear benzene layer was decanted off, the residue washed with solvent and after redispersion in benzene, it was again centrifuged. The treatment was carried out three times in all to make sure that the benzene-soluble material was completely removed. After this treatment a small water-soluble residue remained undissolved and was discarded since it was found to contain a negligible amount of phosphorus.

(a) Analysis of the Non-Phosphatide Fraction: Known aliquots were taken for the determination of total lipoids and free cholesterol. After this the solvent was distilled off from the remainder, and the residue saponified by heating it for 30 minutes with 30 ml. alcohol and 2ml. of 40% KOH, to which 20 ml. benzene was added to ensure that solution was complete. The fatty acids and unsaponifiable matter were then isolated in the usual way. Aliquots of the unsaponifiable matter were taken for total weight, and for total cholesterol, while aliquots of the fatty acids were removed for the determination of weights, iodine values and molecular weights. Cholesterol determinations were made throughout by weighing the digitonide.

One of the chief difficulties in analysing blood lipoids at the present time is that no satisfactory method has yet been found for separating the cholesteryl

esters from the tri-glycerides, the two main components of the non-phosphatide fraction. It is only possible to estimate the amount of fatty acids which were probably present originally as triglycerides by calculation. To do this it is assumed that the mean molecular weight actually found for the total non-phosphatide fatty acids is also the mean molecular weight of the acids esterified with cholesterol, which, as the latter greatly predominate, will give a very close approximation to the truth. The weight of fatty acids combined with cholesterol is thus readily obtained and the difference between this value and that found for the total fatty acids is taken as the amount present as tri-glycerides. From the amount of glyceride fatty acids so estimated it is possible to calculate the amount of glycerol which must have been required for their esterification. Then, by adding together the values for total fatty acids, total unsaponifiable matter and for glycerol, it is possible to estimate the percentage of the original non-phosphatide fraction which is accounted for by the various constituents. It will be observed from Tables X, XI and XII that this figure was usually well over 90%, a fact to which reference is made later.

(b) Analysis of Phosphatides: Aliquots were taken for estimating total lipoids and phosphorus in the acetone-insoluble fraction. The remainder was then saponified, the fatty acids and unsaponifiable matter isolated and examined as already described for the

corresponding components of the non-phosphatide fraction.

The variations in concentration of the different blood lipoids from the three animals are shown in Tables X - XIV. For plasma phosphatides and for corpuscle lipoids, only pooled samples obtained before, during and after the thyroxine treatment were analysed.

TABLE X : The analytical data for the plasma non-phosphatides from cow No.1. (mg./100ml. plasma).

Days of thyroxine treatment	Total non-phosphatides	Cholesterol		Unsaponi-fiable matter	Total fatty acids				Glyceride fatty acids	% recovery@
		Free	Ester		Weight	I.	V.	M.	W.	
Before: 9	451	48	175	270	158	140	294		26	96
7	415	45	168	234	156	139	292		29	95
5	415	42	167	247	151	138	294		24	97
3	404	40	166	238	151	139	292		26	97
Average (A)	421	45	168	247	154	139	293		26	96
During: 4	378	36	151	212	138	140	294		28	93
6	352	36	146	199	131	149	288		22	94
8	373	38	145	225	126	149	291		17	95
10	351	35	142	196	131	150	290		25	94
Average (B)	363	36	146	208	131	147	291		22	94
After: 7	385	34	156	198	154	140	290		37	92
9	389	34	162	215	150	144	288		25	95
Average	387	34	162	206	152	142	289		31	94
% decrease (A-B)100/A	13.8	20.0	13.1	15.8	14.9					

@ Sum of fatty acid, unsaponifiable matter and glycerol as per cent. of original non-phosphatide fraction.

TABLE XI : The analytical data for the plasma non-phosphatides from Cow No.2. (mg./100ml. plasma).

Days of thyroxine treatment	Total non-phosphatides	Cholesterol		Unsaponi-fiable matter	Total fatty acids			Glyceride fatty acids	% recovery@
		Free	Ester		Weight	I. V.	M. W.		
Before: 9	352	33	141	186	140	143	298	32	94
7	325	30	132	176	125	139	308	22	93
5	324	29	130	171	122	144	289	24	91
3	309	29	128	164	114	150	288	19	91
Average (A)	327	30	133	174	125	144	294	24	92
During: 4	296	27	120	154	113	149	292	22	91
6	287	30	118	157	108	147	293	18	93
8	285	29	116	156	108	147	292	21	94
10	292	29	118	157	113	146	292	24	93
Average (B)	290	29	118	156	110	147	292	21	92
After: 7	296	28	119	154	115	143	294	24	92
9	283	28	118	154	110	147	293	20	94
Average	289	28	118	154	112	145	293	22	93
%decrease (A-B)x100/A	11.3	3.3	11.3	10.4	12.0				

@Sum of fatty acid, unsaponifiable matter and glycerol as per cent. of original non-phosphatide-fraction.

TABLE XII. The analytical data for the plasma non-phosphatides from cow No.4. (mg./100ml. plasma).

Days of thyroxine treatment	Total non-phosphatides	Cholestrol		Unsaponi-fiable matter	Total fatty acids			Glyceride fatty acids	% recovery©
		Free	Ester		Weight	I. V.	M.W.		
Before: 11	285	25	100	149	117			41	95
8	273	24	100	141	112			36	94
6	275	24	99	135	114	125	295	38	92
4	266	24	103	134	109			30	93
Average (A)	275	24	100	140	113			36	94
During: 4	255	21	103	136	109			31	98
8	253	26	79	118	91			31	91
11	242	25	80	117	99	127	295	38	91
14	235	24	81	115	102			40	94
Average (B)	241	24	86	121	100			35	93
After: 8	256	25	91	130	105			35	93
14	250	25	86	125	105	124	299	38	94
17	247	25	86	122	105			38	93
Average	251	24	88	126	105			37	93
% decrease (A-B)x100/A	12.4	0	16.3	13.6	11.5				

©Sum of fatty acid, unsaponifiable matter and glycerol as per cent. of original non-phosphatide fraction.

TABLE XIII : The analytical data for the plasma phosphatides from cows 1, 2 and 4. (mg./100ml. plasma).

Days of thyroxine treatment	Total lipoids	Phosphorus in alcohol-ether extracts	Phosphorus in acetone insoluble material	Total fatty acids		
				Weight	I. V.	M. W.
		Cow No. 1.				
Before (A)	286	8.9	7.2	136	83	324
During (B)	230	7.7	6.0	109	82	321
After	254	8.5	6.7	123	82	321
% decrease (A-B)x100/A	19.6	13.5	16.7	19.9		
		Cow No. 2.				
Before (A)	248	7.4	5.9	105	81	315
During (B)	225	6.5	5.2	89	79	321
After	246	6.7	5.4	92	83	335
% decrease (A-B)x100/A	10.1	12.2	11.9	15.2		
		Cow No. 4.				
Before (A)	212	5.5	-	-	79	309
During (B)	187	4.7	-	-	77	310
After	190	4.7	-	-	79	316
% decrease (A-B)x100/A	11.8	14.5				

TABLE XIV : The analytical data for the corpuscle lipoids from cows 1, 2 and 4. (mg./100ml. plasma).

Sample No.	Cow No. 1. Total cholesterol	Cow No. 1. Phosphorus in alcohol-ether extracts	Cow No. 2. Total cholesterol	Cow No. 2. Phosphorus in alcohol-ether extracts	Cow No. 4. Total cholesterol	Cow No. 4. Phosphorus in alcohol-ether extracts.
Before: 1		16.8		15.1		14.2
2		15.8		16.5		14.5
3	169	15.6	165	17.1	154	14.9
4		15.9		16.1		14.5
Average		16.0		16.4		14.5
During: 1		16.0		16.1		14.4
2		15.6		16.5		14.8
3	159	16.1	169	16.4	139	15.0
4		16.3		16.5		15.1
Average		16.0		16.4		14.8
After : 1		16.1		16.6		15.2
2	171	16.2	164	16.7	151	14.9
3						15.1
Average		16.1		16.6		15.1

DISCUSSION.

The Milk Yields: In order to estimate the effect of thyroxine on the milk yields, it is necessary to compute what the yield would have been approximately had no hormone been given. Such values have been arrived at from Figs. 3 and 6 by drawing a straight line through the points representing the yields before and after thyroxine treatment. It will be observed that the slope of the lines so drawn is somewhat similar to the lactation curve for the control group except that in the latter the decline appears to be very slightly steeper. This is probably due to the fact that after the hormone treatment had ceased the yield did not return to normal for a considerable time, so that with the experimental cows the gradient is not quite so great. It will be seen from Figs. 3 and 6 that towards the end of the thyroxine period the yields for cows 1, 2, 3 and 4 were 8.9, 15.3, 14.3 and 13.6 litres, whereas the approximate values which would normally have been expected were 6.1, 10.2, 10.2 and 10.6 respectively. Therefore the corresponding increases in yield due to thyroxine were 46, 50, 40 and 28%. The average yield for cows 1, 2 and 3 on the 10th day of thyroxine administration was enhanced from a probable value of 8.6, to 13.0, i.e. 51%.

The Amount of Milk Fat Secreted: From a general inspection of the curves for the fat percentages shown in Figs. 4, 5 and 6, it will be seen that, although the milk yield was markedly increased during thyroxine

treatment, the level of the fat in the milk did not decrease but was well maintained at or even slightly above the average pre-thyroxine value. After hormone administration had ceased a rise in fat percentage was observed in some instances, but this was probably contributed to by the fall in milk yield which took place during that period.

With regard to the total daily yield of fat, for which the values are also shown in Figs. 4, 5 and 6, an average increase of some 48% was observed for cows 1, 2 and 3 over the amount which would have been expected. In fact with cow No.3 there was even an increase of 39% over the highest pre-thyroxine value. With cow No.4 the yield of fat on the last day of the hormone treatment was 510 g. as against the computed value of 365 g., an increase of 40%. These findings with regard to the yield of milk and fat are in general accordance with those of the previous workers already cited.

Yield of Solids-not-Fat: Folley and White (64) obtained a 4% increase in the level of solids-not-fat as a result of thyroxine administration, while in the experiments of Jack and Bechdel (73) and Herman et al. (71, 77) the effect of thyroxine in this direction was not so marked, if indeed it existed at all. It was therefore thought worth while in the present experiments to estimate the total and some of the individual non-fatty solids in the milk. The results, shown in Figs. 5 and 7, suggest very clearly that in the present

instance no consistent measurable change was observed in the concentration of any of these constituents. In this respect therefore the present results do not confirm those of Folley and White. At the same time it may be observed that the total daily yield of non-fatty solids, as distinct from their concentration, increased during the thyroxine treatment as a result of the increase in milk yield..

The Nature of the Milk Fat: As indicated in Part I, changes in milk fat can readily be detected by studying the iodine and Reichert Meissl values of the fat. For the present experiments these are recorded in Fig. 8, from which two conclusions may be drawn. In the first place it is perfectly clear that no very marked change in the nature of the fat took place as a result of thyroxine treatment even though the total daily yield was so much enhanced. Thus for cows 1 and 2 on the last day of hormone administration, the iodine and Reichert Meissl values were almost identical with those recorded in the pre-thyroxine period. With cows 3 and 4 on the other hand a slight increase in iodine value with a corresponding decrease in R.M. value was observed but these changes were so small as to be of little, if any, significance. In the second place, it will be seen that with all four cows a temporary change in iodine value was accompanied by a temporary but opposite change in the Reichert Meissl value during the first few days of the treatment,

suggesting that there was a slight alteration in the nature of the fat, which tended to become a little more unsaturated and to contain very slightly less of the lower fatty acids during the period when the gland was becoming accustomed to the increase in milk production. It is important to observe that these facts were true not only for cows 1, 2 and 3 which yielded butterfat possessing somewhat unusual characteristics (p. 63) and which were treated in the autumn, but also for cow No.4 for which the butterfat was quite normal and which differed from the others in that it was not so far advanced in its lactation and was treated in the summer.

Changes in the Blood:

(a) Sugar: Although the plasma-sugar level even in the ruminant is subject to some variation as a result of environmental and other factors, the results given in Table IX are sufficiently consistent to provide a clear indication that the general level tended to rise during the period of thyroxine treatment and to approach the normal value once again when the injections were stopped. The average increases for cows 1, 2 and 4 over pre-thyroxine levels were 10, 15 and 26% respectively. In this connexion it is interesting to point out that Graham et al. (68) suggested from their work that there might be a close relationship between the arterial blood sugar level on the one hand and the amount of sugar taken up by

the gland on the other, a value which itself would probably be directly related to the milk yield. In the present experiments, in which the plasma of the venous blood was used, the increase in the yield of milk during hormone treatment for each individual cow was accompanied by an increase in its blood sugar level. But it is worth observing in passing that this relationship did not hold from animal to animal. Thus when the normal average blood sugar values for cows 1, 2 and 4 are compared with their pre-thyroxine milk yields, it is seen that there is no correlation between the concentration of plasma sugar and the yield. Obviously the rate of blood flow, the amount of active mammary tissue in the gland and the stage of lactation will all be factors of importance in a matter such as this.

(b) Corpuscle Lipoids: Smith (79) found a decrease of considerably less than 10% in the corpuscle lipoids of lactating cows during a 12 days inanition period as compared with a reduction of 40 - 50% for those of the plasma, and concluded that it is highly improbable that alterations can readily take place in corpuscle lipoids even under such drastic conditions as those of a twelve day fast. The figures for the lipoid phosphorus are given in Table XIV and it will be seen that the hormone did not appear to bring about any noticeable change. Thus for cow No.1, the average values before, during and after the thyroxine treatment

were 16.0, 16.0 and 16.1 mg. per cent. respectively. Similarly for total cholesterol the small variations existing between the values recorded in the same table were neither sufficiently great nor consistent to be of any significance, particularly when the difficulties involved in making a complete extraction of the corpuscle lipoids is taken into consideration. It may therefore be concluded that during hyperthyroidism as in inanition the corpuscle lipoid level of the lactating cow does not alter to any measurable extent. For this reason it is important in studies which involve the determination of blood lipoids that plasma or serum should always be used rather than whole blood, for the corpuscles contain a large proportion of the total lipoids and the volume of the red cells in the blood may vary within fairly wide limits. Changes in the plasma lipoids may thus be masked.

(c) Plasma Lipoids: Unlike the corpuscles, the plasma lipoids showed a definite reduction in the level of the various constituents as a result of thyroxine treatment. The lipoid phosphorus values for cows 1, 2 and 4 are shown in Table XIII and are typical of the general effects of thyroxine on the other lipoid fractions of the plasma. For the three animals the average content of alcohol-ether soluble phosphorus dropped from 8.9, 7.4 and 5.5 mg. per cent. to 7.7, 6.5 and 4.7 respectively, decreases of 14, 12 and 15%. For the acetone precipitated lipoid phosphorus decreases of a similar magnitude were also observed.

From Tables X - XIII it will be seen that the average fall in total non-phosphatides during hormone administration for each of the three cows was 14, 11 and 12%, while for total phosphatides it was 20, 10 and 12% respectively. The fact that the lipoid level remained low even several days after the thyroxine injections had ceased is of considerable interest, though no definite explanation can at present be given.

The decrease in most of the other constituents was of about the same order as that given for the total lipoids. For example, the ester cholesterol for cows 1, 2 and 4 decreased by 13, 11 and 16% and the non-phosphatide fatty acids by 15, 12 and 13% respecitvely. Similarly the phosphatide fatty acids from the plasma of cows 1 and 2 diminished by 20 and 15%. The changes in the free cholesterol and glyceride fatty acids were very irregular. Thus, free cholesterol, which is in any event very small and therefore difficult to estimate accurately, appeared to decrease by 20, 5 and 0%, and the glyceride fatty acids by 15, 16 and 5%. The variations in the values of the glyceride fatty acids might have been a normal characteristic of the plasma, but on the other hand, they may have been due to the fact that these values were obtained, as already described, by calculation from the figures found for free and total cholesterol and for total non-phosphatide fatty acids. Any unavoidable errors in the estimation of these

latter fractions, particularly of the free cholesterol, may therefore have combined to give a somewhat greater error in the relatively small values calculated for the tri-glyceride acids.

In Tables X - XII it will be observed that for each sample, the total non-phosphatide fatty acids and unsaponifiable matter determined by direct weighing have been added to the small amount of glycerol calculated to be present (usually about 3 mg./100 ml. plasma), and the sum has been expressed as a percentage of the original non-phosphatide fraction, also determined by weight. Most of the figures so obtained were found to be well above 93%. What proportion of the remainder is water-soluble fatty acid it is difficult to say. Stewart and Hendry (80) found that the water-soluble fatty acids of whole human blood did not exceed 3% of the total fatty acids.

It is also of interest to notice from the figures recorded in the tables that the phosphatide fatty acids tended to be affected to a slightly greater extent than the other lipoid constituents. This has been pointed out by Smith (79) as also taking place both during inanition and in one instance of milk fever. He suggests that this effect might possibly be due to a greater degree of metabolic activity on the part of these particular acids.

The possibility of a relationship existing between blood lipoid level and the amount of milk fat produced

is worthy of particular attention in view of the fact that suggestions have occasionally been made in the literature that the milk fat producing capacity of a cow may be judged to some extent from the concentration of fatty substances in its blood, and that the future capacity of a calf in this direction may even be foretold from its blood lipoid level. However true such suggestions may be (and at present, confirmation is lacking), it is clear from the results just recorded that there are certain endocrinological factors which are of much greater importance than the lipoid level per se. Thus in the present work an enhanced capacity to secrete milk fat was not only associated with hyperthyroidism but also with a general reduction in blood lipoid level. It follows, therefore, that little reliance can be placed on the total plasma lipoid concentration in itself as a guide to the cow's capacity to produce milk fat.

It is now well established that the main, if not the only precursors of milk fat in the blood plasma are the tri-glyceride fatty acids. These form a very small proportion of the total lipoids of the blood and might readily rise or fall independently of the other fatty components. It is realised here that these particular fatty acids are so difficult to estimate that no definite conclusion can be drawn with certainty as to whether they actually fell or remained constant during the hormone treatment, but whatever change

occurred must have been very slight. From Tables X - XII, it certainly appears as though there had been a slight decrease in this particular fraction with each cow, but a study of the individual figures will show that it would be unwise to draw such a conclusion without due reserve. Thus with cow No.1, for example, there appears to be a 15% decrease, but actually the tri-glyceride fatty acids only fell from a pre-thyroxine average of 26 mg./100 ml. to one of 22 during the experimental period, and it is impossible to estimate this fraction accurately within a few milligrams. It is also of interest to observe that for cow No.4, which was giving the highest daily yield of fat, the tri-glycerides amounted to 36 mg./100 ml. of plasma in the pre-thyroxine period as compared with 26 and 24 for the other two animals, in spite of the fact that in cow No.4 the non-phosphatide fraction of the plasma, taken as a whole, is actually less than in that of the other two cows. In other words, for the higher yielding cow, the tri-glyceride fatty acids of the plasma were at a higher level than in the other two cows, although the total plasma lipoids were actually lower. A much greater collection of similar data will certainly be required to establish whether this suggestive observation holds for lactating cows in general.

With regard to the effect of thyroxine on the actual nature as distinct from the amounts of the various lipoid fractions in the blood, it may be con-

cluded from the iodine values and molecular weights recorded in Tables X - XIII that there was little change in the non-phosphatide fatty acids. The slight increase in the iodine value recorded in the tables might suggest that the fatty acids during hormone treatment tended to be a little less saturated, but the difference must have been very small indeed. For the phosphatide fatty acids, the iodine values and molecular weights show too much variations amongst themselves for definite conclusions to be drawn, but considered as a whole, they do not suggest any noticeable alteration as a result of thyroxine treatment in the general type of fatty acids present. At the same time it must be observed that here, as in the earlier work (Smith, 79), considerable difficulty was experienced in estimating the molecular weights of the phosphatide fatty acids, so that variations shown for them are thought to be due mainly to the experimental difficulties involved in their estimation rather than to changes in the acids themselves. As is generally found for blood lipoids, the phosphatide fatty acids have higher molecular weights and much lower iodine values than those from the non-phosphatide fractions.

The fact that there was no appreciable change in the composition of the milk fat during hormone treatment suggests that the same precursors were readily available to the gland in much the same proportions throughout all the three periods of the experiment, except perhaps during the first few days of the hormone

treatment when the marked increase in the milk yield was actually taking place. This fact is not necessarily inconsistent with a decrease in the lipoid constituents of the plasma, for it is known that thyroxine causes an increase in the general rate of blood flow. Consequently, in any given time, the gland might still be supplied with the same amount of the same precursors, although their concentration in the blood had actually decreased.

There are so many factors involved in the physiological processes which lead to the secretion of milk fat, that it is impossible at present to decide whether routine administration of thyroxine or breeding for animals of high thyroid activity would ever be likely in the course of time to have desirable results, but it may be concluded from the present work, taken in conjunction with the previous experiments of others, that no general alteration in the nature of the butterfat would be expected to take place, and that the decrease in plasma lipoids, which so frequently appears to accompany hyperthyroidism, would not have any deleterious effect on the yield of milk or on the concentration of its constituents.

SUMMARY.

1. Thyroxine has been administered to three cows in the 6 - 9th month of their lactation under autumn conditions and to a fourth cow in the 4th month of lactation during the summer. The blood lipoids and milk fat of these animals have been studied before, during and after the hormone treatment.

2. In confirmation of the results of other workers, it was found that the yields of milk and of milk fat were very markedly increased. There was, however, no consistent change in the level of the non-fatty solids in the milk.

3. There was no significant alteration in the actual nature of the milk fat during the period when its yield was enhanced except for a slight temporary change at the beginning of the hyperthyroid period, when the gland was becoming accustomed to the increased production;

4. The concentration of sugar in the plasma was increased by some 10 - 26% during the period of the hormone administration and this was accompanied by a general decrease of some 10 - 20% in the lipoid level.

5. The relationship between the lipoid level of the plasma on the one hand and the yield of milk fat on the other is discussed.

6. No measurable change took place in the lipoid level of the corpuscles.

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