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With 4 *plates*

An electron microscope study of the ultrastructure of bovine and human casein micelles in fresh and acidified milk

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(*Received* 23 *January* 1967)

SUMMARY. When examined under the electron microscope, bovine casein micelles were seen as aggregates of spheroidal granules arranged in spherical symmetry. The granules were of 2 kinds—one transparent in the electron beam and the other relatively opaque. With increasing acidity of the milk the regular arrangements of granules tended to break down, and bridges composed of granules, formed between neighbouring micelles. The average diameter of the granules was about 8 mμ, from which a molecular weight of about 225000 was calculated. Evidence was adduced for the identity of these granules with the macromolecules of native casein in equilibrium with whey.

Human casein micelles showed the same structural features. The average diameter of the granules was about 6 mμ, from which a molecular weight of about 100000 was calculated.

This paper describes a study of the fine structure of the casein micelles of human and bovine milk, as revealed under the electron microscope by the negative staining procedure of Brenner & Horne (1959). A preliminary account of the work has been reported elsewhere (Calapaj, 1966).

In earlier studies, shadow-casting with gold (Baud, Morand & Pernoux, 1951) or chromium (Nitschmann, 1949; Hostettler & Imhof, 1951, 1952; D'Agostino Barbaro & Calapaj, 1958) or with palladium or platinum (G. G. Calapaj, unpublished) has for technical reasons proved unsatisfactory for revealing the inner structure of the casein micelle, as also have the procedures of staining with osmium vapour (Huth, 1957) and of sectioning methacrylate-embedded samples to a thickness of 30–50 mμ (Knoop & Wortmann, 1960). More recently Shimmin & Hill (1964) examined sections, measuring only 10 mμ in thickness, of bovine casein micelles embedded in Araldite, and observed intensely osmiophilic granules of diameter c. 10 mμ, which they considered to be macromolecules.

METHODS

Samples of bovine or human milk were freed from fat by centrifugation, and then fixed by addition of 1 *V* 38–40 % formaldehyde solution to 3 *V* milk. After standing for 15 min the mixture was diluted with distilled water until a slight opalescence appeared. An equal volume was then added of 2 % (w/v) phosphotungstic acid solution (PTA) of pH 7·2. A drop of this mixture was transferred to a 'formvar'

covered grid, and allowed to stand for 5 min. Excess liquid was then drained off with a filter paper and the residual film allowed to dry at room temperature. Electron micrographs were made with a Siemens model 1 A electron microscope equipped with a double condenser, at an objective aperture of 20 μ. The magnification was $\times$ 20000–40000.

Observations were made on fresh milk samples, and also on samples containing acid equivalent to about 5 g lactic acid/l. The acidity was contributed either through the action of the natural bacterial flora of the milk during incubation at room temperature, or by the addition, with stirring, of a 1 % aqueous solution of lactic acid.

At this concentration of 5 g lactic acid/l, the milk casein formed a flocculent precipitate which was removed by centrifugation. Specimens for electron microscopy were prepared from supernatant fluid which still contained micro-particles of casein in suspension.

For purposes of comparison, preparations shadowed with palladium and platinum were made from both fresh and acidified milk samples.

RESULTS

In negatively stained preparations from fresh cow's milk, the casein micelles are composed of spheroids, transparent in the electron beam, and closely packed in a dark background of PTA in which, on careful examination, other more opaque

Table 1. *Distribution of the diameters of 722 transparent granules of bovine casein in class intervals of 1 mμ*

Class intervals, mμ	Frequency
$\leqslant$ 5·4	1
5·5–6·4	56
6·5–7·4	119
7·5–8·4	312
8·5–9·4	180
9·5–10·4	47
$\geqslant$ 10·5	7
	722

granules are detectable (Plate 1, fig. 1). Both the transparent and the opaque granules appear to be arranged in spherical symmetry, especially in the larger micelles, which probably offer greater resistance to deformation by the mechanical stresses involved in the preparation of the samples for examination. This pattern remains broadly unchanged until the acidity in the milk approaches 3 g/l, when thin 'bridges' of material, transparent in the electron beam, begin to link neighbouring micelles (Plate 1 fig. 2). After flocculation of the casein, and its removal by centrifugation, only large and shapeless aggregates are left behind in the supernatant (Plate 2).

The bridges mentioned above have been observed by Baud *et al.* (1951) in cow's milk coagulated by the action of rennet, and by Hostettler & Imhof (1951, 1952) in cow's milk coagulated either with rennet or by the action of milk-souring bacteria.

In casein micelles, and in the aggregates found in the acid supernatant, the transparent and the opaque granules were still detectable in the bridges, and may

be regarded as basic constituents of both these morphological configurations of the casein. In order to measure the average diameter of the granules, electron microscope plates were enlarged photographically, to a total magnification of × 500000. Measurements on 722 transparent granules gave the frequency distribution shown in Table 1. The distribution is, apparently, Gaussian, but it is not possible to conclude that the variation is indicative of the real existence of granules of different diameter, and is not rather a reflexion of the cumulative errors of measurement. There is the further possibility that the apparent diameter of the granules might vary according to the extent to which they were covered by the PTA.

Table 2. *Distribution of the diameter of* 504 *dark granules of bovine casein in class intervals of* 1 mμ

Class intervals, mμ	Frequency
$\leqslant$5·4	3
5·5–6·4	50
6·5–7·4	106
7·5–8·4	219
8·5–9·4	87
9·5–10·4	31
$\geqslant$10·5	8
	504

The average diameter of the transparent granules was 8·1 ± 1·1 mμ.

On the same photographic enlargements, measurement of 504 opaque granules gave the frequency distribution shown in Table 2, from which an average diameter of 7·9 ± 1·06 mμ may be calculated. This is not significantly different from the value of 8·1 found for the transparent granules, and it is reasonable to suppose that there is no essential difference between the granules, and that they may appear opaque or transparent according to the thickness of the PTA deposit in which they are embedded. If this is so, then we may assign to all the granules an average diameter of 8·0 ± 1·1 mμ.

This value of 8·0 mμ relates to the micelles as they exist under the conditions of high vacuum in the electron microscope. If the casein is to be considered in its hydrated condition, the average value of the diameter of the granules would be greater. Hankinson & Briggs (1941) found that 1 g dry casein having a specific volume of 0·8 ml hydrated to a volume of 3·2 ml when dispersed in a solution of calcium hydroxide at a final pH of 6·6. Thus:

$$\frac{D_{\text{hyd}}}{D_{\text{anhyd}}} = \sqrt[3]{\frac{V_{\text{hyd}}}{V_{\text{anhyd}}}} = \sqrt[3]{\frac{3{\cdot}2}{0{\cdot}8}} \simeq 1{\cdot}58,$$

where V_{hyd}, D_{hyd}, V_{anhyd} and D_{anhyd} are the volumes and diameters, respectively, of the hydrated and anhydrous, supposedly spherical, casein particles.

Applying this value of 1·58, we obtain for granules of hydrated casein an average diameter of $8{\cdot}0 \times 1{\cdot}58 = 12{\cdot}64$ mμ.

Negatively stained casein micelles from fresh human milk appear smaller than those from cow's milk, thus confirming earlier findings with shadowed (Calapaj, 1962) or osmium-stained (Huth, 1958) preparations, or with ultra-thin sections (Knoop & Wortmann, 1960). They have the same appearance of being composed of closely packed transparent and dark granules, arranged in spherical symmetry. With in-

creasing acidity, the human casein micelles examined in shadowed preparations seemed to increase in diameter and to become linked by bridges of varying thickness. With lactic acid in excess of 4 g/l only shapeless aggregates were observed.

Similarly, in negatively stained preparations, the same characteristic features were observed. Here, in addition, the granules were clearly detectable within the isolated micelles, and in the bridges and the aggregates.

Measurement of the photographically enlarged images of 354 granules gave the values listed in Table 3, from which the average value of the diameter of the elementary granule in human casein was calculated to be $6{\cdot}1 \pm 1{\cdot}2$ mμ. If we assume that the factor 1·58 (see above) applies equally to human milk casein, we can calculate for hydrated granules an approximate diameter of 9·6 mμ.

Table 3. *Distribution of the diameters of* 354 *granules of human casein in class intervals of* 1 *mμ*

Class intervals, mμ	Frequency
$\leqslant$ 4·4	5
4·5–5·4	55
5·5–6·4	197
6·5–7·4	91
$\geqslant$ 7·5	6
	354

DISCUSSION

The molecular weight of the granules can be approximately estimated from the specific gravity of the casein. Literature values for the specific gravity of the casein of cow's milk range from 1·335 (Svedberg, Carpenter & Carpenter, 1930) to 1·400 (Nitschmann, 1949) and 1·504 (Nichols, Bailey, Hahn, Greenbank & Deysher, 1931), giving an average value of 1·41. The mean weight of the bovine casein granules can be put at approximately 378×10^{-21} g. No value could be found for the specific gravity of the casein of human milk, but assuming the same value of 1·41, then the mean weight of the casein granules from human milk would have been 159×10^{-21} g. From these data we may calculate for the casein of cow's milk a molecular weight of 225000 and for that of human milk of approximately 100000.

In the light of present knowledge, some of the older literature values should now be disregarded. Thus, Svedberg *et al.* (1930) assigned to the acid-alcohol soluble fraction of casein a molecular weight of 375000, and reported that the granule is ellipsoidal in shape. Ford & Ramsdell (1949) found, surprisingly, a diameter of 64 mμ and a molecular weight of 33×10^6 for casein in the dry state, and of 94×10^6 for hydrated casein. However, Nichols *et al.* (1931) reported spheroidal granules of diameter 8 mμ.

The most recent reports in the literature indicate that the casein macromolecule is a spheroid of diameter about 9 mμ, and of molecular weight between 250000 and 300000.

Waugh & von Hippel (1956) calculated that the κ fraction of casein, upon which rennin acts, is associated with 4 times its weight of the α-fraction. They ascribe to the κ fraction a molecular weight of about 15000, and thus imply a molecular weight of about 80000 for the α–κ complex. More recently, Beeby (1963) suggested that the

above molecular weight of 15000 relates not to the protein in its natural state but to a monomer formed during the processes of isolation. In the natural state, κ-casein would be comprised of 3 molecules of this monomer, and its molecular weight would be 45000–50000. Accordingly, the α-κ complex should have a molecular weight of 225000–250000. Similarly, assuming a molecular weight between 15000 and 25000 for β-casein, then the molecular weight of the α–κ–β complex would lie between 250000 and 300000. According to Birbeck (1961) the macromolecule should be spherical and have a diameter of about 9 mμ. This evidence strongly suggests that the granules observed in the micelles and bridges of the negatively stained preparations are macromolecules of the casein complex as it occurs in its native state. Shimmin & Hill (1964) came to the same conclusion concerning the osmiophilic granules which they observed in their ultra sections.

There is, as yet, insufficient evidence to support the same conclusion concerning the nature of the elementary granules in human milk casein.

It may be supposed that, in human milk and in cow's milk, the casein micelles are made up of closely packed macromolecules. With increasing acidity, the spherical micelles become elongated into rods, whose presence causes the streaming birefringence of acid casein.

The different diameters of the macromolecules of human and bovine casein are of interest in relation to the observation (Knoop & Wortmann, 1960; Calapaj, 1962) that the frequency versus diameter curves for the micelles of the 2 caseins are similar. To explain this similarity it may be supposed that the law governing the aggregation of the macromolecules into the micelles is the same for these 2 mammalian species. Thus, micelles of the same frequency class, and especially those in the highest frequency class, would contain the same number of macromolecules. Calapaj (1962) found that the most frequent micelle diameter was 65 mμ in human milk, and 95 mμ in cow's milk. Taking 6 and 8 mμ as being the diameters of the respective macromolecules, the human casein micelle would contain about $(\frac{65}{6})^3 \simeq 1200$ as against $(\frac{95}{8})^3 \simeq 1600$; clearly the 2 figures are at least of the same order of magnitude. However, it should be borne in mind that my value for the most frequent diameter of the human casein micelle, namely 65 mμ, does not agree with that of 42 mμ found by Knoop & Wortmann (1960).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Bovine milk casein; natural acidity; negative staining; × 100000.

Fig. 2. Bovine milk casein; acidity 2·5 g/l; negative staining; × 100000.

PLATE 2

Bovine milk casein; acidity 4·8 g/l; negative staining; × 100000.

PLATE 3

Fig. 1. Human milk casein; natural acidity; negative staining; × 100000.

Fig. 2. Human milk casein; acidity 3 g/l; negative staining; × 100000.

PLATE 4

Human milk casein; acidity 4·5 g/l; negative staining; × 100000.

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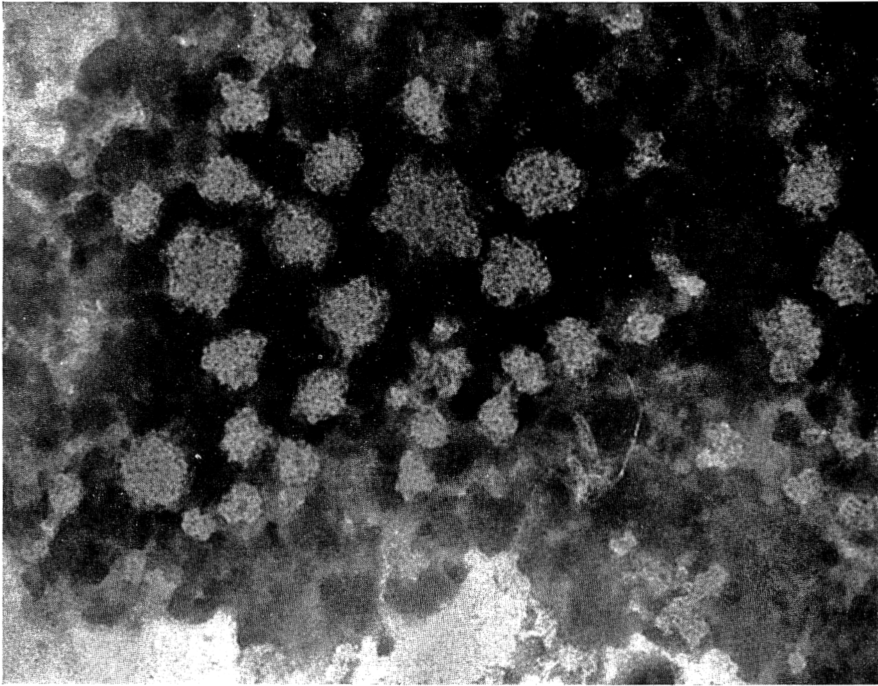


Fig. 1

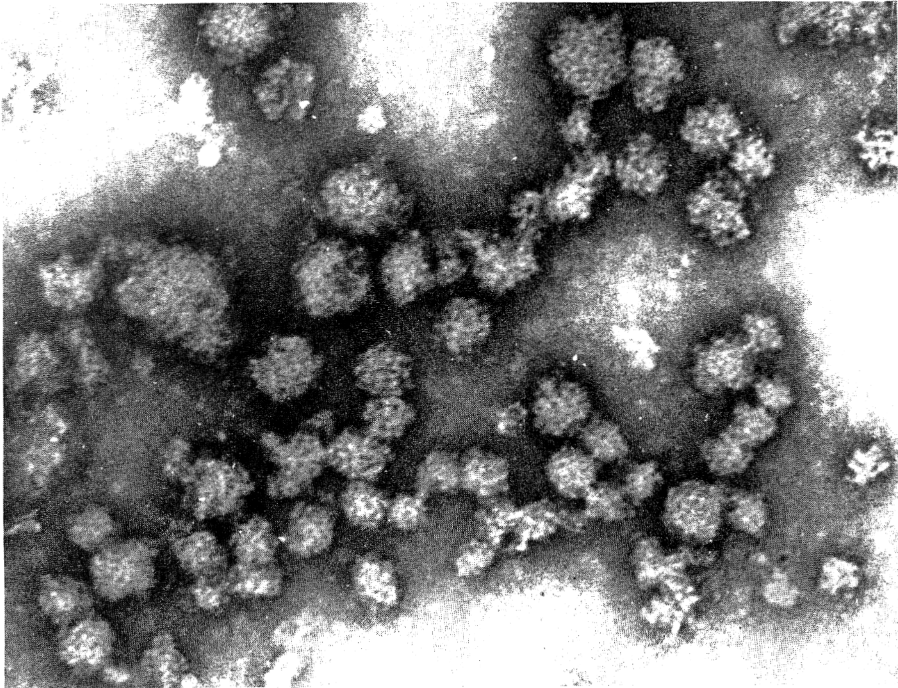
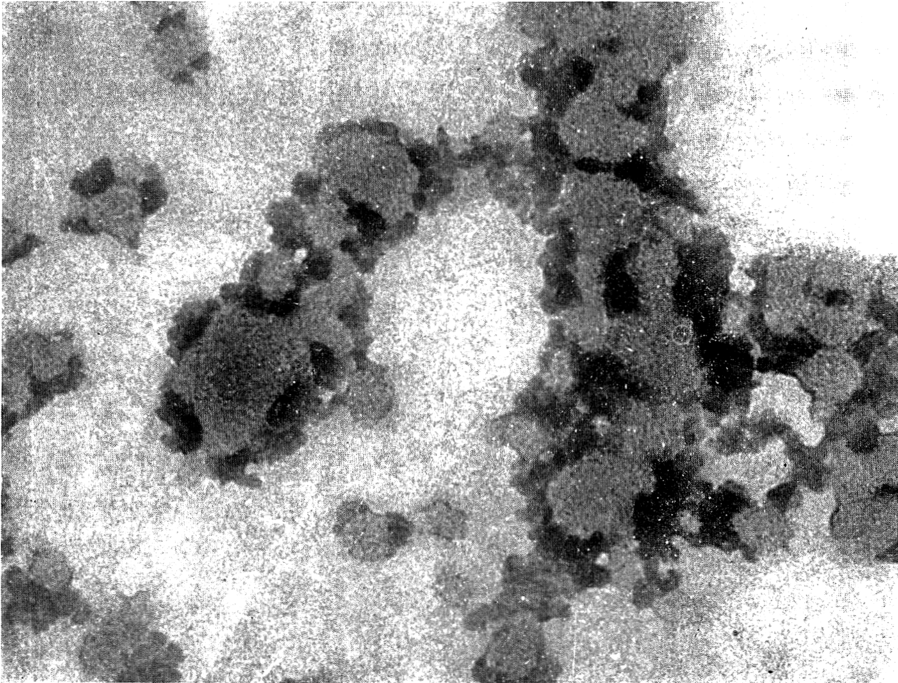


Fig. 2



G. G. CALAPAJ

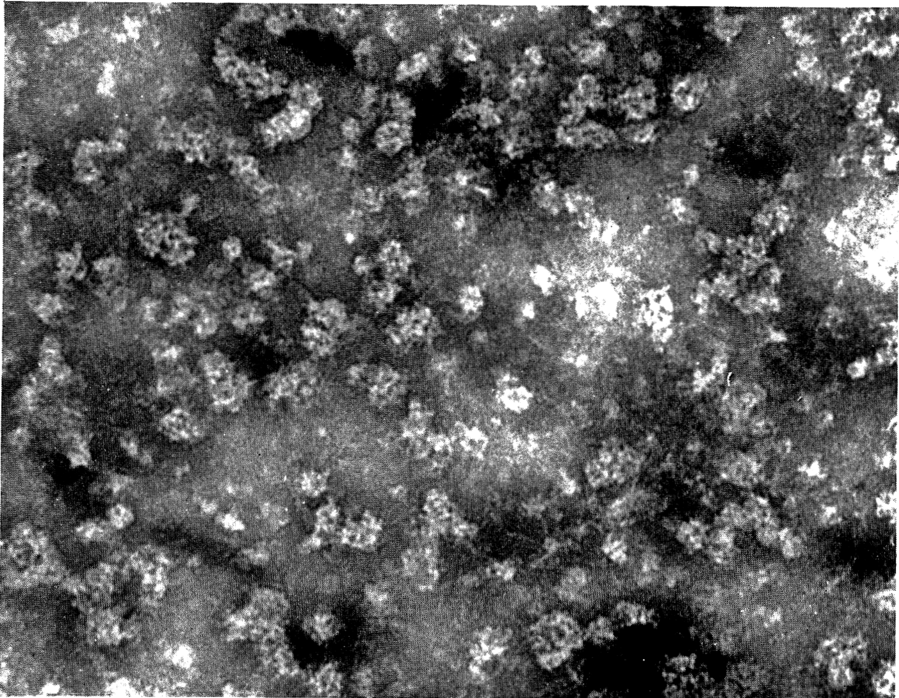


Fig. 1

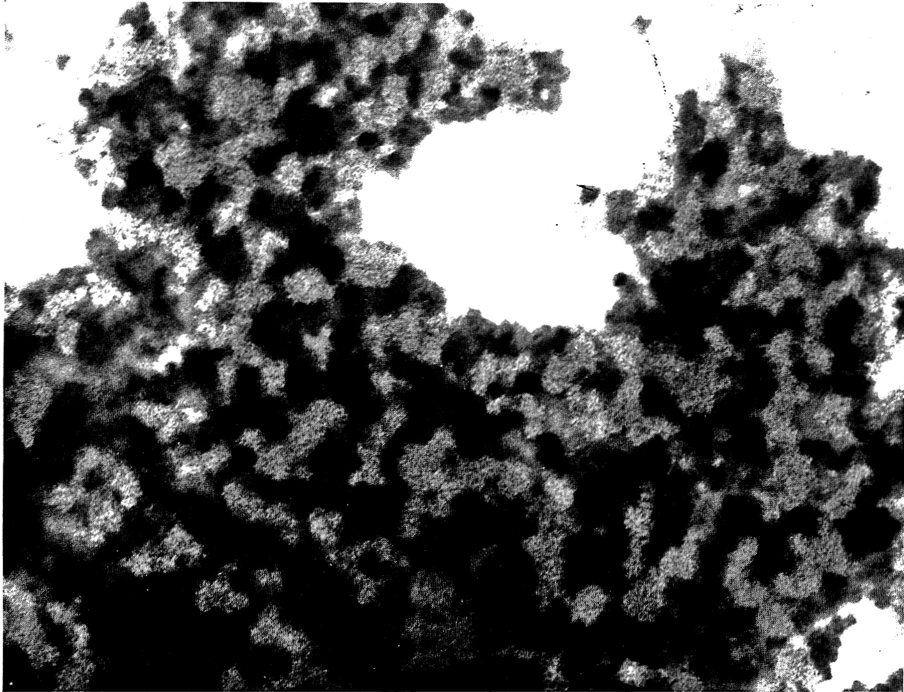


Fig. 2

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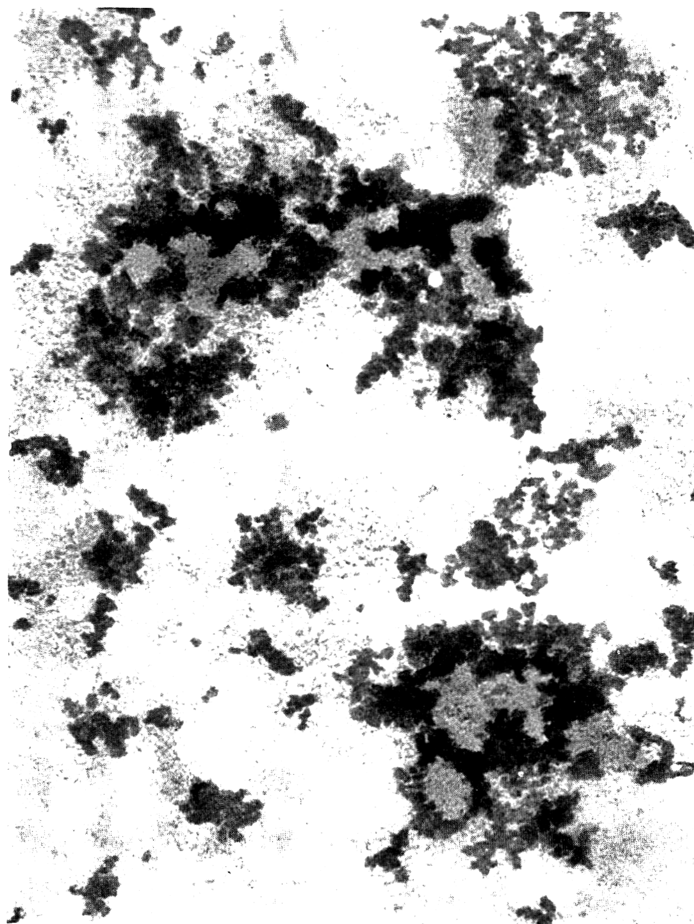

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Anti-O chaining titre in colostral serum from cows infused prepartum with *Salmonella pullorum*-H

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(*Received* 27 *March* 1967)

SUMMARY. A high anti-O chaining titre against *Salmonella pullorum*-H can be obtained in colostral serum when the killed antigen is introduced into the mammary gland during the dry period. A significant titre, several times the normal level, is maintained in the milk serum for 3 weeks postpartum. There was a definite residual titre in the milk serum of non-infused cows against the O and H antigens of *Salmonella typhosa* and the O antigen of *S. pullorum*-H.

Studies by Larson & Kendall (1957), Larson & Gillespie (1957), and Larson (1958) have indicated that the milk serum proteins, γ-casein, immune globulins and albumin, have a common origin in the blood. The change in milk protein levels postpartum was examined in relation to the rapid decrease in total proteins during the first 36 h and the changes in the relative quantity of the milk serum protein fractions. Porter & Conrad (1967) made a more detailed study of the change in the milk serum proteins postpartum and considered the change to be a 2-phase process. The first phase is a rapid dilution of the stored proteins during the first 60 h, followed by a gradual adjustment during which the immune globulins become insignificant in quantity and β-lactoglobulin becomes the major fraction. The findings of Kerr, Pearson & Rankin (1959) definitely indicate a local production of antibody against dead *Brucella abortus* antigen within the mammary gland and that the degree of blood-serum immune globulin found in milk serum is related to the period of lactation and to any inflammatory reaction occurring in the udder.

Outteridge, Rock & Lascelles (1965), using ewes that were infused prepartum with killed *S. typhi* O antigen, have definitely shown high titres in the colostrum and milk for 3 months postpartum. A further study with sheep was made by Lascelles, Outteridge & Mackenzie (1966) using *Br. abortus* and *S. typhi* O antigens (cellular antigens). They found that there was a rapid fall in the antibody titre of milk during the first week of lactation. However, the ratio of the titres of specific antibody in the samples from the sides of the udder receiving corresponding specific antigen to those in the plasma was usually well above unity throughout lactation. On the other hand, the milk:plasma ratios for whey samples collected from the sides which were not infused with corresponding antigens rapidly decreased within 2 weeks after parturition to values which varied between 0·03 and 0·25. The results indicate that during lactation most of the specific antibody in milk was of local origin.

Information on the immunological response of the cow when infused during the

dry period with *S. pullorum*-H would be of interest. By using *S. typhosa* as the test antigen, a response could be obtained against the specifically infused antigen (somatic IX and XII O antigens common to both the infused and the test bacteria) and also against the non-infused flagellar antigen (*d*) of *S. typhosa.*

MATERIALS AND METHODS

Animals used

Twelve multiparous dry cows, 7 Jerseys and 5 Holsteins, were selected from the dairy herd of the Ohio Agricultural Research and Development Center. Six of the cows were given intramammary infusions of an antigen preparation 2 or more times at approximately weekly intervals prepartum, and the other 6 cows were used as controls.

Table 1. *Infusion schedule and calving dates for the 12 cows on experiment*

	Treated cows					
Infusion	1427	1487	1525	1535	1577	1621
1	8 Feb.	8 Feb.	11 Feb.	11 Feb.	3 Feb.	11 Feb.
2	15	15	18	18	9	18
3	18	25	4 Mar.			25
4	25		11			
Calved	28	26	25	4 Mar.	13	26
	Control cows					
	1292	1469	1477	1541	1636	1644
Calved	9 Feb.	2 Feb.	7 Feb.	3 Feb.	4 Feb.	7 Feb.

Antigen preparation and infusion rate

The antigen preparation was a suspension of heat-killed *S. pullorum*-H bacteria. The live culture was obtained through the courtesy of Dr J. E. Williams of the Southeast Poultry Research Laboratory, Athens, Georgia. It was grown on tryptose agar medium, harvested at 24 h, heat-killed and suspended in physiological saline to a standard turbidity of 3 on the McFarland index. At 16 days before the expected calving date, and approximately weekly thereafter, the treated cows received 5 ml of the suspended antigen in each quarter (Table 1). All the cows were maintained under the same management conditions, and all except cow 1525 calved within a 31-day period.

Sampling

A sample of milk representative of the complete milking was collected every 12 h for the first 3 days, at the morning milking for days 4–7, and thereafter at the morning milking on alternate days to the 21st day. Milk serums were prepared from each sample by the method of Greene, Olson & Jezeski (1957) and held at −20 °C until used.

Determinations of milk serum proteins

The relative percentages of the milk serum protein fractions were determined by the disc electrophoresis method (Ornstein & Davis, 1962, reprinted by Eastman Kodak Co.). Densicord tracings were made with the Model 542 Densicord (Photovolt Corporation, New York). All tracings were read at the 'L' response setting. A blank gel was used to establish the scale zero (100 % transmission). The areas under the curves were determined with a planimeter.

Nitrogen in the milk serums was determined with a Technicon Auto-Analyser by its manufacturer's modified Kjeldahl nitrogen method (Technicon Controls Inc., Chauncey, New York). A standard nitrogen curve was made using graded concentrations of urea, and a factor of 6·38 was used to convert nitrogen to protein.

Titre measurements

The anti-somatic (anti-O) chaining titres and anti-flagellar (anti-H) immobilization titres of the antiserums prepared from all the milk samples were determined by the method of Nossal (1958) as modified by Smith (1966). The endpoint of the chaining titre was that dilution of antiserum which caused numerous chains of 4 bacteria/chain or more. These chains were readily observed under the microscope. The chaining titre is approximately 50 times as sensitive as the standard agglutination titre.

RESULTS AND DISCUSSION

All cows in the experiment calved within a 31-day period except for cow 1525 (see Table 1).

The number of infusions prepartum varied as it was impossible to predict the exact date of calving. There were no external indications of an inflammatory reaction from any of the infusions and there were no cases of mastitis during the experimental period.

From inspection of Fig. 1 it is clear that there was no marked difference in the total protein content of the milk serum from the infused and the non-infused cows. However, it is shown in Table 2 that for the first 24 h the level of immune globulin protein was somewhat higher in the milk serum from the infused cows than in that from the non-infused cows. For the remainder of the experimental period there was very little difference in the relative level of immune globulin protein between the 2 groups.

It was of interest to observe the relative percentage of immune globulin protein in the milk serum of infused and non-infused cows and to compare this with the titre levels of the respective milk serums. The increase in titre for the infused cows does not seem to be related to an increase in the relative amount of immune globulin compared to the other milk serum proteins.

The anti-H immobilization titre of the infused group was very similar to that of the non-infused group, as is shown in Fig. 2. An analysis of variance gave an F-value of 1·755 (for $P = 0{\cdot}01$, $F\,1, 167 = 6{\cdot}81$). This definite titre in the non-infused cows confirms the findings of Smith (1966) that there is a residual *S. typhosa* anti-H titre in cow's milk.

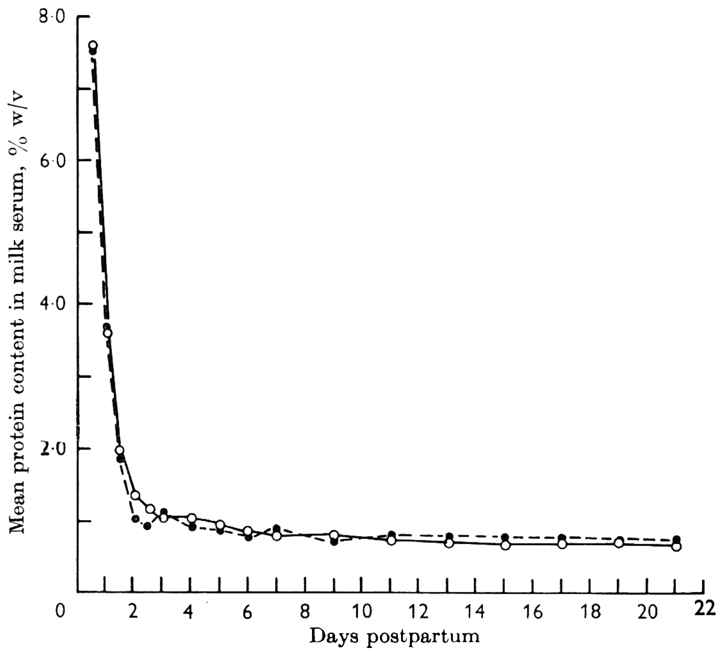

Fig. 1. Change in the mean protein content of serum from the milk of 6 infused and 6 non-infused cows during the first 21 days postpartum. ●---●, Infused; ○——○, non-infused.

Table 2. *Level of immune globulin in the milk serum of* 6 *infused and* 6 *non-infused cows expressed as a percentage of the total milk serum protein and as gram percentage protein in milk serum*

	Infused cows		Non-infused cows	
Sample	$\bar{X}$*, %	$\bar{X}$, g %	$\bar{X}$, %	$\bar{X}$, g %
1	59·2 ± 6·68	4·44	47·9 ± 12·89	3·63
2	50·3 ± 13·07	1·83	47·1 ± 4·41	1·69
3	33·1 ± 12·12	0·62	33·6 ± 9·98	0·66
4	26·3 ± 9·80	0·26	27·2 ± 6·32	0·35
5	21·0 ± 5·62	0·19	18·5 ± 5·93	0·21
6	20·3 ± 4·86	0·22	19·6 ± 14·90	0·21
7	15·5 ± 4·91	0·13	16·6 ± 7·73	0·17
8	10·9 ± 4·23	0·08	13·4 ± 7·34	0·12
9	10·8 ± 2·96	0·09	10·8 ± 4·96	0·09
10	8·3 ± 3·08	0·07	8·3 ± 3·11	0·06
11	7·8 ± 2·82	0·05	7·7 ± 3·28	0·06
12	7·5 ± 3·63	0·06	6·1 ± 1·97	0·04
13	6·1 ± 3·46	0·05	5·1 ± 2·01	0·03
14	7·5 ± 4·20	0·06	5·8 ± 3·46	0·04
15	6·2 ± 3·02	0·05	8·1 ± 3·48	0·05
16	6·2 ± 2·20	0·04	5·5 ± 2·99	0·04
17	6·0 ± 2·90	0·04	5·3 ± 3·30	0·03

* $\bar{X}$ Arithmetical mean.

Figure 3 shows that there was a significant and marked difference in the anti-O chaining titre of the milk serum of the infused and non-infused groups. An analysis of variance gave an F-value of 501·7 (for $P = 0{\cdot}01$, $F\,1{,}167 = 6{\cdot}81$). The $\bar{X}$ titre of the infused group was 15·4 times as great as that of the non-infused group at calving and

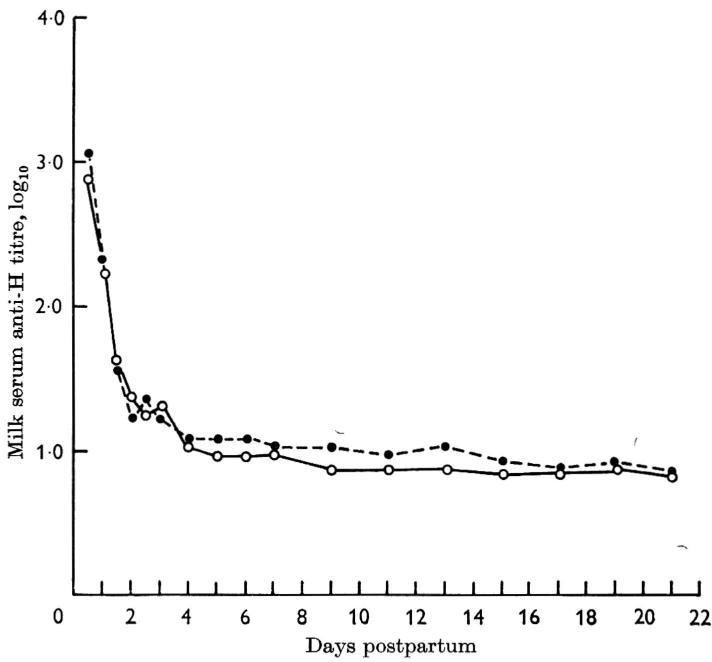

Fig. 2. *S. typhosa* anti-H immobilization titre of serum from the milk of 6 infused and 6 non-infused cows for the first 21 days postpartum. ●– – –●, Infused; ○——○, non-infused.

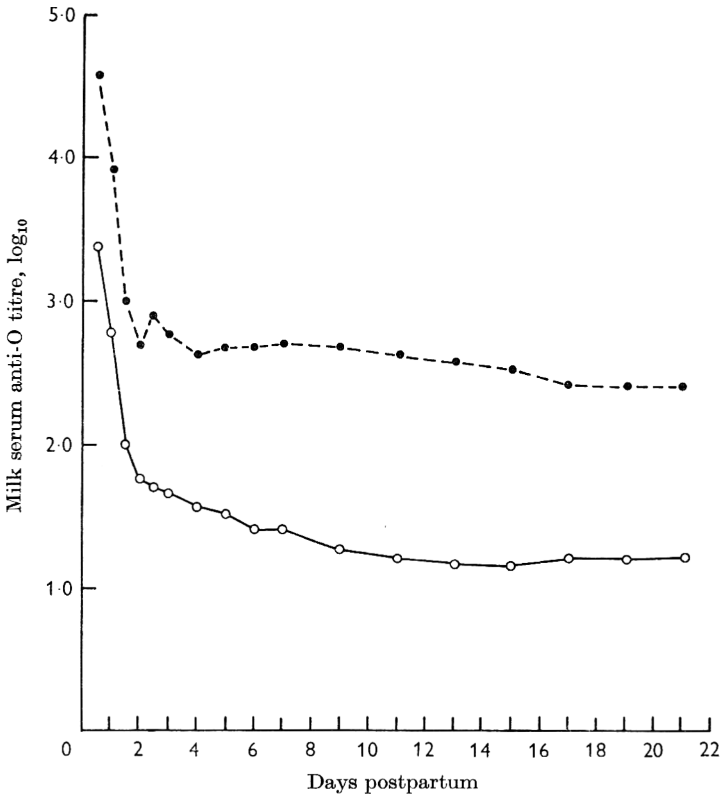

Fig. 3. *S. typhosa* anti-O chaining titre of serum from the milk of 6 infused and 6 non-infused cows for the first 21 days postpartum. ●– – –●, Infused; ○——○, non-infused.

increased to 18·9 times at day 21. The sustained anti-O titre of the infused group indicates an active and continued production of antibody during the period of time when there was a marked increase in daily milk production. The increase in anti-O titre of the infused group is maintained without a noticeable difference between the 2 groups in the amount of immune globulin present after 24 h postpartum. The higher anti-O than anti-H titre of the control group confirms the finding of Smith (1966) that cows respond with a higher titre to the O than to the H antigen of *Salmonella*. In making this comparison one must remember that the anti-H titre is an immobilization titre, whereas the anti-O is a chaining titre.

Our findings are in agreement with those of Kerr *et al.* (1959) from their study with *S. pullorum* in one dry cow, and with those of Outteridge *et al.* (1965) for *S. typhi* in the pre-parturient ewe. Our conclusions would agree with those of Kerr *et al.* (1959), that the sustained milk serum titre against *S. pullorum*-H must be of local origin within the mammary gland, as the normal udder barrier would not permit passage of so large a quantity of immune globulin from the blood without an inflammatory reaction having changed the permeability of the udder epithelium.

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The role of lysine residues in the coagulation of casein

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SUMMARY. Coagulation of rennin-treated casein was inhibited by treatment of the casein with dimethylaminonaphthalene sulphonyl chloride. The effect was caused by substitution on lysine side chains of the κ-casein fraction, and inhibition was complete when 2–3 lysine residues/molecule were blocked. This level of substitution did not affect other properties of the κ-casein, such as the release from it of non-protein nitrogen (NPN) by rennin and its ability to stabilize α_s- and β-caseins in the presence of Ca^{++}. The evidence suggests that lysine side chains on κ-casein take part in the coagulation of rennin-treated casein.

Although it is well known that the rennin-induced coagulation of casein depends upon the presence of calcium, which is presumed to form intermolecular links between ester phosphate groups (McFarlane, 1938; Hsu, Anderson, Baldwin, Ernstrom & Swanson, 1958), the roles of other functional groups in the casein molecules are less certain. For investigating this problem, the selective modification on a limited scale of different types of functional group seems to be a promising approach. This is particularly so in view of the increasing variety of reagents now available which are capable of modifying specific groups on proteins.

In previous work along these lines we have shown that modification of tryptophan, methionine and, probably, tyrosine side chains does not interfere with the enzymic stage or with the coagulation of rennin-altered casein, whereas modification of histidine side chains interferes with both of these reactions (Hill & Laing, 1965). Continuing this work we have found that casein which has been given a quite limited treatment with 1-dimethylaminonaphthalene 5-sulphonyl chloride (dansyl chloride) does not coagulate when treated with rennin. Dansyl chloride reagent was introduced by Gray & Hartley (1963) as a fluorescent label for determining amino terminal groups. It will also react with the ϵ-NH_2 groups of lysine and less rapidly with phenolic and alcoholic OH and imidazole NH groups. The present paper reports the effects of dansyl chloride treatment on the coagulation of casein, and the results of experiments to determine the nature and location of the groups concerned with these effects.

MATERIALS AND METHODS

The dansyl chloride was a product of Fluke Ltd (Switzerland); all other reagents were of analytical grade. Sodium caseinate and 'crude' κ-casein were prepared as described by Hill & Hansen (1963), and α_s-casein by the method of Zittle & Custer

(1963). β-casein was prepared by twice-repeated fractionation of the mixture of mainly α_s- and β-caseins obtained during the preparation of κ-casein, and taking advantage of the difference in solubility at 3 °C of α_s- and β-caseins in the presence of Ca^{++} (0·2 M). The preparations of α_{s1}- β- and κ-caseins were only about 90 % pure, as judged by starch gel electrophoresis, but pure preparations were not essential for this work.

In initial trials, casein solutions of pH 8·0 were treated with dansyl chloride in solution in acetone. To 10 ml solution containing 120–280 mg casein was added 1 mg dansyl chloride in 1 ml acetone. Control solutions of casein were treated with acetone only. The mixtures were stirred for 1 h, and the reagents were then separated from the protein by filtration in G25 Sephadex gel, in 0·03N sodium acetate buffer at pH 6·25. In later experiments, in which it was desired to estimate by means of UV difference spectroscopy the extent of the reaction between casein and dansyl chloride, 200 mg portions of casein in 10 ml solution of pH 8·0 were treated with 0·3–1·2 ml of a 1 % (w/v) solution of dansyl chloride in isopropanol. Controls were treated with isopropanol only. The solutions were stirred for 1 h, following which the casein was separated from reagents by precipitation with HCl at pH 4·5. The precipitate was centrifuged out, washed with water and redissolved with addition of NaOH, care being taken to keep the pH of the solution below 8·0.

For the tests with rennin, portions of the control and treated solutions were adjusted to pH 6·5 after addition to them of 0·01–0·025 M-$CaCl_2$ and diluted to equal protein concentration. The solutions were treated with rennin (1/20000 by weight of the casein) and the times determined for coagulation at 30 °C. Other portions of the solutions were diluted to contain 0·125 mg/ml and the UV difference spectra determined. The amount of dansyl bound to the casein was estimated assuming that the absorption of bound dansyl at 245 mμ was the same as that of dansyl chloride in isopropanol (Hill & Laing, 1967). The amount of dansyl which was bound to amine groups was also estimated by comparing the results of formol titrations made at pH 8·3 on both control and dansylated caseins.

In order to identify the dansylated residues, high voltage electrophoresis was used. Portions of the solutions were freeze-dried and 10 mg of the dried material was hydrolysed *in vacuo* in 6N-HCl for 6–18 h at 110 °C. The hydrolysates were freeze-dried to remove the acid and dissolved in 1 ml 50 % acetone–water. These solutions were applied in 2–6 μl spots on Whatman no. 3 MM paper, and electrophoresis runs performed at 3 kV in pyridine-acetate buffer of pH 4·4 (Gray & Hartley, 1963). As standards for electrophoresis ϵ-N-dansyl lysine was prepared as described by Hill & Laing (1967) and other dansylated amino acids as described by Gray & Hartley (1963).

The stabilizing power of κ-casein as compared with that of α_s-casein was tested by mixing 2 ml of κ-casein solution ($\simeq$ 2·5 mg/ml) with 4 ml of a solution of α_s- or β-casein ($\simeq$ 4 mg/ml) and adding 3 ml of 0·06 M-Ca lactate solution: the mixture was then adjusted to pH 6·5. After standing for 15 min at room temperature duplicate preparations were centrifuged at about 3000 *g* for 20 min, after which the protein content (A) of the supernatants was calculated from nitrogen determinations made by a semi-micro Kjeldahl distillation procedure. The amount (B) of κ-casein remaining in solution under the same conditions was determined in a similar experiment in which

water was substituted for the 4 ml of α_s-casein solution. The quantity (A–B) represents the amount of α_s- or β-casein stabilized by the κ-casein. Expressed as a percentage of the α_s- or β-casein originally added, it gives a measure of the stabilizing power of the κ-casein. In addition, the solubility of the individual α_s- and β-caseins in the presence of 0·02 M-calcium was determined by tests in which water was substituted for the 2 ml κ-casein solution. The extent of coagulation following rennin action was determined by treating solutions of κ-α_s-casein or κ-β-casein, of the composition indicated above, with rennin for 30 min at 30 °C before centrifuging and analysing the supernatants described above.

The extent of release by rennin of NPN from sodium caseinate or κ-casein was estimated by determining the proportion of the casein rendered soluble in 12 % TCA as a result of the rennin action.

RESULTS

The effect of the dansyl treatment on time of coagulation

The results for the first 5 preparations listed in Table 1 show that treatment of the caseins with relatively small amounts of dansyl chloride in acetone caused considerable increases in the times of coagulation, and that the effect appears to be a general one.

Table 1. *Setting times of dansylated caseins*

Preparation no.	Amount of caseinate, mg	Amount of dansyl chloride,* mg	Setting time at pH 6·5, min		$CaCl_2$ (M)
			Control	Treated	
1	155	1	1	6	0·025
2	207	1	6·2	17	0·025
3	280	1	0·3	5·5	0·025
4	120	1	3·8	9·2	0·025
5	280	1	3·3	6·8	0·01
6	200	3	3·2	4·5	0·015
6	200	6	2·5	3·2	0·015
7	200	9	3·5	4·2	0·015
7	200	12	4	> 40	0·015
7	200	12	2	> 40	0·015
7	200	12	3·8	> 40	0·015

Preparations 1–5 were treated with dansyl in acetone (1 mg/ml), and excess reagents were separated from the casein by filtration in Sephadex gel G25. Preparations 6 and 7 were treated with dansyl in isopropanol (10 mg/ml). Corresponding amounts of acetone or isopropanol were added to the control solutions. Excess reagents were separated from the casein by precipitation at pH 4·5.

* Treatment was for 1 h at pH 8·0.

Although the magnitude of the effect differed somewhat among these 5 samples which were prepared from different milks, coagulation times were at least doubled in every case; it is worth noting that this effect was achieved with an amount of reagent sufficient on the average to affect only about one protein residue in every 300–400.

The addition of dansyl chloride in acetone appeared to be more effective than adding it in isopropanol (cf. preparations 1–5 and 6–7, Table 1), possibly because the amount of acetone added (9 %) was sufficient to render more accessible for reaction the residues concerned in coagulation. In this connexion it is significant that the effect of adding dansyl chloride in isopropanol also increased considerably when the

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content of isopropanol in the solution reached 11 % (Table 1). Isopropanol was used as the vehicle in the last 6 experiments as it was desired to estimate the extent of binding of the dansyl by means of UV difference spectra. Because of its strong absorption in the UV, it was feared that relatively small amounts of acetone retained with the protein might lead to inaccurate results. However, the effects shown for preparations 6 and 7 were similar to those given by the first 5 in that the treatments caused increased times of coagulation, and this effect was particularly marked as the amount of dansyl chloride was increased from $4\frac{1}{2}$ to 6 % of the casein.

Extent of reaction and nature of affected functional group

The amount of dansyl bound to the casein was 6–7 moles/10^5 g casein for the treatments with 3 % by wt of dansyl chloride and 11–13 moles/10^5 g for samples treated with 6 % of dansyl chloride. For the latter, formol titrations showed 42 moles of reactive amine groups per 10^5 g casein compared with 53/10^5 g for the controls, indicating that 11 moles/10^5 g or virtually all of the dansyl in the casein was bound to amine groups. The results from high-voltage electrophoresis confirmed this and showed in addition (1) that the functional group mainly affected was the ϵNH_2 group of lysine and (2) that the increased dansyl uptake for the caseins treated with 6 % dansyl chloride, as compared with 3 %, was due to reaction with this group. As this was the level of treatment at which the most marked effects on coagulation occurred, these effects must be associated with the dansylation of lysine side chain.

The nature of the inhibition of coagulation

Possible explanations for the observed effects of the dansyl treatment were (1) that the treatment interfered with the release of NPN by rennin and (2) that the reaction between rennin and dansylated casein proceeded normally but that the

Table 2. *Plan of experiment for determining the effects of dansylation on some properties of caseins*

Components of test mixtures, ml	A	B	C	D	E	F	G
H_2O	2	4	0	0	0	2	2
Solutions of:							
α_s-casein (control)	4	0	4	0	0	0	0
κ-casein	0	2	2	2	2	0	0
Ca lactate, 0·06 M	3	3	3	3	3	3	3
α_s(I)-casein	0	0	0	4	0	4	0
α_s(II)-casein	0	0	0	0	4	0	4

Duplicate samples adjusted to pH 6·5 were centrifuged at about 3000 *g* for 20 min and supernatants analysed for protein content.

Combinations C,D and E were also dialysed after treatment with rennin for 30 min at 30 °C before centrifuging.

α_s (I) and α_s (II) were treated with dansyl chloride at the 3 and 6 % levels, respectively.

subsequent polymerization of the casein in the presence of Ca^{++} was inhibited. Tests on the extent of release of NPN by rennin from control and dansylated sodium caseinates and κ-caseins showed that, at the levels of treatment used in these experiments, the release of NPN was not affected. A similar result for *N*-trifluoroacetylated κ-casein has been obtained by Woychik (1966). The failure to coagulate,

therefore, appeared to be caused by an interference with the polymerization of rennin-treated caseins. Since relatively few functional groups were affected by the treatments, particularly in preparations 1–5 (Table 1), it seemed likely that the important residues might be located in one type of casein molecule only. This hypothesis was tested by treating α_s-, β- or κ-casein individually with dansyl chloride at the 3 and 6 % levels as previously described for whole casein and combining, for example, untreated κ-casein and treated α-caseins as in the experimental arrangement which for brevity is set out in Table 2.

Table 3*a*. *Effects of dansylation on some properties of α_s-casein**

Sample	α_s-Casein precipitated by Ca in absence of κ-casein, %	α_s-Casein stabilized by κ-casein, %	Total casein precipitated after rennin action, %
α_s	96	100	85
α_s (I)	95	100	84
α_s (II)	96	100	85

α_s (I) had been treated with dansyl chloride at the 3% level. α_s (II) had been treated with dansyl chloride at the 6% level.

* The dansylation of β-easein alone had similar effects.

Table 3*b*. *Effects of dansylation on some properties of κ-casein*

Sample	κ-Casein soluble in presence of Ca, %	α_s-Casein stabilized, %	Total casein precipitated after rennin action, %
κ	100	100	86
κ (I)	93	92	72
κ (II)	99	85	0

κ (I) had been treated with dansyl chloride at the 3% level. κ (II) had been treated with dansyl chloride at the 6% level.

From this arrangement, information can be obtained about the effects of dansylation on: (1), the solubility of the α_s-caseins in presence of 0·02 M Ca^{++}; (2), the stabilizing power of κ-casein compared with that of α_s-caseins in presence of 0·02 M Ca^{++}; and (3), the extent of coagulation of the κ-α_s-caseins following rennin action. This information is pertinent, since the observed effects could conceivably have been caused by the dansyl treatments rendering α_s-casein soluble in the presence of Ca^{++} (cf. Hoagland, 1966) as much as by the direct blocking of a functional group essential for coagulation.

The results of these experiments are set out in Tables 3*a* and 3*b*. The dansylation of α_s- or β-casein at the 3 and 6 % levels had no effect on their solubility in presence of Ca^{++}, their stabilization by κ-casein or the coagulation of κ- α_s- and κ- β-casein solutions under the action of rennin (Table 3*a*). However, the dansylation of κ-casein, while having little effect on its solubility in presence of Ca^{++} and its power to stabilize α_s-casein, caused complete inhibition of coagulation at the 6 % level of treatment (Table 3*b*). Treatment with dansyl chloride at the 3 % level resulted in the uptake of 7 moles of dansyl/10^5 g κ-casein, as estimated from UV difference spectra.

At the 6% level of treatment 12–13 moles of dansyl were taken up. Assuming a molecular weight of 20000 for κ-casein (Swaisgood & Brunner, 1963) this represents 2–3 moles of dansyl bound per mole of κ-casein.

DISCUSSION

Because the blocking of lysine side chains on the α- and β-caseins has no effect on their stabilization by κ-casein, nor on the coagulation of rennin-treated κ- α- and κ- β-caseins, it can be concluded that lysine side chains do not take part in these processes. Similarly, it must be concluded that lysine residues on the κ-casein are not involved in the reaction between rennin and casein. However, interpretation of the finding that the dansylation of κ-casein does cause inhibition of coagulation is less simple. The direct interpretation is that lysine side chains on the κ-casein take part in essential intermolecular reactions leading to coagulation. Other possible explanations are that the blocking of the lysine side chains causes a conformational change in the κ-casein sterically unfavourable to coagulation, and that the increased net negative charge caused by the substitutions makes it more difficult for the micelles to interact. In these experiments, inhibition of coagulation was virtually complete when 2–3 lysine residues/κ-casein molecule were blocked. In view of this relatively low level of substitution on accessible residues, and because of the native disordered structure of κ-casein (Herskovits & Mescanti, 1965) it does not seem likely that the treatment would have caused conformational changes in the protein molecule. Woychik (1966) has reported that in *N*-trifluoroacetylated κ-casein there was evidence of conformational change which was associated with an almost complete loss of ability to stabilize α_s-casein. The lack of such effects in our experiments supports the view that no important conformational changes occurred. Change of charge is also unlikely to have been responsible for these effects, as the much greater changes of charge occasioned by the use of dansylated α_s- and β-caseins were without effect on the coagulation. The direct interpretation, therefore, is the one most likely to be correct. It is that one or more of the lysine side chains on the κ-casein fraction take part in the coagulation of rennin-treated whole casein.

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A modification of the acid degree value test for lipolytic rancidity in milk

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SUMMARY. While using an acid degree value test during work on lipolysis in milk, difficulty was encountered in extracting the fat from the milk of certain cows, especially milk of high butterfat content and milk from newly calved cows. This difficulty was overcome by increasing the quantity of reagent used. A study was made of the quantities of reagent required for milks of different butterfat content and for colostrum. No effect on the acid degree value occurred as a result of increasing the quantity of reagent. A modification of the test is recommended, using an increased quantity of reagent.

The acid degree value (ADV) test of Thomas, Nielsen & Olson (1955) is a convenient screening test for the laboratory detection of lipolytic rancidity in milk. The test involves the extraction of fat by heating 35 ml of milk with 10 ml of BDI reagent for 15 min in a boiling water bath. BDI reagent is a mixture of a non-ionic surface active agent and sodium tetraphosphate (or hexametaphosphate). The extracted fat is titrated using 0·02 N alcoholic potassium hydroxide. The ADV is expressed as the 'number of ml of 1N base required to titrate 100 g fat'.

This test was used by one of the authors (Hunter, 1966) in a recent survey of lipolysis in raw milk supplies in north-east Scotland. During this survey, and especially during more recent work on milk samples from individual cows, difficulty was experienced in extracting the fat from certain samples. This was most noticeable in milk from Jersey cows and newly calved cows. With some samples heating for as long as 4 h was necessary before the amount of fat required for the titration (1 ml) was available. With other samples no fat could be extracted even after such prolonged heat treatment. It was thought that a modification of the technique, possibly by increasing the volume of BDI reagent, might resolve this difficulty.

Accordingly, an investigation was made to ascertain whether: (1) an increase in the volume of BDI reagent used would extract the fat more readily; (2) any increase that occurred in the volume of fat extracted was proportional to the volume of BDI reagent used; (3) any relationship could be observed between the total fat content of the sample and the quantity of BDI reagent required to extract the fat; (4) the ADV would be affected by variations in the quantities and proportions of the reagents used; (5) an increase in the volume of BDI reagent would increase the volume of fat extracted from the milk of newly calved cows; and (6) a more suitable ratio of BDI reagent to milk than that recommended by Thomas *et al.* (1955) could be established.

METHODS

Milk samples of about 250 ml each were taken from 52 cows. The samples consisted of mixed milk taken from the milking machine bucket. In order to give a range of butterfat percentages the animals comprised 18 Jersey, 17 Friesian and 17 Ayrshire cows and heifers. The samples were taken at afternoon milkings, quickly cooled to 4–5 °C, stored at that temperature overnight, and tested the following morning.

Six fat extractions were made on each sample using quantities of BDI reagent ranging from 10 to 15 ml. Owing to the size of the test bottles, which was governed by the size of centrifuge available, it was necessary that the total contents of each test bottle should not exceed 45 ml. Consequently the quantities of milk used ranged from 35 to 30 ml in order to maintain a uniform volume in each bottle.

A note was made of the volume of fat extracted after immersion for 15 min in boiling water, and of the ratio of the volume of BDI reagent to the volume of milk used. Where the volume of fat extracted exceeded 1 ml the ADV test was completed to detect whether variations in results occurred due to variations in the proportions of milk and reagent.

Four samples of milk were taken at 24-h intervals from each of 6 Ayrshire cows, commencing at the first or second milking after calving. The first samples were tested using quantities of milk ranging from 35 to 15 ml with quantities of BDI reagent ranging from 10 to 30 ml. The remaining samples were tested using quantities of milk ranging from 35 to 25 ml and quantities of BDI reagent ranging from 10 to 20 ml. Those samples yielding more than 1 ml of fat in 15 min were noted. No titrations were made on the extracted fat.

A butterfat determination was made on each milk sample by the Gerber method.

The term 'BDI: milk ratio' is used to indicate the ratio of the volume of BDI reagent to the volume of milk.

RESULTS

Relationship of BDI: milk ratio to the numbers of samples yielding at least 1 *ml fat in* 15 min.

The results shown in Table 1 indicate that, using a BDI: milk ratio of 10:35 as recommended by Thomas *et al.* (1955), less than half the samples tested yielded, within 15 min, sufficient fat to complete the test. A proportion of 14 ml of BDI reagent to 31 ml of milk was required before fat extraction was satisfactory in all the samples.

Table 1. *The effect of increasing the BDI: milk ratio on the extraction of fat*

BDI:milk ratio	No. and % of samples having their fat extracted within 15 min
10:35	18 (34)
11:34	34 (65·4)
12:33	46 (88·5)
13:32	51 (98·1)
14:31	52 (100)
15:30	52 (100)

Relationship of BDI : milk ratio to volume of fat extracted

A substantial rise in the volume of extracted fat occurred at a critical volume of BDI reagent, which varied from sample to sample. After this point was reached, only slight differences in the volume of extracted fat occurred with increased quantities of BDI reagent. The volume of fat extracted was therefore not in simple proportion to the quantities of BDI reagent or milk which were used.

The samples have been arranged in 5 groups according to the critical volume of BDI reagent required to extract the bulk of the fat. The average volumes of fat extracted in each group have been plotted against the BDI: milk ratios used and are shown in Fig. 1.

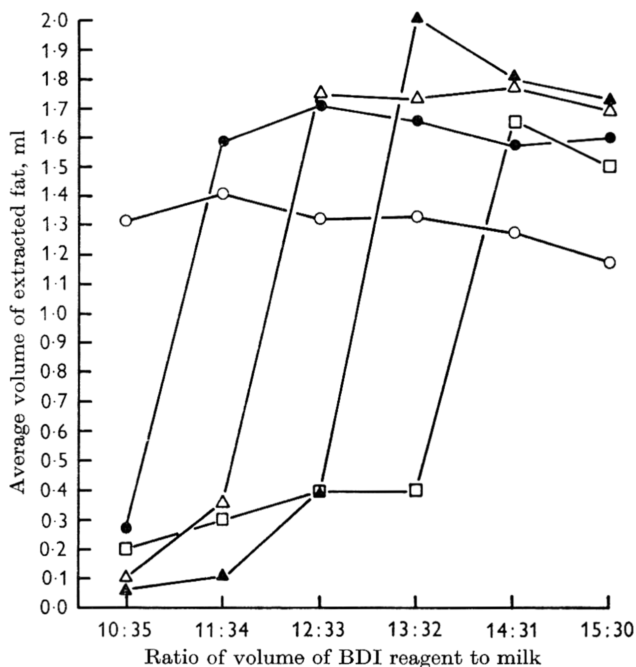

Fig. 1. The relationship of the volume of fat extracted to the ratio of BDI reagent and milk used. ○, Group 1, 18 samples, fat extracted using 10 ml BDI reagent; ●, Group 2, 16 samples, fat extracted using 11 ml BDI reagent; △, Group 3, 12 samples, fat extracted using 12 ml BDI reagent; ▲, Group 4, 5 samples, fat extracted using 13 ml BDI reagent; □, Group 5, 1 sample, fat extracted using 14 ml BDI reagent.

In group 1 the average volume of fat extracted from 18 samples, using 10 ml BDI reagent and 35 ml milk, was 1·32 ml. Increasing the volume of BDI reagent in 1-ml stages up to 15 ml did not result in any substantial increase in the quantity of fat extracted. In fact, a fall of 0·14 ml was observed when 15 ml BDI reagent were used, but this could be accounted for by the correspondingly smaller volume of milk used. In this group the quantity of BDI reagent recommended by Thomas *et al.* (1955) was adequate to extract the fat from 35-ml quantities of milk.

In groups 2–5 the average volumes of fat extracted using 10 ml BDI reagent were 0·27, 0·01, 0·06 and 0·2 ml, respectively. These quantities of fat were insufficient for completion of the test.

In group 2, the use of 11 ml BDI reagent gave an increase of 1·31 ml in fat volume. In group 3, an increase in BDI reagent from 11 to 12 ml raised the fat volume by 1·39 ml. In group 4, an increase from 12 to 13 ml raised the fat volume by 1·60 ml and in group 5 an increase from 13 to 14 ml raised the fat volume by 1·25 ml.

These sharp increases in fat volume resulting from the use of an additional 1 ml of BDI reagent were observed in all samples in groups 2–5. Further increase in the quantity of BDI reagent resulted in little change in the volume of fat extracted.

Relationship of the BDI : milk ratio necessary to extract the bulk of the fat to the butterfat content of the milk sample

As trouble was experienced with some milk samples of high fat content, especially of Jersey milk, the minimum BDI:milk ratio required to extract the bulk of the fat and the fat content of the milk samples were examined to determine whether there

Table 2. *The effect of the butterfat content of the milk on the BDI : milk ratio required to extract the bulk of the fat*

BDI:milk ratio required to extract bulk of fat	No. of samples in each group				Average and range of butterfat percentages in each group			
	Friesian	Ayrshire	Jersey	Total	Friesian	Ayrshire	Jersey	Over-all average
10:35	8	7	3	18	3·7 (2·8–4·6)	4·4 (4·1–5·05)	5·4 (3·8–7·3)	4·32
11:34	3	7	6	16	4·4 (3·9–4·9)	4·6 (4·5–4·75)	6·5 (5·9–6·9)	5·30
12:33	5	3	4	12	4·9 (4·15–5·6)	5·3 (4·85–5·95)	6·7 (6·0–7·3)	5·63
13:32	1	—	4	5	3·9	—	6·9 (6·35–8·1)	6·35
14:31	—	—	1	1	—	—	6·3	6·3

was any correlation between them. The distribution of milk samples according to breed, butterfat percentage and BDI:milk ratio required for fat extraction is shown in Table 2. From this table it can be seen that as the quantity of BDI reagent required to extract the fat increased, the average butterfat percentages of the groups of Friesian, Ayrshire and Jersey milk samples all tended to rise, with the exception of the 2 groups comprised of only one sample each. There would therefore appear to be some positive correlation between these 2 factors. Due to the considerable variations in individual butterfat percentages within groups, it was thought that such a correlation would not be a strong one. The correlation coefficient was calculated and found to be +0·514. This figure indicates that only about 25 % of the differences in BDI : milk ratio required for fat extraction could be accounted for by the differences in butterfat percentage.

The effect of varying the BDI : milk ratio on the ADV of the fat

Where sufficient fat was extracted, 1 ml was dissolved in 5 ml of fat solvent (2 parts of light petroleum to one part absolute ethanol) and titrated against a

standardized alcoholic solution of KOH (approx. 0·02 N) using 1% phenolphthalein in ethanol as indicator. The ADVs were then calculated.

Table 3 illustrates the effect on the ADVs of increasing the quantity of BDI reagent beyond that necessary to extract the bulk of the fat. It is apparent that no significant changes in ADV occurred as a result of varying the quantities of BDI reagent and milk.

Effect of increasing the volume of BDI reagent on the extraction of fat from the milk of newly calved cows

Although it is illegal to sell milk from newly calved cows for a period of 4 days after calving, it was thought that sometimes more than 4 days might elapse before the fat could be extracted if a BDI:milk ratio of 10:35 were used. The effect of increasing this ratio to 15:30 is shown in Table 4.

Table 3. *The effect on the ADV of varying the BDI: milk ratio*

BDI:milk ratio required to extract bulk of fat	No. of samples	Average ADVs obtained using different BDI:milk ratios					
		10:35	11:34	12:33	13:32	14:31	15:30
10:35	17	0·80	0·75	0·80	0·74	0·73	0·75
11:34	16	—	0·66	0·66	0·65	0·64	0·66
12:33	12	—	—	0·57	0·61	0·58	0·56
13:32	4	—	—	—	0·88	0·85	0·84
14:31	1	—	—	—	—	0·73	0·66

Table 4. *The effect of increasing the BDI: milk ratio on the extraction of fat from the milk of newly calved cows*

Cow no.	Days after calving before fat could be extracted from milk: BDI:milk ratio 10:35	BDI:milk ratio 15:30
1	3	2
2	4	2
3	> 4	> 4*
4	2½	2
5	3	2½
6	3½	1½

* Fat was extracted at 4½ days using a BDI:milk ratio of 16:29.

From the milk of 5 of the 6 cows the fat was extracted within 4 days using the quantities recommended by Thomas *et al.* (1955). However, the increased BDI:milk ratio enabled the fat to be extracted from these milks from ½ to 2 days earlier.

The milk from the remaining animal, cow no. 3, was considered abnormal. A well-mixed bulk sample of evening milk taken 3 days after this cow calved had a butterfat content of over 10%, and the animal showed loss of appetite and greatly reduced yield. The fat was, however, extracted at 4½ days using a BDI:milk ratio of 16:29.

CONCLUSIONS

From the foregoing results it appears that the quantity of BDI reagent recommended by Thomas *et al.* (1955) for the extraction of fat from milk, though usually adequate for bulk milk supplies, is not always sufficient for milk of individual cows. Larger quantities are required for the milk from many animals, especially for milk of high butterfat content, and these larger quantities enable the fat to be extracted earlier from the milk of newly calved cows.

We recommend that the test be modified by the use of BDI reagent and milk in the proportion of 15:30 instead of 10:35.

The authors gratefully acknowledge the assistance given by Mr R. Ross, dairy cattleman from the North of Scotland College of Agriculture, Craibstone, and by a number of local dairy farmers whose animals were made available for this investigation.

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Seasonal variation in the viscosity index and adhesive strength of casein from the milk of individual cows

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SUMMARY. The viscosity index and adhesive strength of casein from the milk of 6 individual cows were determined on samples collected regularly throughout the 9-month dairying season. The viscosity index of the casein from each cow showed a seasonal decline while the adhesive strength showed a general increase during this time. Casein from one cow had a consistently low viscosity index attributed to the presence in it of a rare genetic variant of α-casein. It is suggested that the variations in viscosity index and adhesive strength are lactational rather than seasonal.

Casein has for many years been used in large quantities as a binder for the pigments used in coating paper (Sutermeister & Browne, 1939; Salzberg, Georgevits & Cobb, 1961; Salzberg & Britton, 1963). In this respect, 2 properties of the casein are of special importance, namely the viscosity index and the adhesive strength. For coating paper a low viscosity casein combined with good adhesive strength is preferred. The viscosity index of New Zealand commercial lactic casein has been shown (Dolby, 1964) to decline during the dairying season from August to May. Casein manufactured during the latter half of the season would, therefore, be more acceptable for coating paper provided that the adhesive strength was not significantly affected.

The estimation of the adhesive strength of the binder used in paper coatings is not easy as it is necessary first to produce a sample of coated paper using the particular adhesive and then to obtain some measure of the force required to pull or 'pick' the coating from the paper. An early and still widely used method, the Dennison wax pick test (Sutermeister, 1914; TAPPI Specification T 459 m-42), which uses a series of standard waxes, has poor reproducibility. Tests by this method on laboratory-coated papers containing New Zealand commercial caseins (R. M. Dolby, unpublished) showed no significant seasonal trend.

Greater precision is obtainable by use of the IGT Printability Tester, manufactured by the Institute for Graphic and Allied Industries, Amsterdam, and described in detail by Hemstock & Swanson (1957). This instrument simulates the printing operation by running the paper to be tested past a roller (printing disc) coated with printing ink or a standard oil of similar viscosity and tackiness. The paper, which is mounted on the surface of a quadrant, is driven at increasing velocity by a weight or spring and the velocity at which picking commences can be determined. This method was used in the present investigation.

The present paper describes the seasonal variation in viscosity index and in adhesive strength of casein obtained from the milk of 6 individual cows.

EXPERIMENTAL

Experimental design

One Friesian, 1 Friesian–Jersey cross and 4 Jersey cows were chosen from an experimental herd of identical twins. The cows had calved between 1 and 8 weeks before the experiment began. Two of the Jersey cows (nos. 95 and 96) comprised a twin pair (Table 1).

For sampling purposes the cows were divided into 3 pairs. As it was not possible to make more than 2 casein preparations in one day, 1 pair of cows was sampled on each of 3 successive days at the beginning of each fortnight (Table 1).

Table 1. *Data from cows supplying milk for trial*

Sampling order	Cow	Breed	Calving date 1965
1	95	Jersey } twin pair	16 July
	96	Jersey } twin pair	22 June
2	23	Jersey	12 July
	133	Jersey	8 August
3	26	Friesian	6 July
	48	Friesian–Jersey	9 August

Preparation of casein

Combined night and morning milk from each cow was separated and a 1-gal sample of the resulting skim-milk was used for preparation of casein.

One gal skim-milk was placed in a water bath maintained at 30–32 °C. The milk was stirred mechanically while 0·5 N-hydrochloric acid was added to bring the pH value to 4·4. The resultant coagulum was then warmed to 51 °C and held for 15 min to complete the curd formation. The whey was drained off and the curd washed 3 times with 4 l distilled water, at 55, 70 and 45 °C, respectively. At each washing the casein was held in the wash water for 15 min. The casein was dried on trays for 3–4 h in a well-ventilated oven, ground to pass a B.S. 30-mesh screen and thoroughly mixed.

Determination of viscosity of casein

Viscosity was determined on a 15% solution of casein in dilute aqueous ammonia by the method of Dolby (1964). Casein (45 g on a moisture-free basis) was dissolved at 60 °C in a mixture of 8 ml 10 N aqueous ammonia and sufficient water to bring the total weight of solution to 300 g. After 30 min stirring at 60 °C, the solution was cooled to 25 °C for a further 30 min and its viscosity then measured with a Ferranti viscometer at a shear rate of 50 s^{-1}.

Reproducibility. Previous work (Dolby, 1961) has shown that provided the casein is precipitated at a pH below 4·6 and thus contains a negligible content of calcium, changes in pH of precipitation or in temperatures and other conditions in the preparation of the casein have a negligible effect on the viscosity index of the product. The standardized conditions of casein preparation should thus yield a representative sample. The standard error of the viscosity determination on a given sample of casein was of the order of ± 1 poise.

Laboratory paper coating

A mixture of 88 ml water, 9 ml 5 % (w/v) sodium hexametaphosphate and 2 ml 1 N-sodium hydroxide was stirred mechanically while 150 g of a spray-dried paper coating clay ('Spray Satin') was slowly added. The clay was dispersed by stirring at high speed for 15 min, after which 135 g of the casein solution from the viscosity determination was added. The dispersion was mixed for a further 15 min. This coating mix contained approximately 45 % solids and had a casein:clay ratio of 0·135.

A sheet of strong 18 lb quarto paper (Wiggins Teape, Abermill Bond) was placed on a 'Bird' vacuum plate with the wire side of the paper uppermost, and held there by applying suction to the plate. The coating mix was applied in the machine direction of the paper by means of a 'Bird' applicator to give a coating with a wet thickness of 0·038 mm (0·0015 in.). Sheets of paper coated in this manner were then allowed to dry for several hours on a flat surface.

Adhesive strength of casein in paper coatings

Each sheet of paper to be examined for adhesive or 'pick' strength was cut into 5 strips measuring 2·5 × 25 cm, which were placed in a testing room maintained at 20·5 ± 1 °C and 55 ± 3 % relative humidity for at least 24 h before testing.

In the determination of the 'pick' strength of the coated papers, 'normal' pick test oil of known viscosity (supplied by the Institute for Graphic and Allied Industries, Amsterdam) was used at a pressure of 35 kg with both the pendulum and spring drive (speed A) according to the velocity range required to produce picking of the coating.

The point at which picking of the coating began on each strip of paper was measured in order to determine the pick velocity, i.e. the velocity of the strip at the point where picking began. The product of the viscosity (in poises) of the pick test oil (data supplied by the manufacturers of the instrument) and the pick velocity (in cm/s) of the sheet of coated paper, expressed in viscosity-velocity units (v.v.u.), was a measure of the adhesive strength of the coating applied to that particular paper and therefore of the casein in that coating under the conditions of the test. Standard error of the variation was of the order of ± 5 v.v.u. or ± 5 %.

RESULTS AND DISCUSSION

Viscosity

The viscosity of casein samples from the 6 cows all showed a general decline from August to May (Fig. 1), as was earlier shown by Dolby (1964). The twin Jersey cows 95 and 96 gave very similar graphs which showed a number of simultaneous rises or falls on the same sampling dates. Short-term changes with the other cows were more irregular but all samples taken in early December tended to have low viscosity values. Variations with cow 26 were less pronounced but the proportionate decline over the season was similar.

The values for viscosity index of caseins from 5 cows were rather higher than those reported for commercial casein (Dolby, 1964), while those for the 6th cow (no. 26) were very much lower, approximately one-third of the mean value of the caseins from the other cows.

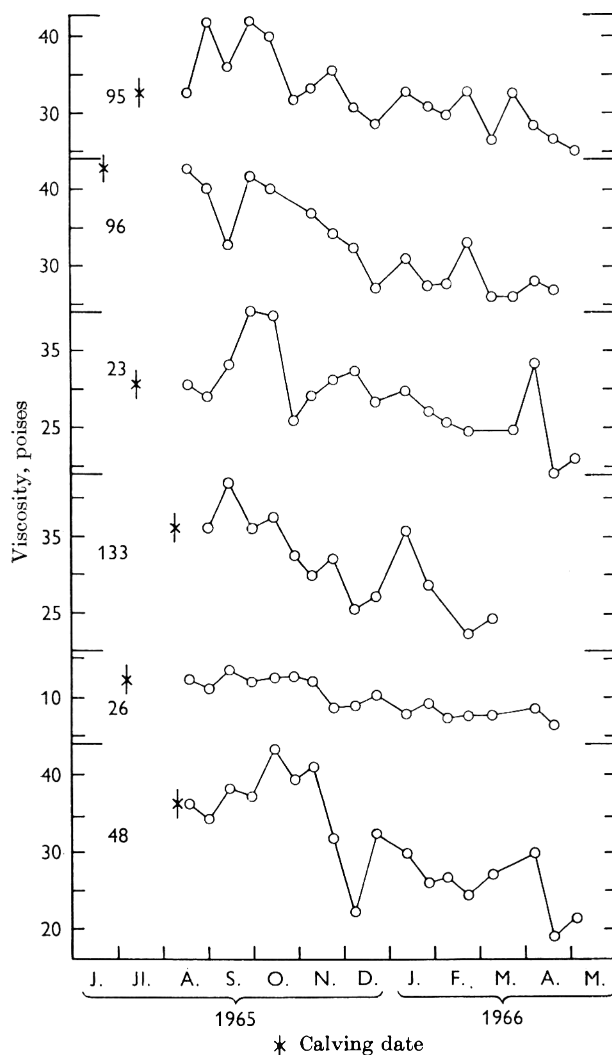

Fig. 1. Variation in the viscosity index of caseins from individual cows during a lactation. Values (in poises) are for a 15 % (w/w) solution of casein in ammonia, measured at a shear rate of 50 s^{-1} at 25 °C.

Table 2. *Genetic variants of α_{s1}-casein from the milk of individual cows*

Cow	Breed	Genetic variants A	B	C
96	Jersey	.	+	+
23	Jersey	.	+	.
48	Jersey–Friesian	.	+	.
26	Friesian } twin pair	+	+	.
25	Friesian } twin pair	+	+	.

The consistently low viscosity of the casein from the milk of cow 26 prompted investigation of the casein from its identical twin, cow 25. This, too, was of similar low viscosity. Electrophoretic examination showed the presence in these low viscosity caseins of the α_{s1}-A genetic variant (Table 2), that has been shown to be present in a

blood line of Holstein (Friesian) cows (Thompson, Kiddy, Pepper & Zittle, 1962). This variant was not present in the milk of cows 96, 23 and 48, which were examined at the same time for comparison. A sample of casein from cow 26 was submitted to Dr M. P. Thompson of Eastern Utilization Research and Development Division, U.S. Department of Agriculture, who confirmed the presence of this genetic variant by electrophoretic comparison with an authentic sample of α_{s1}-A casein.

This finding implies that α_{s1}-A casein has a lower viscosity, presumably through forming smaller aggregates in solution.

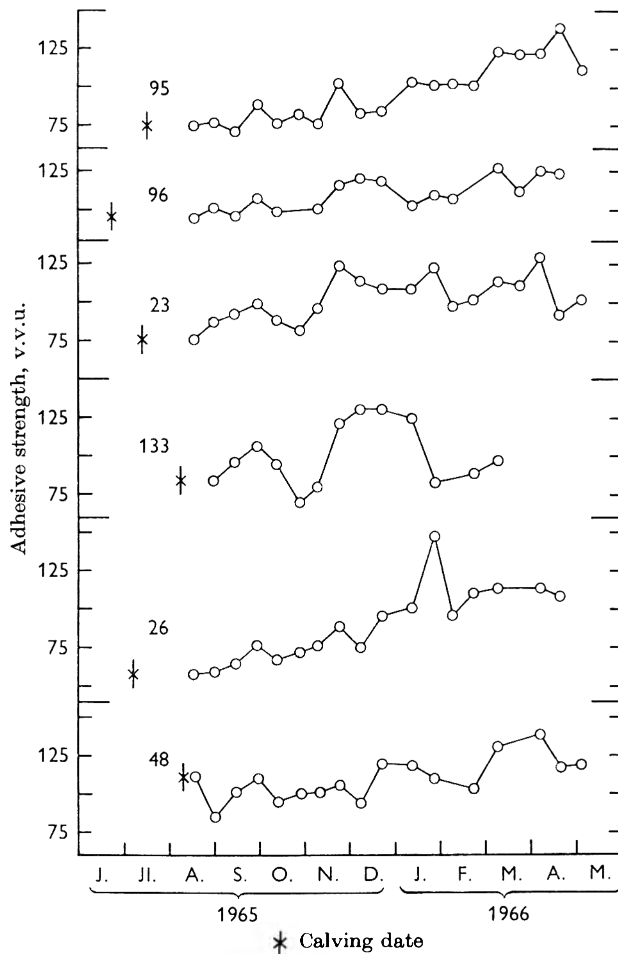

Fig. 2. Variation in the adhesive strength of caseins from individual cows during a lactation. Values (in v.v.u.: kilopoise, cm/s) are expressed in terms of the product of the viscosity of the pick test oil and the pick velocity of paper (coated with a mixture of clay and casein) at 20·5 °C and 55 % R.H.

Adhesive strength

Figure 2 shows the variation during lactation in the adhesive strength of caseins from the milk of individual cows. Each value is the mean of 5 separate determinations. For 5 of the cows the adhesive strength showed a gradual increase from August to May. For the remaining cow, no. 133, the values showed no such clear trend, and

produced a rather indeterminate pattern, which proved reproducible with a further set of test papers. This pattern cannot readily be interpreted. The mean values for the adhesive strength of casein from cow 26 are slightly lower than the corresponding values for the other cows, although the adhesive strength: viscosity ratio for this casein is still much greater than the ratios for any of the other caseins examined.

The combined 'seasonal' decline in viscosity and increase in adhesive strength resulted in a small but steady increase in adhesive power: viscosity ratio through the season for 5 of the cows and a steeper increase for the 6th cow (no. 26). The paper-coating properties of the caseins therefore improved as the season progressed.

The question now arises, whether this variation is seasonal and dependent on pasture growth and consequently on the weather, or whether it is lactational. This cannot be answered directly from the data available at present as the calving dates of the experimental cows had a range of only 7 weeks. The steady overall variations in the 2 properties of casein investigated do not follow any pattern that can be associated with a spring–summer–autumn weather cycle. It seems likely, therefore, that the steady decline in viscosity and increase in adhesive power are due to lactational rather than seasonal changes.

The authors are grateful to Mr G. F. Wilson for providing samples of milk, to Dr L. K. Creamer for the electrophoretic analysis of the casein samples and to Miss E. A. Pinfold for skilled technical assistance. The assistance of Dr M. P. Thompson in confirming the presence of α_{s1}-A casein is gratefully acknowledged.

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Factors affecting the viscosity of caseinates in dispersions of high concentrations

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SUMMARY. The physical effects of various cations in caseinate dispersions of high concentrations were investigated over a range of temperature and pH.

With calcium and strontium the temperature–viscosity relationships of the caseinates were abnormal in that the viscosity decreased rapidly from 30 to about 40 °C and a gel formed at temperatures in the region of 50–60 °C. On cooling, the gel reliquefied. No gel formed with barium, aluminium or magnesium. On cooling, magnesium preparations separated into 2 phases.

The supernatant phase from the magnesium caseinate and a corresponding phase prepared by centrifuging the calcium caseinate showed depletion of α-casein and enrichment of κ-casein and β-casein. The supernatant phase from the calcium caseinate showed the reversible gel formation on heating. The magnesium supernatant phase did not. κ-Casein and a mixture of κ- and β-caseins gave reversible gels at similar levels of calcium and pH.

For reversible gel formation to occur, calcium caseinate was required to be in fairly high concentration, to have a calcium content of about 1·0 % of the protein and to be within the pH limits 5·2–6·0. The temperature at which gelation occurred was affected by the concentration of calcium and protein and by pH.

The behaviour of the material was compared with that of methyl cellulose with and without addition of urea.

Some potential commercial applications of the findings on viscosity relationships are outlined.

INTRODUCTION

In many industrial applications of casein the high viscosity of concentrated casein dispersions at neutral or alkaline pH values presents difficulties. For example, in paper coating this high viscosity restricts the concentration of total solids that can be used in the coating mixture. In spray-drying of caseinates the viscosity limits the concentration and so increases the cost of drying.

In an earlier investigation (Hayes & Muller, 1961) it was noted that as the pH of a concentrated caseinate solution was reduced below 6·0, the viscosity dropped sharply when there was sufficient calcium present. It was, therefore, decided to investigate in more detail the effects of varying calcium concentrations and pH in caseinates. in order to produce more concentrated dispersions of low viscosity. In the course of

this investigation an interesting phenomenon of a reversible gel formation was observed in calcium caseinate solutions of low pH. The investigation was therefore extended to explore this phenomenon in more detail, and to compare the behaviour of calcium with that of other metallic ions. The 2 phases of the work are reported separately below.

METHODS

Dispersion technique

In preliminary trials it was found difficult when normal stirring techniques were used to prepare stable dispersions of caseinates in the presence of calcium at pH values below about 6·0. Thewlis (1961) had used high shearing forces to prepare graft polymers of glycerol and casein. Tarassuk & Palmer (1939) used homogenization at 3000 p.s.i. at 160 °F in preparing calcium caseinate. These approaches suggested the need for the application of considerable mechanical energy to disperse the casein. A successful laboratory technique resulted from the use of an Ultra-Turrax stirrer (Janke & Kunkel, K. G., Staufen, Germany) which applied high energy with a shearing action. On pilot and commercial scale a colloid mill was found satisfactory. For a typical 900 g preparation the dry casein was added to water at room temperature with the salt of the appropriate metallic ion, and the preparation stirred with the Ultra-Turrax for several minutes. The energy from the stirring caused the temperature to rise and the casein to take on a flocculated appearance. At this stage any sodium hydroxide solution required for pH adjustment was added gradually with constant stirring. At pH values between 5·2 and 6·2 where the casein was dispersed in a calcium hydroxide solution, sodium hydroxide was often not needed to obtain the desired pH. Stirring was continued until the casein had dispersed and a temperature of 70 °C was reached.

A similar technique was found suitable in preparing methyl cellulose solutions.

A single batch of high-quality commercial hydrochloric acid casein was used throughout the studies, except for occasional confirmation of results with other batches of casein.

The concentrations given in this paper refer to commercial casein. As the sample contained 10 % moisture the protein concentration would be approximately 90 % of the figures given.

Analytical methods

Viscosity determinations were made with a 'Rotovisko' coaxial cylinder viscometer (Gebrüder Haake, K. G., Berlin) which included facilities for control of sample temperature by means of a water jacket. A shear rate of 50^{-1} s was usually employed but higher shear rates were sometimes used to obtain readings with viscous samples, a procedure which slightly altered the absolute viscosities but gave the required information concerning the shape of the temperature–viscosity curves.

Light transmission-temperature curves were obtained using a Beckman D.K. Spectrophotometer in a 2·0 mm cell at a wavelength of 600 mμ.

Calcium was determined by the method of Sawyer & Hayes (1961), magnesium by the method of Hildebrand & Reilley (1957), protein from micro-Kjeldahl nitrogens using a factor of 6·38 and sialic acid by the method of Warren (1959). Starch gel electrophoresis was carried out by the procedure of Wake & Baldwin (1961). Centri-

fuging was done in a Servall refrigerated centrifuge at 15000 rev/min for 30 min at 35 °C with head no. S.S. 34.

Preparation of casein fractions

The α_s-, β- and κ-fractions of casein were prepared from skim-milk. The κ-casein was prepared by the method of Hill & Hansen (1963) but without a final chromatographic separation. The α_s- and β-caseins were obtained by repeated fractionation of the remaining material from the κ-casein separation, taking advantage of the difference in solubility at 3 °C of α_s- and β-caseins in the presence of 0·2 M calcium (cf. Waugh & von Hippel, 1956). The α_s- and κ-fractions appeared fairly pure electrophoretically although, in the absence of a chromatographic separation, a small proportion of β-casein would be expected in the κ-fraction. The β-fraction was slightly contaminated with α_s.

I. STUDIES ON CALCIUM CASEINATE

Results

Viscosity–temperature relationships

Kruyt (1952) pointed out that the temperature–viscosity relationship of a colloidal solution may be linear when viscosity is plotted on a logarithmic scale against

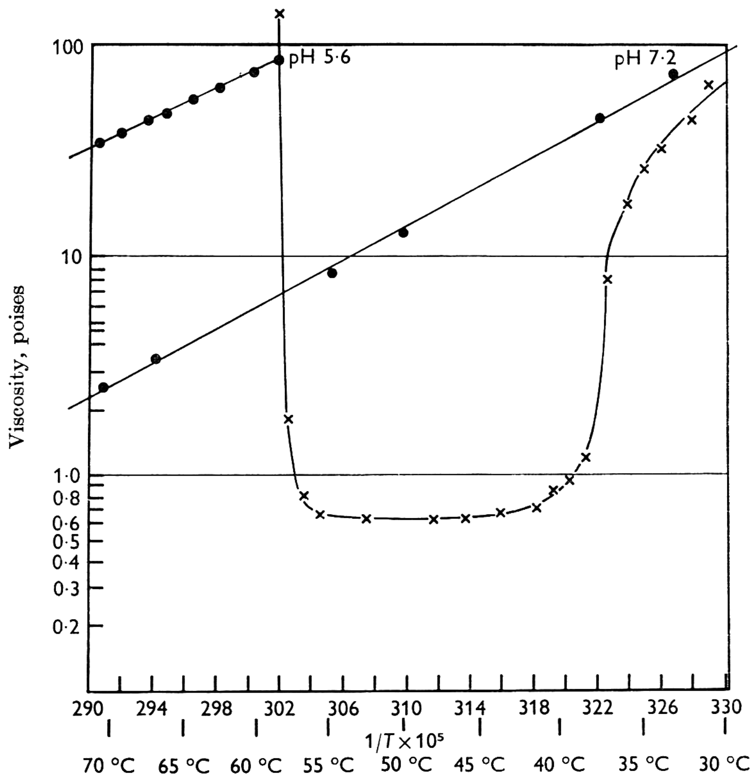

Fig. 1. Temperature–viscosity relationship of 20 % caseinate preparations. ●, Sodium caseinate at pH 5·6 and 7·2; ×, calcium caseinate with 1 % calcium at pH 5·4.

the reciprocal of the absolute temperature. This was shown (Hayes & Muller, 1961) to apply to solutions of sodium caseinate over a concentration range of 11–20 %.

Figure 1 shows that this straight-line relationship applied for 20% sodium caseinate at pH 5·6 and 7·2. However, the results at pH 5·4 for the 20 % solutions of calcium caseinate with 1 % added calcium showed a considerable curvature; the viscosity decreased sharply as the temperature was raised from 30 to 38 °C and then remained fairly constant until 57 °C, when a gel formed very rapidly.

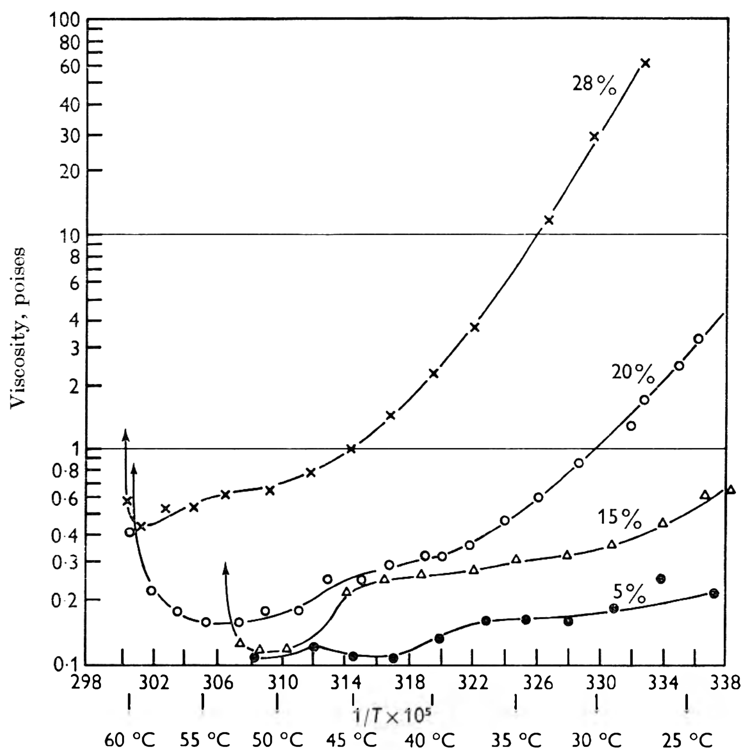

Fig. 2. Effect of casein concentration on temperature–viscosity relationships of calcium caseinate with 2·0 % calcium; pH, approximately 5·4. Casein concentration, %: ●, 5; △, 15; ○, 20; ×, 28.

On cooling, the gel redispersed and could be reformed at the same temperature during successive heating and cooling cycles. It was noted that some preparations of this type when first prepared did not show the gelation phenomenon. Cooling to room temperature and reheating, or storage for some time at refrigeration temperatures, gave gel formation on subsequent heating. Incomplete hydration of the protein in the freshly made preparations may have been responsible.

Effect of casein concentration

The effect of varying the concentration of casein and calcium so as to maintain the calcium content at 2·0 % of the casein is illustrated in Fig. 2. The composition of the solutions is set out in Table 1.

The curves in Fig. 2 show increasing gradient with casein concentrations from 5 to

28 %—the highest concentration found possible. Earlier studies with sodium caseinate at concentrations from 11 to 20 % showed only very slight change in gradient.

The gelation phenomenon was shown by the 15, 20 and 28 % dispersions. The exact temperature at which gelation occurred was difficult to determine in the visco-

Table 1. *Effect of concentration of casein containing* 2·0 % *calcium on gelation temperatures*

Casein, %	Coagulation temperature, °C	pH value
28	60	5·5
20	57	5·3
15	54	5·3
5	43	5·3

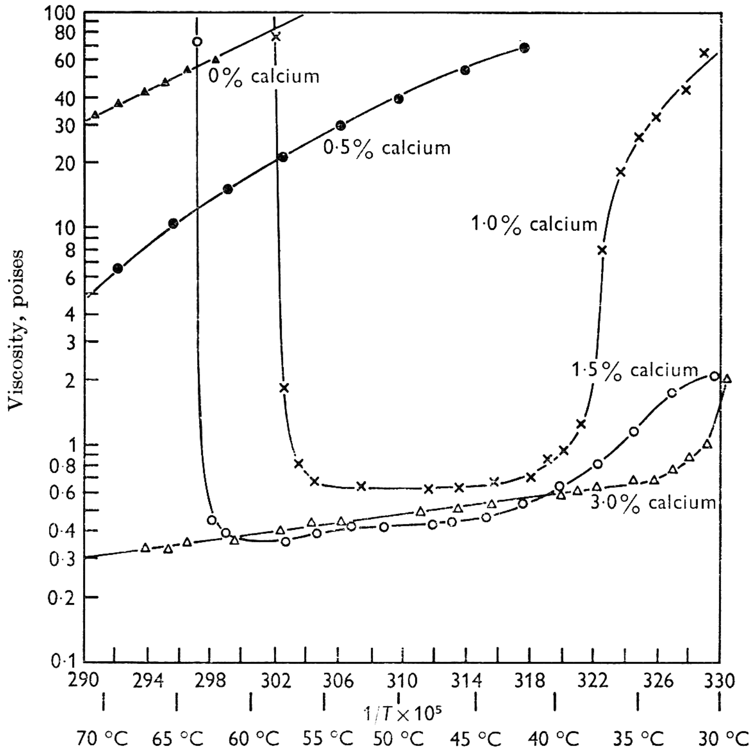

Fig. 3. Effect of increasing calcium content on temperature–viscosity relationships of 20 % caseinate preparations at pH approximately 5·4. Calcium concentration, %: ▲, 0; ●, 0·5; ×, 1·0; ○, 1·5; △, 3·0.

meter because of slippage at this point. The use of profiled rotors failed to increase the accuracy. However, visual observations of the coagulation point such as were made to obtain the results given in Table 1 confirmed the information from viscometry that the temperature at which gelation occurred increased with increasing concentration of protein.

At 5 % casein concentration a precipitate formed rather than a gel. There was apparently insufficient protein to immobilize all the water. A similar result was

observed by Zittle, DellaMonica & Custer (1956), who, on heating a 2 % caseinate preparation containing 0·012 M calcium (2·4 % of the casein), obtained a precipitate which redissolved on cooling.

Effect of calcium content

Figure 3 shows the change in the viscosity–temperature relationship of a 20 % sodium caseinate preparation at pH 5·6 when calcium was added. With 0·5 % calcium, the viscosity was lower than when no calcium was present but the relationship was

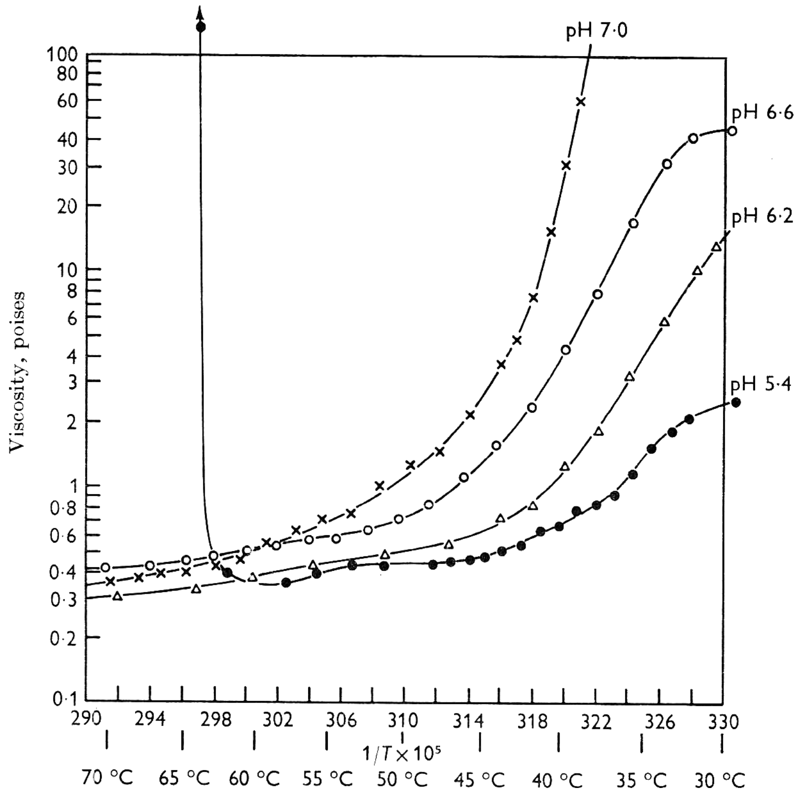

Fig. 4. Temperature–viscosity relationships for 20 % calcium caseinate preparations (1·5 % calcium) at varying pH values. pH value: ●, 5·4; △, 6·2; ○, 6·6; ×, 7·0.

still virtually a straight line. With 1·0 % calcium the sharply curved gel-forming system appeared. With 1·5 and 2·0 % calcium (the latter curve is not shown since it almost coincided with that for 1·5 %) the preparations showed a much smaller gradient from 30 °C until they formed a gel at a temperature somewhat higher than for the 1·0 % preparation. At the 3 and 4 % calcium levels the preparations were of very low viscosity and showed a straight-line temperature–viscosity relationship up to at least 70 °C. These high-calcium preparations settled out slowly on standing.

The effect of calcium level on the gelation temperature was observed visually in a series of 20 % caseinate preparations at about pH 5·4. The calcium levels as a percentage of the casein, and the corresponding gelation temperatures, were: 0·5 %, no

gel formed; 1·0 %, 57°; 1·5 %, 65°; 2·0 %, 75°; 2·5 %, 80°; 3·0 %, 90°, and 3·5 %, 100 °C.

The results clearly showed that increasing the calcium percentage increased the gelation temperature. It appeared that about 0·8 % calcium was the minimum necessary to induce gelation on heating.

Effect of pH

Figure 4 illustrates the effect of varying the pH in preparations with 20 % casein and 1·5 % calcium. The gradient of the viscosity curves between 30 and 45 °C became steeper as the pH increased. At the higher temperatures, the curves for pH values above 6·0 showed practically a straight-line relationship of gentle gradient. At pH values above about 6·0 gels did not form at temperatures up to 100 °C. Through observations in sealed McCartney bottles it was found that at 120 °C a coagulum which was not reversible formed, in 3 min at pH 6·2 and in 6 min at pH 6·6. At pH 7·0 the material had not coagulated after 18 min at 120 °C.

Table 2. *Effect of* pH *on gelation temperature of* 20 % *caseinates with* 1·5 % *calcium*

pH value	Gelation temperature, °C
5·4	65–70
5·55	75
5·63	80
5·70	85
5·85	90

Because of the difficulty, caused by slippage, in accurately determining gelation points with the viscometer, more detailed information was sought through visual observation in the pH range 5·4–5·85 (Table 2).

These results illustrated the rise in gelation temperature with small increases in pH and confirmed the observations made with the viscometer. It was observed under the phase-contrast microscope that, in general, the aggregates became smaller and less numerous as the pH was raised towards 7·0.

The shape of the viscosity–temperature curves appeared to be little affected by the use of ammonium hydroxide instead of sodium hydroxide for adjustment of pH. The use of calcium hydroxide, calcium chloride or calcium lactate as the source of calcium did not alter the shape of the curves, but calcium oxide gave a sharper minimum in the viscosity curve.

Practical applications

The results outlined above indicated the range of concentration, temperature, pH and calcium levels at which it would be possible to prepare dispersions of calcium caseinate with viscosities permitting their handling in industrial equipment. Use was made of this information in commercial-scale trial manufacture of spray-dried calcium caseinate.

In these trials, lime and water added to fresh wet casein curd gave a pH of 6·5 at

concentrations of about 20 % protein and 1·3 % calcium. This material spray-dried satisfactorily.

The low viscosity and the formation of a gel on heating with calcium caseinate dispersions of low pH were considered to have potential usefulness in paper-coating. Recent patent applications such as that of Nakajima & Shimizu (1962) suggested that casein-based coating colours at acid pH values would be practical.

Laboratory tests were made with paper-coating colours at 55 % solids in which calcium caseinate at pH 5·8 was used as the adhesive in the ratio of 15 g casein : 100 g clay. The coated test sheets of paper showed a fairly high gloss and, as judged by 'wax pick' tests of 8–10, had fairly good adhesive strength.

II. STUDIES OF THE HIGH-TEMPERATURE GELATION

The formation of a gel at high temperatures which redisperses on cooling is an unusual phenomenon. To gain further information studies were undertaken on the effects of metallic ions other than calcium and on the role of the major casein fractions. The behaviour of calcium caseinate was also examined in relation to that of other gelling systems.

Table 3. *Magnesium levels and* pH *values used in experiments on magnesium caseinates*

Trial no.	Molarity of magnesium in water	Magnesium as percentage of casein	pH value
1	0·05	0·31	5·70
2	0·09	0·60	5·80
3	0·14	0·92	5·80
4	0·19	1·20	5·75
5	0·51	3·10	5·75

Low pH *caseinates with other cations*

Preparations with barium chloride or aluminium ammonium sulphate showed no resemblance in their temperature–viscosity relationships to those of the calcium caseinates of the same molarity.

Preparations with strontium chloride, on the other hand, behaved very similarly to the calcium caseinates. The strontium caseinates showed reversible gelation on heating although the temperature–viscosity curves did not appear to be as reproducible as those for calcium caseinates.

Magnesium, as magnesium chloride, was added to 15 % caseinate dispersions at the concentrations and pH values shown in Table 3. The pH was adjusted with sodium hydroxide.

At magnesium concentrations of 0·31, 0·60 and 0·92 % the preparations gave temperature–viscosity curves which showed only a small gradient in the region from 10 to 4 poise. No gel formed, although there was a tendency for a small increase in viscosity at 55 °C with 0·60 % magnesium and at 48 °C with 0·92 %. In both instances, the viscosity decreased to normal levels as the temperature rose further. Similar results were obtained at pH values down to 5·3. At 1·20 and 3·10 % magnesium, the

systems appeared fairly normal and homogeneous at the temperature of 70 °C which was reached during stirring in the course of their preparation (p. 32). However, when allowed to cool they separated into 2 phases. The supernatant phase so formed was very low in viscosity and contained approximately 20 % of the original protein. The lower phase was a very viscous liquid which showed a fairly straight temperature–viscosity curve similar to that of sodium caseinate.

The temperature–viscosity curve of the supernatant phase was similar to that of sodium caseinate except for a slightly steeper gradient at low temperatures and a tendency to curve upwards as the temperature rose. No gel was formed.

Microscopic examination of the supernatant phase showed the presence of numerous micelles of diameter up to about 0·6 μ.

Comparison of calcium and magnesium caseinates

To compare with the components separating from the magnesium caseinate preparations, sediments and supernatant liquids were prepared from a number of calcium caseinates in the Servall centrifuge. The proportion of the original protein in the supernatant phase was higher in the more dilute caseinate preparations and varied from about 36 % in a 10 % caseinate to about 20 % in a 25 % preparation. The super-

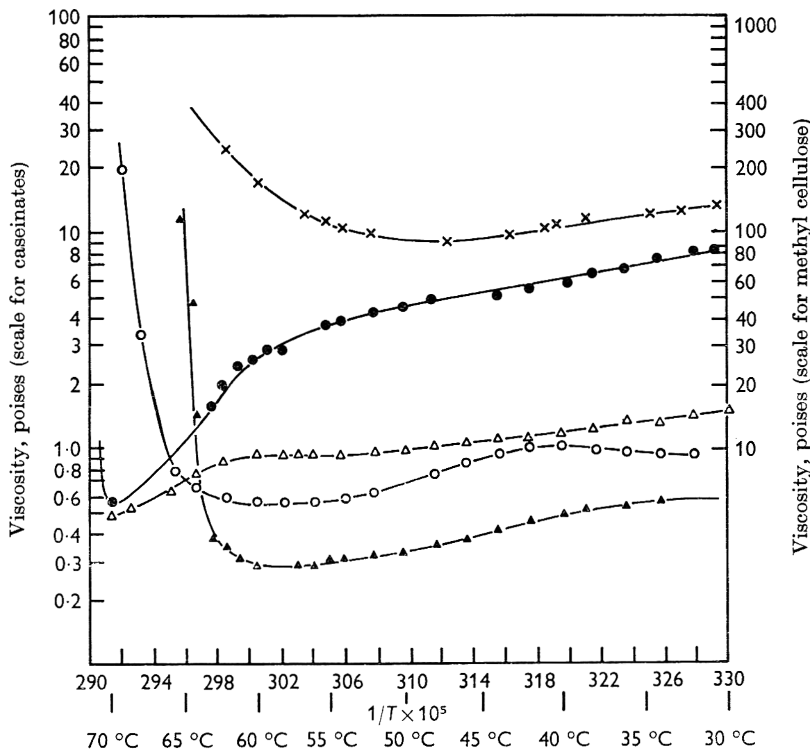

Fig. 5. Comparison of temperature–viscosity relationships of methyl cellulose solutions and supernatant liquids from calcium caseinate preparations. ×, Methyl cellulose 3·0 %, ●, methyl cellulose 3·0 % in 4 M urea; △, supernatant liquid from 25 % calcium caseinate in 4 M urea; ○, supernatant liquid from 25 % calcium caseinate (8·0 % protein); ▲, supernatant liquid from 20 % calcium caseinate (7·0 % protein).

natant liquids were almost clear but showed fairly numerous micelles of maximum size 2·3 μ when examined under the phase-contrast microscope.

Figure 5 shows temperature–viscosity curves for supernatant liquids with 7 % protein from a 20 % preparation of calcium caseinate, and with 8 % protein from a 25 % preparation. It will be seen that these supernatant liquids exhibited the gelation phenomenon even at these fairly low protein concentrations. From comparison with the curve for a 5 % calcium caseinate (Fig. 2) it is apparent that the supernatant liquids were of low viscosity. The gradient of the curve for the supernatant liquid with 7 % protein was much flatter than that for the original 20 % calcium caseinate shown in Fig. 2, suggesting that aggregation of the components from the sediment may have been the main factor in the sharp decrease of viscosity in the complete system.

Light-transmission–temperature curves for the 7 % protein supernatant liquid (Fig. 6) show clearly that the gelation occurred on heating and that it was reversible on cooling. The reversion occurred in 2–3 min.

Role of casein fractions

Sialic acid determinations were made on preparations of both calcium and magnesium caseinates and on their supernatant liquids and sediments. The sialic acid levels, expressed as a percentage of the protein, were similar in the calcium and magnesium preparations. Typical figures were: original material, 0·19 %; sediment, 0·12 %; supernatant, 0·43 %.

The enrichment in sialic acid of the supernatant phase and the depletion of the sediment suggested concentration of κ-casein in the supernatant liquid. Starch gel electrophoresis showed that the supernatant liquid was depleted in α-casein and that β-casein represented a greater proportion of the stainable material than was present in the original preparation.

The finding that the supernatant liquids were enriched in κ- and β-casein suggested that these may be the fractions primarily involved in the gel formation. Preliminary studies on the temperature–gelation behaviour of α_s-, β- and κ-caseins, alone and in admixture, were inconclusive when ordinary laboratory stirrers were used for preparations. Using a micro Ultra-Turrax stirrer, which permitted high-energy stirring of small volumes, reproducible results were obtained. The fractions prepared were not dialysed to remove calcium as the levels present—in α_s, 1·8 %; in β, 1·8 %; and in κ, 1·3 %—were in the range desired for observation of the gelation phenomenon. Some checks were made, however, on dialysed samples with added calcium, when similar results were obtained.

α_s Fraction

A 20 % preparation of α_s fraction at pH 6·2 was almost impossible to disperse but showed a strong tendency to form sticky masses. When the pH was reduced to 5·4 a dispersion of highly aggregated particles was obtained. This tended to revert to sticky masses as the temperature was raised to about 50 °C.

α_s–κ Mixture

A 20 % preparation of α_s and κ fractions mixed in the ratio of approximately 4:1 readily gave a white, low-viscosity dispersion at pH 5·3 which showed no tendency to gel up to 85 °C, and no stickiness. Some stickiness was noticeable at pH 5·9 and 85 °C. At pH 6·9 the preparation was very viscous at room temperature but showed a rapid decrease in viscosity as the temperature rose. This behaviour was similar to that of whole casein preparations at this level of calcium but at lower pH.

β Fraction

β-Casein at 20 % concentration dispersed readily at pH 5·3, and as the temperature rose the preparation became quite sticky and fibrous. At about 40 °C it formed a gummy mass in a clear liquid. The β-casein would not disperse at pH 6·8.

β–κ Mixture

A 20 % mixture of β- and κ-caseins in the ratio of approximately 4:1 dispersed readily at pH 5·6 and gelled at 50 °C. The gel redispersed on cooling. When more κ-casein was added to give a ratio of 2:1 the preparation gelled at a lower temperature.

κ Fraction

At 20 % concentration and pH 7·0 the κ-casein preparation was too viscous for pH adjustment and had to be diluted to approximately 10 %. At pH 5·4 it dispersed well and gelled when the temperature reached 30 °C. In this preparation the calcium was 0·03 M. When diluted with water to give 5 % κ-casein in 0·016 M calcium no gel formed. When diluted in 0·1 M calcium chloride to give 5 % κ-casein in 0·06 M calcium, a precipitate formed at 40 °C which redispersed on cooling. A preparation with 5 % κ-casein in 0·5-M calcium did not form a gel or precipitate even on gentle boiling.

It thus appears that κ-casein was the principal casein component involved in the gelation phenomenon. The gelation phenomenon was given by the κ-casein preparation on its own or in admixture with β-casein. The behaviour of α_s- and mixed α_s- and κ-caseins suggests that α_s-casein was not involved in the gelation. These findings are in accordance with expectations from the results with the supernatant liquids.

Comparable gelling systems

In an effort to obtain information concerning the nature of the interactions leading to the formation of reversible gels, the literature was searched for examples of systems in which viscosity increased on heating and reversible gels formed. Very few such examples were found. The viscosity of liquid sulphur increases with temperature (van Wazer, 1966), the increase being ascribed to the formation of a chain or ring sulphur at elevated temperatures. Bence-Jones proteins, according to Neet & Putnam (1966), precipitate at 45–55 °C and disperse again at higher temperatures. Murayama (1957) reported reversible gelation of sickle-cell haemoglobin with increasing temperature.

The behaviour of methyl cellulose (Young & Kin, 1946) appears to resemble more closely that of calcium caseinates. In Fig. 5 the temperature–viscosity relationships of 3 % aqueous methyl cellulose solution are shown. While the level of viscosity for

methyl cellulose is much higher, there is a general similarity in the shape of the curves to those for supernatant liquids from calcium caseinates. The similarity is also seen in the light-transmission–temperature curve in Fig. 6 which shows that the gelation on heating and its reversibility on cooling followed a similar pattern to those of the supernatant liquid from calcium caseinate.

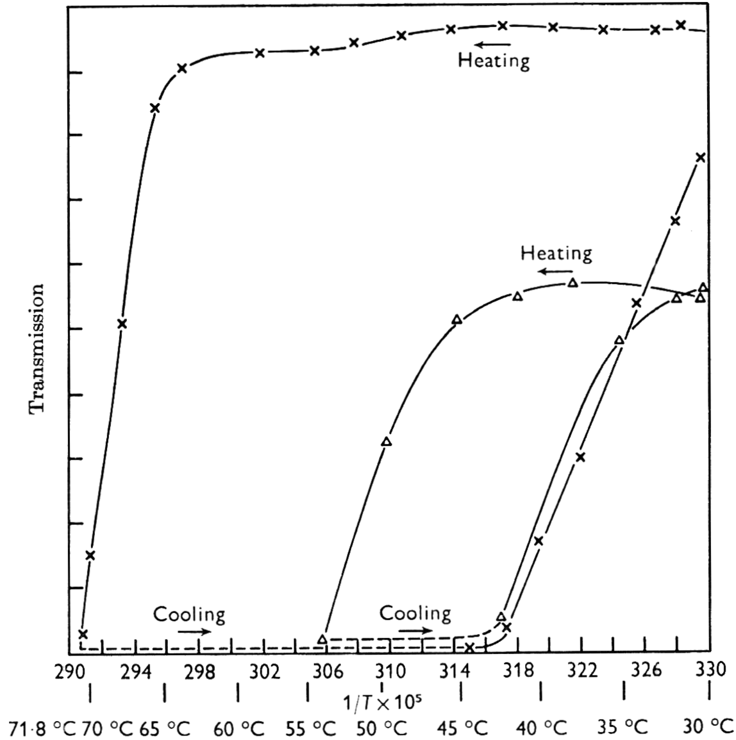

Fig. 6. Light-transmission–temperature relationships of methyl cellulose and supernatant liquid from calcium caseinate preparations. ×, 3 % Methyl cellulose; △, supernatant liquid from 20 % calcium caseinate (7 % protein).

Also shown in Fig. 5 are curves for methyl cellulose and the caseinate supernatant liquid in the presence of 4 M urea. It will be seen that gelation was suppressed for both. The methyl cellulose did, however, show some viscosity increase at 74 °C. The effect of lower concentrations of urea on the complete system of 20 % caseinate with 2 % calcium is shown in Fig. 7. It can be seen that, at lower temperatures, urea additions up to 0·8 M increased the viscosity of the preparation. As the temperature increased over about 50 °C the curves apparently crossed as increasing urea concentration increased the gelation temperature. With 1·2-M urea the trend was the same—higher viscosity at the lower temperatures and no gel formation up to 80 °C.

Nature of bonding in gel

A number of authors have suggested that hydrophobic bonding is a factor in aggregation of proteins. These authors include Kenkara & Hansen (1967) with κ-casein, Payens (1966) in a paper on the association of caseins, Neet & Putnam (1966)

on Bence-Jones protein and Murayama (1957, 1966) working with sickle-cell haemoglobin. However, little experimental evidence that hydrophobic bonding is involved appears to have been published. An exception may be the studies of Murayama (1966), who showed that the sickling was prevented by the presence of propane.

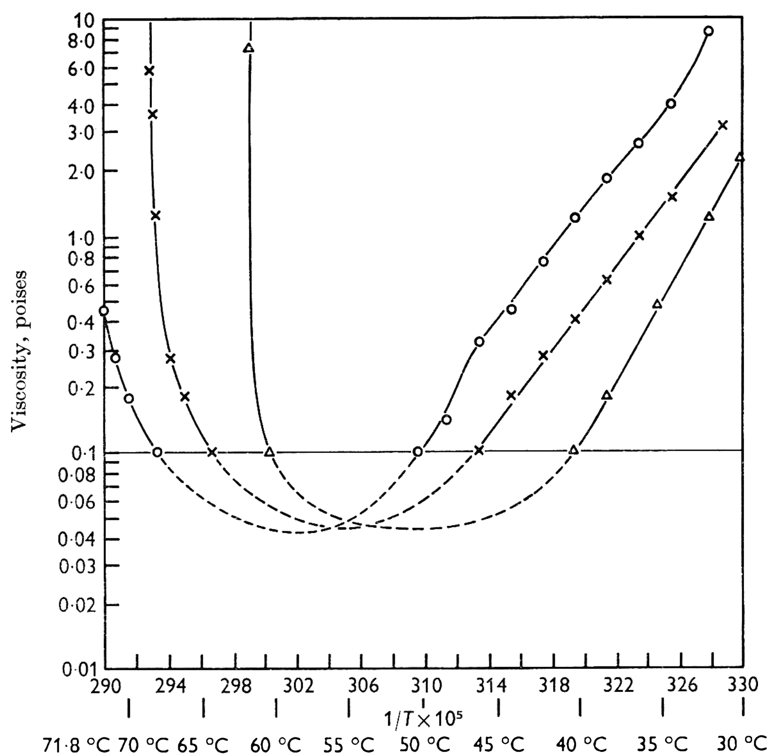

Fig. 7. Temperature–viscosity relationships of 20 % casein solutions containing 2 % calcium with varying concentrations of urea. △, Control; ×, 0·4 M urea; ○, 0·8 M urea.

In the present studies an attempt was made to demonstrate whether hydrophobic bonding is involved in the gel formation. The addition of pentane up to 0·3 ml/10 g of protein to a 20 % calcium caseinate at pH 5·6 had no effect on the gelation temperature. When 0·8 ml/10 g was reached the gelation temperature dropped from 60 to approximately 54 °C. The exact temperature was difficult to determine because of some foam formation. At similar levels of addition pentane had no effect on the supernatant liquid from the calcium caseinate preparation. There was an apparent drop to 54 °C in the gelation temperature of the supernatant liquid after 2·5 ml/10 g protein had been added but the production of a considerable amount of foam during stirring made it difficult to be certain of the occurrence of gelation. On removal of pentane under reduced pressure, the gelation temperature increased, to 65 °C. Similar results were obtained when hexane was added instead of pentane.

DISCUSSION

Methyl cellulose analogy

The general similarity of the temperature–viscosity relationships of the concentrated calcium caseinates and of methyl cellulose suggests that similar mechanisms may be involved. Young & Kin (1946) consider that, on increasing the temperature, dehydration brings the methyl cellulose chains into closer relationship with each other so that forces of attraction between them are increased. If there is a sufficient concentration of methyl cellulose a gel structure results.

However, there are some notable differences in behaviour between methyl cellulose and the calcium caseinates:

(1) Concentration has opposite effects—Young & Kin (1946) showed that the gelation temperature decreases as the methyl cellulose concentration increases; with the calcium caseinates the gelation temperature increases as the concentration increases.

(2) The rate of change in viscosity on approaching the gelation point is more gradual with methyl cellulose.

(3) Stirring of methyl cellulose solutions near the gelation point could destroy the gel structure, whereas fairly strong stirring of calcium caseinate preparations could cause the gel to form at 2–3 °C below the normal temperature.

These differences can possibly be explained in terms of the distribution of sites for the bonds or forces of attraction responsible for the gel. These sites on casein are probably of relatively infrequent occurrence and a conformational change may be necessary to bring them into positions where the interactions can give rise to gels. The effect of stirring suggests that a conformational change may be involved—for instance a stretching or elongation of spherical aggregates. This may be interfered with by increasing casein concentration so leading to higher gelation temperatures.

With methyl cellulose it seems likely that the reactive sites would be more frequent throughout the molecule. The effect of stirring indicates that the bonds which are involved in gel formation are weaker than those involved in the casein gels and are probably different.

Effect of urea

The effect of urea in high concentration in suppressing gelation could suggest that hydrogen bonding is involved, but as Tanford (1962), Wetlaufer, Malik, Stoller & Coffin (1964) and others have pointed out, urea can also act as a hydrophobic bond-breaker. The effects of urea in increasing the viscosity of calcium caseinate preparations at temperatures between 30 and 50 °C may be explained in terms of suppressing hydrogen-bonding as part of the aggregation mechanism at these temperatures. The crossing of the curves as the temperature rises above 50 °C and hydrogen bonds become less important, and the fact that as the urea concentration increases the gelation temperature is increased suggests that at the higher temperatures urea is acting to break hydrophobic bonds.

The known temperature patterns of hydrophobic bonding (Scheraga, 1965) correspond well with the range of temperatures of gelation of calcium caseinate preparations. The lack of positive effect of pentane and hexane on gelation may be taken to

suggest that hydrophobic bonding is not important, but the results cannot be regarded as conclusive because of the difficulty of incorporating the hydrocarbons and the uncertainty surrounding their mode of action in these highly concentrated protein systems.

Ionic bonding

The observation that reversible gel formation occurs only with calcium and strontium—cations of similar size—and not with the dissimilar barium, aluminium and magnesium, suggests that a steric factor may be important. Gel formation may involve the formation of bridges which bring the protein molecules or aggregates close enough together to favour the formation of other linkages, or it may be associated with the possible conformational change suggested by the effects of stirring.

A concentration of between 0·5 and 1·0 % calcium (0·03–0·06 M) has been shown necessary to induce gelation on heating a 20 % caseinate preparation. The amount of calcium needed to induce gelation at pH 5·4 is similar to the amount of calcium found by Zittle (1957) to be bound to the casein near this pH. As the calcium concentration is raised above this level the gelation temperature increases until at 3 % calcium (0·185 M) no gel forms.

Casein fractions

The observations on the distribution of casein fractions between the centrifugally separated phases of calcium caseinates, and the evidence from interactions of α_s-, β- and κ-caseins, indicate that κ-casein is the fraction primarily involved in gel formation. In the presence of 0·03–0·06 M calcium high concentrations of κ-casein lead to the formation of gels, and low concentrations to the formation of precipitate. The absence of gel or precipitate with κ-casein in presence of 0·016 or 0·5 M calcium accords with the findings of the effect of calcium on the whole caseinate. It appears that the effect of increasing the levels of calcium above 0·06 M in raising the gelation temperature and finally in preventing gelation is associated with stabilization of the κ-casein to heat. While no direct data are available on this point, it could be inferred from the findings of Hill & Hansen (1963) that increasing the calcium concentration should increase the proportion of κ-casein split from the casein. A changing proportion of free κ-casein could influence the gelation temperature.

pH

The pH limits within which reversible gel formation occurred were 5·2–6·0. The upper limit approaches the pH value of 6·3 below which Chanutin, Ludewig & Masket (1942) reported the dissociation of calcium caseinates to increase sharply. The lower limit is close to the value of pH 5·0 below which no calcium is bound by casein (Zittle, DellaMonica, Rudd & Custer, 1958).

The effect of increasing pH in raising the gelation temperature and in finally preventing gel formation may result from the direct effect on the electrical charges on the protein molecules. The viscosity curves at various pH levels similarly show low viscosities at the higher temperatures, suggesting that the pH value has little effect on the state of hydration of the protein. However, at higher pH values there is likely to be sufficient electrostatic repulsion between the particles to interfere with bonding.

Hydration

Whitaker, Sherman & Sharp (1927) showed from data on specific gravity that the hydration of milk solids decreases with increase of temperature up to 40 °C. Hankinson & Briggs (1941) studied preparations containing 2·5 % caseinate and up to 0·005 M calcium and ascribed the low viscosity they observed to reduction of the electro-viscous effects and the lower water-combining capacity of calcium caseinate.

The initially high viscosity of a 20 % caseinate preparation with 1·0 % calcium at pH 5·4 suggests that the protein was fairly well hydrated at temperatures around 30 °C. As the temperature increased to 40 °C, the very steep drop in viscosity suggested rapid dehydration. However, a similar drop over the range 30–40 °C did not occur in the supernatant liquids in which the protein was mainly β- and κ-casein. The difference between the 2 curves could, therefore, be explained in terms of dehydration of α_s-casein aggregated through calcium linkages. At higher levels of calcium the aggregation or dehydration was apparently fairly complete at 30 °C. However, all the preparations were very viscous and therefore well hydrated at refrigeration temperatures.

CONCLUSIONS

The evidence suggests that the most likely explanation for the behaviour of those caseinate dispersions which show the gelation phenomenon is as follows. The initial rapid decrease in viscosity as the temperature is raised is due to dehydration and the breaking of hydrogen bonds. The gelation on further heating is due to the diminution of the dispersive forces of hydration accompanied by the strengthening of hydrophobic forces, the effectiveness of which depends on the steric control of the molecule by the particular divalent ionic bonding. The balance of these forces is reversed as the temperature is again lowered.

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Oxidation of some milk lipid materials in model systems in presence of copper and ascorbic acid

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SUMMARY. Some milk lipid materials representing the phospholipid protein complex —fat globule membrane from buttermilk (FGMI) and from butter serum (FGMII), intact fat globules (dialysed cream) and triglycerides (butter oil)—were subjected to copper-induced oxidation in systems containing distilled water, phosphate buffer of pH 6·60 or milk dialysate. These model systems usually contained 2 % lipid material and were allowed to oxidize for 48 h at 3–5 °C. Oxidation intensity was measured by means of the 2-thiobarbituric acid test.

The oxidation intensity was dependent on the composition of the lipid material and of the aqueous phase. Ascorbic acid in absence of added copper catalysed the oxidation of the membrane materials and of globular fat. The oxidation intensity produced by FGMI (from buttermilk) differed significantly from that produced by FGM II (from butter serum) and the difference was well correlated with differences in their composition. Butter-oil preparations gave the lowest oxidation intensities, indicating that they are not the site for development of oxidative defects in aqueous media.

Research on the development of oxidized flavour in aqueous dairy products is complicated by the fact that these products show extreme variations in susceptibility (Greenbank, 1940; Jenness & Patton, 1959). El-Negoumy (1965) has shown that oxidation of milk fat is markedly influenced by the composition of the aqueous phase. Phospholipids in the milk fat globule membrane have been suspected as the most likely site for the development of oxidized flavour (King, 1963; Palmer & Samuelsson, 1924; Swanson & Sommer, 1940). This is presumably due to their higher content of polyunsaturated fatty acids (Smith & Lowry, 1962), and especially of linoleic and linolenic acids, which were shown to be the precursors of the breakdown carbonyl compounds responsible for this flavour defect (El-Negoumy, Miles & Hammond, 1961; El-Negoumy, de Puchal & Hammond, 1962).

The present paper reports an investigation of the effect of added copper and ascorbic acid on the oxidation intensity developed by various milk lipid materials. An attempt was made to correlate their oxidative behaviour with some of their compositional features. Variations arising from compositional differences in the aqueous phase were eliminated by the use of identical model systems.

EXPERIMENTAL

Materials

Uncooled, fresh, raw morning milk, collected from the University herd, was separated with a McCormick Model 3G cream separator. The cream was washed and reseparated with about 4 vol distilled water at 22 °C.

Preparation of the fat globule membrane. The washed cream was cooled to 5–10 °C and churned in a laboratory churn. The buttermilk was adjusted to pH 4·90 with 0·10 N-HCl and dialysed for 48 h against distilled water at 2–4 °C. The dialysis water was changed every 6 hr. The membrane material was recovered by centrifugation at 10000*g* for 30 min at 5 °C in a model B-20 International refrigerated centrifuge and lyophilized. This preparation was designated FGM I.

The butter was melted at 50 °C and separated into butter oil and butter serum, by the use of a separatory funnel. The membrane material was recovered from the butter serum by the same technique as is described for buttermilk. This preparation was called FGM II.

Extraction of lipids from the membrane material. 5-g samples from each membrane preparation were extracted with chloroform-methanol, 2:1 (v/v), according to Folch, Lees & Sloane Stanley (1957). The protein residue was dried and saved.

Preparation of globular milk fat (dialysed cream). This material was prepared as described by El-Negoumy (1965).

Preparation of butter oil. Three different preparations of butter oil were made as described by El-Rafey, Richardson & Henderson (1944) as follows: (1) water was boiled off by heating butter at a temperature not exceeding 110 °C and the residual oil centrifuged (B.O. I); (2) butter was melted at 70 °C and the oily layer decanted and centrifuged (B.O. II); (3) water was boiled off from the butter by heating to 110 °C. The temperature was then increased to 130 °C, and the hot oil immediately centrifuged (B.O. III). El-Rafey *et al.* (1944) found that the —SH groups are released from the proteins in the cream at this latter temperature.

Preparation of the aqueous media. Three aqueous media were used in this work: (1) milk dialysate: prepared by dialysing 2 l of 5 % lactose solution in doubly glass-distilled water against 24 gal of skim-milk for 48 h at 3–5 °C (the dialysate was divided into 100-ml portions, frozen at −20 °C and thawed as needed); (2) fresh doubly distilled water; (3) phosphate buffer of pH 6·60, containing 0·032 M-NaH_2PO_4 and 0·018 M-Na_2HPO_4.

Preparation of model systems. Unless otherwise stated, all the model systems contained 2 % lipid material and 1 ppm. Cu as $CuSO_4.5H_2O$. The other components were added at concentrations indicated in the individual experiments. Reagent grade L-ascorbic acid solution was freshly prepared immediately before use. All the systems used in each comparative experiment were adjusted to the same volume, using doubly distilled water. Whenever necessary the pH value was adjusted to 6·60–6·80 with 0·10 N solutions of either NaOH or HCl. Oxidation was allowed to proceed at 3–5 °C for 48 h before samples were drawn for analysis.

Methods

Analysis of linoleic, linolenic and arachidonic acids in the membrane materials

Saponification. A 3-g sample of membrane material was saponified according to Holman & Widmer (1959). The non-saponifiable residue was extracted with three 15-ml portions of Skellysolve B and was then discarded. The aqueous phase was acidified to pH 3 with HCl and free fatty acids were extracted with 3 consecutive 15-ml portions of Skellysolve B. The solvent was removed by rotary evaporation. The free fatty acids were taken up in Skellysolve B and stored under nitrogen at −20 °C until esterification.

Esterification. A 50-mg sample of the free fatty acids was esterified according to Radin, Hajra & Akahori (1960) and the esters stored under nitrogen at −20 °C until analysed by gas–liquid chromatography.

Gas–liquid chromatography was carried out on an Aerograph model A-90-P gas chromatograph (manufactured by Wilkens Instrument and Research, Inc., Walnut Creek, California, U.S.A.), equipped with a thermal conductivity detector. The column, in a stainless steel tube measuring 6 ft in length and of $\frac{1}{8}$ in. bore, was of 10 % diethylene glycol succinate on 60–80 mesh chromosorb-W, which had been treated with hexamethyldisilazine. The column temperature was 190 °C, the helium flow rate 75 ml/min and the sample size 3 μl.

The methyl esters of linoleic, linolenic and arachidonic acids were identified by comparing their retention times with those of known standards and by semilog plots in which the logarithm of the relative retention time was plotted against the carbon number. Quantification was by counting the squares under the individual peaks (Moore, Richardson & Amundson, 1964).

Determination of phospholipid content. Phosphorus was determined in a sample of the lipid material according to King (1932). Phospholipids were calculated according to the equation:

$$\text{phospholipid} = \text{lipid phosphorus} \times 25.$$

Copper analysis. Copper was determined by the method of King & Dunkley (1959).

Measurement of oxidation intensity. The 2-thiobarbituric acid (TBA) test of King (1962) was used. Absorbance of the reaction mixture was measured at 532 mu by the method of King (1962). With each set of experiments blank determinations were made on non-oxidized identical systems. The absorbance for each blank was subtracted from that of the corresponding oxidized samples.

Starch gel electrophoresis of membrane proteins. The proteins recovered from the extraction of lipids from the membrane materials were solubilized in a solution containing 4·5 M urea, 1 % sodium dodecyl sulphate and 1 % of the non-ionic detergent, Amidox-10-L an ethyoxylated alkyloamide. A 4 % solution was electrophorized as described by El-Negoumy (1966*a*, *b*).

RESULTS

The data in Table 1 present some of the compositional properties of the milk lipid materials used in the present work. It is evident that FGM II, from butter serum, was much richer in phospholipids and polyunsaturated fatty acids than was FGM I,

from buttermilk. The total lipid contents reported here are within the limits of those reported by Brunner & Thompson (1961) for the high and low density fractions from the fat globule membrane. FGMII contained only half as much protein as did FGMI. These proteins also differed significantly in their electrophoretic composition as indicated in Plate 1. The protein from FGMII (patterns 1, 3, 5) showed practically no migration in the gel, whereas that from FGMI (patterns 2, 4, 6) showed one major and several minor components.

Table 1. *Some compositional properties of the milk lipid materials used*

(Averages from 5 samples each.)

	FGMI (buttermilk)	FGMII (butter serum)	Dialysed cream	Butter oils B.O.I	B.O.II	B.O.III
Lipid, %	14·80	55·30	—	—	—	—
Phospholipids,* wt. % of lipids	22·10	43·2	0·114	0·062	0·007	0·112
Non-polar lipid,† wt. % of lipids	77·90	57·80	—	99·938	99·993	99·888
Protein, %	46·80	22·90	—	—	—	—
Polyunsaturated fatty acids‡						
Linoleic (18:2)	25·42	30·10	—	—	—	—
Linolenic (18:3)	3·91	8·42	—	—	—	—
Arachidonic (18:4)	1·10	2·82	—	—	—	—
Total	30·43	41·33	—	—	—	—
Copper, ppm.	192	207	—	—	—	—

* Phosphorus × 25.
† 100 − phospholipids content.
‡ Determined by gas–liquid chromatography; representing percentage of total fatty acids.

Table 2. *Effect of Cu^{++} (1 ppm.) and ascorbic acid (1 mg/100 ml) on the autoxidation of milk lipid fractions in different aqueous media*

(Average TBA absorbance from triplicate analysis.)

	Reaction medium: Distilled water			Phosphate buffer (pH 6·60)			Milk dialysate		
Milk lipid material	Cu^{++}	Ascorbic acid	Cu^{++} + ascorbic acid	Cu^{++}	Ascorbic acid	Cu^{++} + ascorbic acid	Cu^{++}	Ascorbic acid	Cu^{++} + ascorbic acid
FGMI (from buttermilk)	0·228	0·125	0·565	0·270	0·290	0·720	0·242	0·157	0·317
FGMII (from butter serum)	0·217	0·477	0·477	0·430	0·135	0·835	0·298	0·181	0·478
Dialysed cream	0·042	0·042	0·177	0·092	0·128	0·195	0·105	0·155	0·310
B.O.I	0·014	0·024	0·053	0·001	0·014	0·031	0·017	0·017	0·026
B.O.II	0·006	0·008	0·040	0·006	0·018	0·106	0·020	0·013	0·042
B.O.III	0·015	0·008	0·017	0·004	0·004	0·035	0·010	0·011	0·032

There was no significant difference in copper content between the 2 membrane preparations. However, both preparations were quite rich in natural copper. The copper content was similar to that reported by Richardson & Guss (1965).

Butter oil preparations I and III, prepared by the boiling-off method, were much higher in phospholipids content than was preparation II, which was prepared by the decantation method.

The effect of copper (1 ppm.) and ascorbic acid (2 mg/100 ml), tested separately or together, on the oxidation intensity developed by the various milk lipid materials in distilled water, phosphate buffer of pH 6·60 and milk dialysate is shown in Table 2.

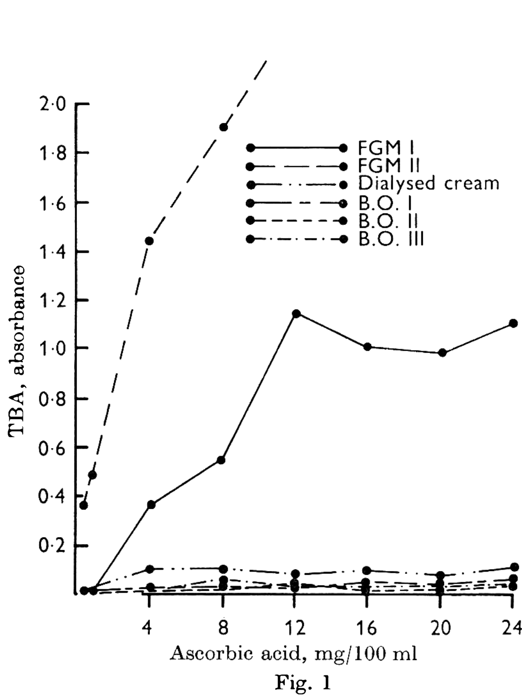

Fig. 1

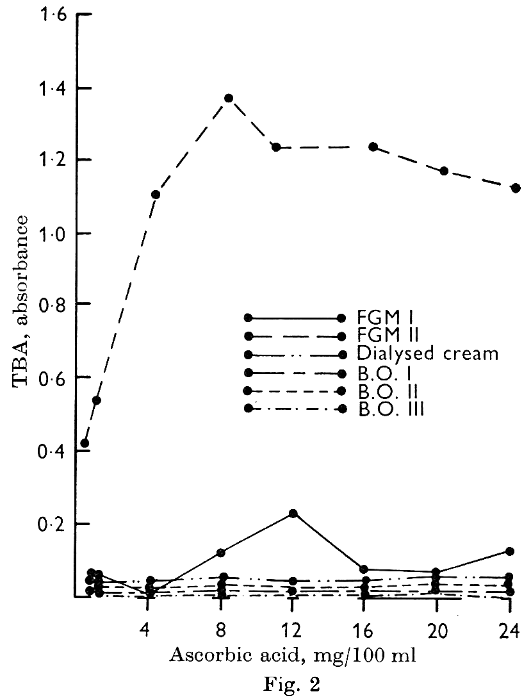

Fig. 2

Fig. 1. The effect of increasing ascorbic acid concentration in absence of added copper on the oxidation of various milk lipid materials in distilled water systems.

Fig. 2. The effect of increasing the concentration of ascorbic acid in absence of added copper on the oxidation of various milk lipid materials in milk dialysate systems.

The membrane material from buttermilk and butter serum gave the highest oxidation intensities under all conditions, regardless of the aqueous medium used. In phosphate buffer and in presence of copper alone, FGM II, from butter serum, gave an oxidation intensity more than twice that given by FGM I, from buttermilk. Ascorbic acid, in absence of copper, resulted in a high oxidation intensity in presence of both membrane materials in all 3 aqueous media. In water, FGM II gave an oxidation intensity which was about 4 times that given by FGM I. This trend was reversed in phosphate buffer, where FGM I gave more than twice the oxidation intensity given by FGM II. The highest oxidation intensities were given by the membrane materials in presence of both copper and ascorbic acid. The intensity was highest in phosphate buffer and lowest in milk dialysate.

The oxidation intensity given by globular fat (dialysed cream) in presence of copper was dependent on the composition of the aqueous phase. Water systems gave the lowest intensity, followed by phosphate buffer, then by milk dialysate. Ascorbic acid

alone in absence of added copper initiated the oxidation of globular fat, especially in phosphate buffer and milk dialysate, but the highest oxidation intensity was recorded in presence of both copper and ascorbic acid.

Virtually no oxidation took place in the 3 different butter oil preparations in presence of copper or ascorbic acid alone. B.O. II, which was devoid of phospholipids, developed in phosphate buffer a relatively high oxidation intensity in presence of both copper and ascorbic acid. This behaviour was quite different from that of B.O. I and B.O. III, which were relatively rich in phospholipids.

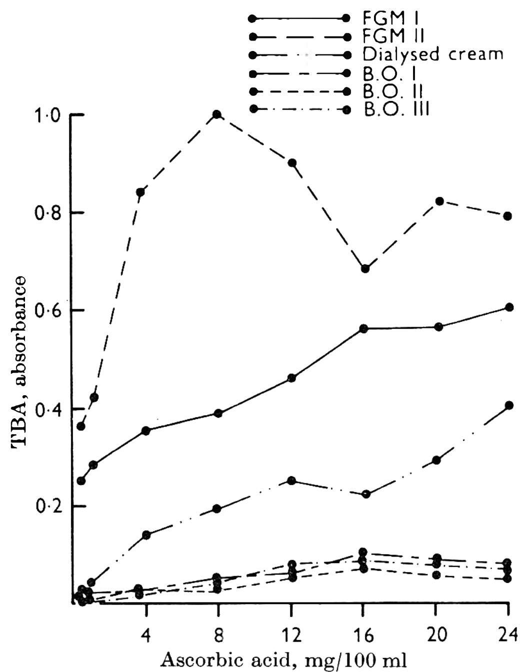

Fig. 3

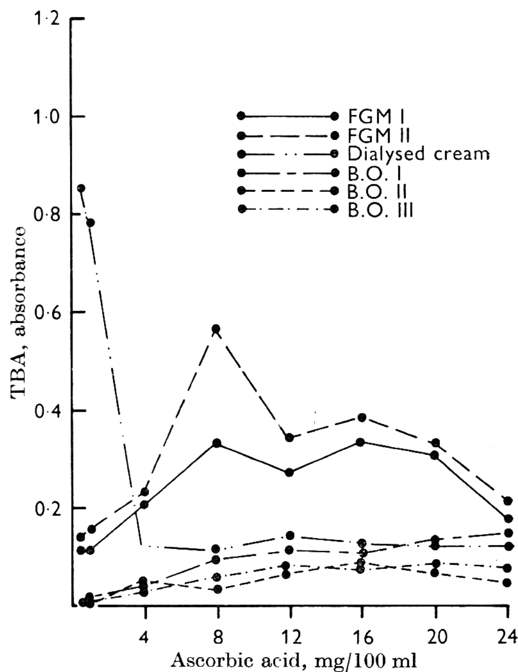

Fig. 4

Fig. 3. The effect of increasing the concentration of ascorbic acid in presence of 1 ppm. added copper on the oxidation of various milk lipid materials in distilled water systems.

Fig. 4. The effect of increasing the concentration of ascorbic acid in presence of 1 ppm. added copper on the oxidation of various milk lipid materials in milk dialysate systems.

The effect of increasing the concentration of ascorbic acid from 0·50 to 24 mg/100 ml in model systems devoid of added copper is shown in Figs. 1 and 2 for aqueous phases of water and milk dialysate, respectively. Concentrations higher than 5 mg ascorbic acid/100 ml caused an oxidation intensity in FGMII more than twice that given by FGMI in the water system and more than 5 times that given by FGMI in the milk dialysate system. With increasing ascorbic acid concentration, there was in the water system a progressive increase in oxidation intensity for both membrane preparations; in the milk dialysate system there was a levelling off or a slight decrease in oxidation intensity at ascorbic acid concentrations above 8 mg/100 ml. No significant change in oxidation intensity with increase in ascorbic acid concentration was observed in systems containing globular fat or butter oil. The magnitude of oxidation was much lower in milk dialysate systems than in the water systems. FGMII gave

an absorbance greater than 2·0 in water systems (Fig. 1), while in milk dialysate systems the absorbance reached a peak of about 1·4 at 8 mg ascorbic acid/100 ml, followed by a slight, gradual decrease at higher concentrations.

The effect of ascorbic acid over the concentration range from 0·50 to 24 mg/100 ml on the oxidation intensity of systems containing 1 ppm. copper is illustrated in Fig. 3 for water systems and Fig. 4 for milk dialysate systems. The oxidation intensity produced by FGM I and dialysed cream in water systems increased progressively as the concentration of ascorbic acid increased, while that produced by FGM II increased to a peak at 8 mg ascorbic acid/100 ml but decreased gradually at higher levels of ascorbic acid. In milk dialysate (Fig. 4) concentrations higher than 4 mg ascorbic acid/100 ml caused a substantial lowering and a levelling-off in oxidation intensity for globular fat. In FGM I and FGM II, concentrations of ascorbic acid ranging from 0·50 to 8 mg/100 ml caused a substantial increase in oxidation intensity, while higher concentrations caused a significant gradual decrease in oxidation intensity. A slight gradual increase in oxidation intensity occurred with the 3 butter oil preparations at ascorbic acid concentrations ranging from 0·50 to 20 mg ascorbic acid/100 ml.

DISCUSSION AND CONCLUSIONS

The fat globule membrane produced the highest oxidation intensity in all systems in comparison to globular fat or butter oil preparations (see Table 2 and Figs. 1–4). This finding supports the conclusion that the fat globule membrane is the main source of flavour defects resulting from lipid oxidation in aqueous dairy products, doubtless because of its higher content of phospholipids and natural copper as compared with butter oil, which is composed mainly of non-polar lipids (Table 1). These phospholipids are rich in polyunsaturated fatty acids, especially linoleic and linolenic acids, which are the precursors for the carbonyl compounds responsible for oxidized flavour defects (El-Negoumy *et al.* 1961, 1962). This is indicated by the early findings of Swanson & Sommer (1940), who reported the iodine number of the fatty acids in milk phospholipids to be substantially lowered through autoxidation while that of the non-polar lipids remained unchanged. The strongly pro-oxidant effect of ascorbic acid in absence of added copper is due to the presence of high concentrations of natural copper in these membrane materials. This accounts for the development of lipid oxidation flavours in commercial milks that are free from external metallic contamination.

The substantial difference in oxidation intensity between FGM I, from buttermilk, and FGM II, from butter serum, is probably a reflexion of compositional differences indicated in Table 1. FGM II consistently gave much higher oxidation intensities, except in phosphate buffer (Table 2). This reversal of behaviour in phosphate buffer is surprising and clearly indicates the influence of the composition of the aqueous phase on lipid oxidation. As indicated in Plate 1, these 2 membrane preparations differ significantly in the electrophoretic composition of their protein residues. It is conceivable that because of unknown configurational differences in the protein of FGM I, it resulted in the formation of more of the highly catalytic cuprous phosphate (El-Negoumy, 1965) and thus gave higher oxidation intensities than did FGM II in phosphate buffer.

Richardson & Guss (1965) prepared the fat globule membrane solely from buttermilk, and apparently assumed that it was identical with that in butter serum. The present findings indicate that this assumption was unwarranted.

The much higher oxidation intensity resulting from the addition of both copper and ascorbic acid is due to the conversion of cupric to cuprous ions by the reduced ascorbic acid (Filtman & Frieden, 1957; Frieden & Alles, 1958; Kelley & Watts, 1957; King & Dunkley, 1959). The much lower oxidation intensities observed in milk dialysate systems is probably due to the interaction of natural or added copper with milk salts forming cupric and cuprous complexes. The catalytic effect of copper is dependent on the kinds and amounts of anions in these complexes (El-Negoumy, 1965). Cuprous ions were reported as the active catalyst (Smith & Lowry, 1962). The predominance of cupric complexes and the presence of copper chelators such as sodium citrate may be responsible for lowering the oxidation intensity in milk dialysate.

The observation that, with increasing concentrations higher than 4 mg ascorbic acid/100 ml, oxidation intensities in water systems increased (Figs. 1, 3) whereas those in milk dialysate systems decreased (Figs. 2, 4), probably reflects interrelationships of the copper, milk salts and ascorbic acid. Ascorbic acid converts cupric to cuprous copper in amounts proportional to its concentration (King & Dunkley, 1959). Complexes between cuprous copper and ascorbic acid strongly promoted oxidation in salt-free systems, especially at higher concentrations of ascorbic acid (El-Negoumy, 1965). It is suggested that in milk dialysate the cuprous ion forms complexes with anions of the milk salts, thus sparing the ascorbic acid, which then exerts its antioxidant properties, especially at its higher concentrations.

The level of oxidation intensity produced by globular milk fat is most probably a reflexion of the amount of intact fat globule membrane surrounding it. This is indicated by the fact that ascorbic acid in absence of added copper initiated their oxidation, especially in phosphate buffer (see Table 2). The results obtained with globular milk fat in water systems were in good agreement with those previously reported (El-Negoumy, 1965).

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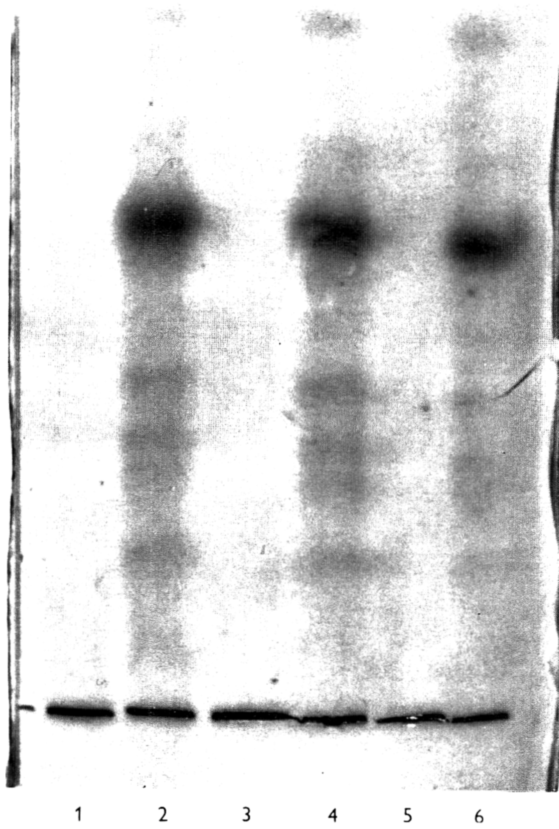

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EXPLANATION OF PLATE

Starch gel electrophoresis of fat globule membrane from buttermilk (patterns 1, 3, 5) and from butter serum (patterns 2, 4, 6). Conditions: 4·5-M urea and tris-citrate buffer in the gel, sodium borate buffer in the electrode vessels. Voltage, 175 V, current 35 mA for 20 h (see El-Negoumy, 1966*a*).

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The cell count of cow's milk and the micro-organisms cultured from the milk

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SUMMARY. Total and differential cell counts were made on a total of 26272 quarter samples of foremilk taken at fortnightly intervals from cows in the Institute herd during the 12 years 1953–65, and the samples were also examined bacteriologically.

The average cell count in each lactation up to the 7th was calculated.

The average total cell count of samples from which no staphylococci, streptococci or coliform organisms were isolated increased from the 1st to the 7th lactations from 0·19 to 0·60 million cells/ml, and from the 2nd lactation onwards the increase was due entirely to an increase in the number of polymorphs. With infected samples, however, changes in the average total cell count were caused by changes in the numbers both of polymorphs and of cells other than polymorphs.

The average total cell count of samples from which coagulase-negative staphylococci were isolated varied between 0·58 and 1·00 million/ml throughout the 7 lactations. For samples from which coagulase-positive staphylococci were isolated the cell count varied between 0·72 and 4·94 million/ml, the highest average total cell count occurring in the 5th lactation.

The average total cell count of samples from which streptococci were isolated varied between 1·48 and 4·62 million cells/ml, the highest value occurring in the 2nd lactation.

The occurrence and control of mastitis in the Hannah Institute herd have been studied for many years. During a 12-year period (1953–65) foremilk samples were taken at fortnightly intervals from most of the cows for cell counts and bacteriological examination. From the results so obtained, the average cell counts for the milk of cows in their 1st–7th lactations were calculated. The differences which occurred in the average total cell count during any one lactation and also from one lactation to the next have already been published (Blackburn, 1966). In the present paper the variations that occurred in the average total cell count in relation to the bacteriological findings are described.

METHOD

The milk samples (a total of 26272) were all quarter samples of foremilk taken just before the evening milking at fornightly intervals throughout lactation. The numbers of cows used were as follows: 1st lactation, 57; 2nd lactation, 64; 3rd lactation, 68;

4th lactation, 67; 5th lactation, 57; 6th lactation, 50; 7th lactation, 47. Samples from cows which were in the herd for fewer than 3 lactations were not included. Before sampling the teat orifices were swabbed with 70 % alcohol.

The total and differential cell counts and bacteriological examinations were made by the methods described by Blackburn (1956), except that during the 2nd half of the experiment sheep blood agar was used in place of horse blood agar. The samples were plated for bacteriological examination within 30 min of being taken. The inoculum consisted of 0·01 ml of milk. The plates were incubated overnight and the presence of 4 or more similar colonies was taken as constituting an infection.

The samples were grouped as follows: (1) samples from which no staphylococci, streptococci or coliform organisms were isolated; (2) samples from which coagulase-negative staphylococci were isolated; (3) samples from which coagulase-positive staphylococci were isolated, and (4) samples from which streptococci were isolated. The herd was free from infection with *Streptococcus agalactiae*, and most of the streptococci isolated split aesculin. In analysing the results, an arbitrary order of precedence was used for samples showing mixed infections. This order was: streptococci, coagulase-positive staphylococci, coagulase-negative staphylococci, coliform organisms. Thus, if streptococci were found in a sample along with other organisms it was considered that the sample was infected with streptococci, the other organisms being ignored. If no streptococci were present but coagulase-positive staphylococci were found along with other organisms it was considered that the sample was infected with coagulase-positive staphylococci. Similarly, if coagulase-negative staphylococci and coliform organisms were found it was considered that the sample was infected with coagulase-negative staphylococci.

RESULTS

Uninfected samples. The average cell counts (arithmetic mean) for samples from which no streptococci, staphylococci or coliform organisms were isolated are shown in Fig. 1. The average total cell count of samples taken from cows in their 2nd lactation (0·37 million cells/ml) was almost double that of samples taken from cows in their 1st lactation (0·19 million cells/ml). In subsequent lactations there was a gradual rise in the average total cell count until in the 7th lactation it reached 0·67 million cells/ml. The increase in the average total cell count in the 2nd lactation was due mainly to a rise in the number of polymorphs and to a lesser degree to a rise in the number of cells other than polymorphs, whereas the increase in subsequent lactations was due entirely to a rise in the number of polymorphs. The percentages of cells that were polymorphs in the milks from the 1st to the 7th lactations were 37, 51, 51, 53, 60, 60 and 63, respectively. The average total cell count of all uninfected samples was 0·41 million cells/ml, of which number 56 % were polymorphs.

Samples infected with coagulase-negative staphylococci. The average cell counts for samples from which coagulase-negative staphylococci were isolated are shown in Fig. 2. The average total cell count rose from 0·58 million/ml in the 1st lactation to 0·83 million/ml in the 2nd lactation. It was 0·61 million/ml in the 3rd and 5th lactations and 0·72, 0·86 and 1·00 million/ml in the 4th, 6th and 7th lactations, respectively. The percentages of cells that were polymorphs from the 1st to the 7th

lactations were 55, 61, 61, 61, 62, 63 and 64, respectively. The average total cell count of all the samples infected with coagulase-negative staphylococci was 0·75 million cells/ml and 63 % of these were polymorphs.

Samples infected with coagulase-positive staphylococci. The average cell counts for samples from which coagulase-positive staphylococci were isolated are shown in Fig. 3. The average total cell count increased from 0·72 million cells/ml in the 1st lactation to 2·73 in the 2nd lactation and increased further to 3·44 in the 3rd lacta-

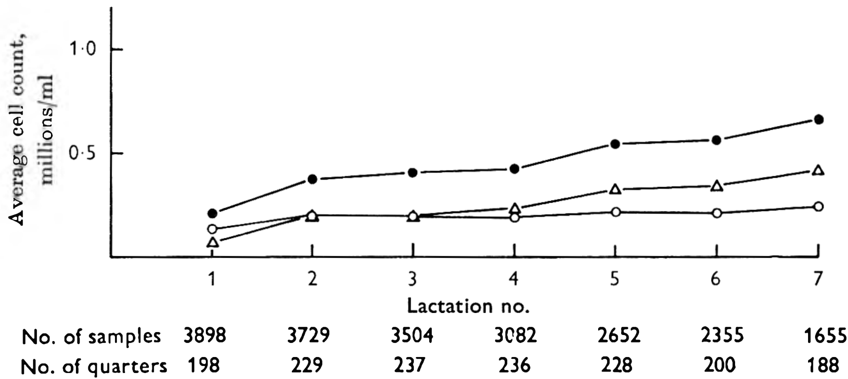

Fig. 1. Average cell count of samples from which no staphylococci or streptococci were isolated for each of 7 lactations. ●, Total cell count; △, polymorph count; ○, count of cells other than polymorphs.

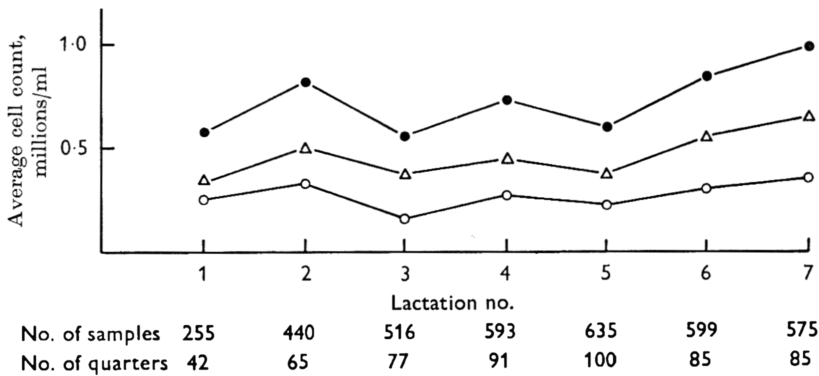

Fig. 2. Average cell count of samples from which coagulase-negative staphylococci were isolated for each of 7 lactations. ●, Total cell count; △, polymorph count; ○, count of cells other than polymorphs.

tion. The count fell to 2·09 million/ml in the 4th lactation and increased to 4·94 in the 5th lactation, after which it fell to 4·24 and 2·58 in the 6th and 7th lactations, respectively. The percentages of cells that were polymorphs from the 1st to the 7th lactations were 57, 70, 74, 70, 76, 75 and 72, respectively. The average total cell count of all the samples infected with coagulase-positive staphylococci was 3·60 million cells/ml, of which number 74 % were polymorphs.

Samples infected with streptococci. The average cell counts for samples from which streptococci were isolated are shown in Fig. 4. The average total cell counts from the 1st to the 7th lactations were 1·48, 4·62, 2·81, 3·14, 1·97, 1·67 and 2·37 million/ml, respectively. The percentages of cells that were polymorphs from the 1st to the 7th lactations were 80, 75, 68, 65, 68, 69 and 69, respectively. The average total cell count of all the samples infected with streptococci was 2·41 million cells/ml, of which number 69 % were polymorphs.

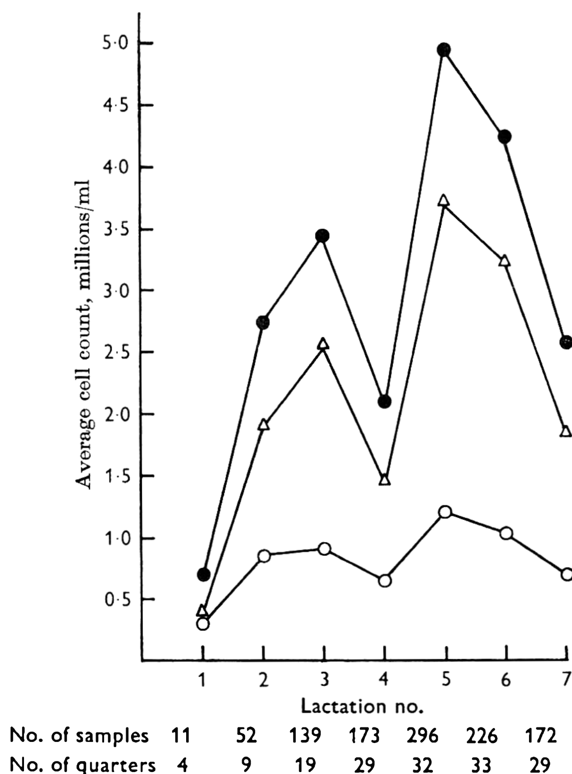

Fig. 3. Average cell count of samples from which coagulase-positive staphylococci were isolated for each of 7 lactations. ●, Total cell count; △, polymorph count; ○, count of cells other than polymorphs.

Samples infected with coliform organisms. Coliform organisms were cultured from 75 samples but staphylococci or streptococci were also cultured from 60 of them. The average total cell count of the remaining 15 samples was 0·68 million/ml, of which number 72 % were polymorphs.

Out of the total of 26 272 samples examined, coagulase-negative staphylococci were isolated from 3613 samples (13·7 %), coagulase-positive staphylococci from 1069 samples (4·1 %), streptococci from 700 samples (2·7 %) and coliform organisms from 15 samples (0·06 %). From 20875 samples (79·5 % of the total) none of the above-mentioned organisms was isolated (see Table 1). The average total cell count of the 26 272 samples examined throughout the experiment was 0·65 million cells/ml and 61 % of these were polymorphs.

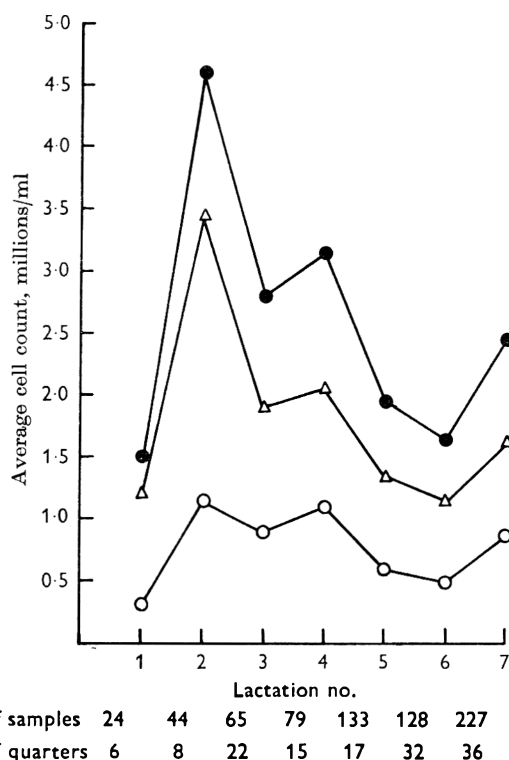

Fig. 4. Average cell count of samples from which streptococci were isolated for each of 7 lactations. ●, Total cell count; △, polymorph count; ○, count of cells other than polymorphs.

Table 1. *The percentage of samples in each of 7 lactations from which were isolated: coagulase-negative staphylococci, coagulase-positive staphylococci, streptococci, and coliform organisms, and from which none of these organisms was isolated*

Lactation no.	No. of samples examined	C− Staph.	C+ Staph.	Str.	Coliform	No organisms isolated
1	4188	6·1	0·3	0·6	—	93·1
2	4267	10·3	1·2	1·0	0·05	87·4
3	4225	12·2	3·3	1·5	0·02	82·9
4	3931	15·1	4·4	2·0	0·10	78·4
5	3717	17·1	8·0	3·6	0·03	71·3
6	3309	18·1	6·8	3·9	0·03	71·2
7	2635	21·8	6·5	8·6	0·23	62·8
All samples	26272	13·7	4·1	2·7	0·06	79·5

DISCUSSION

The average total cell counts of milk samples from which staphylococci were isolated was reported by Campbell (1950), who gave the following figures in millions/ml for samples from which were isolated: no bacteria, 0·069; non-haemolytic staphylococci, 0·0245; weakly haemolytic staphylococci, 0·96, and haemolytic staphylococci, 1·20. Samples of milk from newly calved cows and from cows in the drying-off period

were excluded. MacLeod & Anderson (1952) found a geometric mean cell count of 0·07 million for milk from healthy cows. These figures denote lower cell counts than were observed in the present work.

The average total cell count of the uninfected samples increased from the 1st to the 7th lactation from 0·19 to 0·67 million/ml. Although no staphylococci, streptococci or coliform organisms were isolated from these samples, they were not necessarily free from micro-organisms. The presence or absence of other organisms was not recorded although the so-called milk corynebacteria were isolated from many of the samples. McEwen & Cooper (1947) showed that the milk corynebacteria could be associated with a rise in cell count, and Cobb & Walley (1962) caused mastitis by inoculating cultures of *Corynebacterium bovis* into the udder.

The average total cell counts in the present work were also influenced by the inclusion of samples from quarters which had been treated with antibiotics. It has been shown that such samples can have high cell counts for as long as 5 weeks after the organisms have been eliminated (Blackburn, 1956). From the results obtained, however, it is evident that the average total cell count was lower for samples which were not infected with staphylococci and streptococci than for samples which were so infected.

The rise in the average cell count, from the 2nd to the 7th lactation, of samples which were free from infection with staphylococci and streptococci was accounted for almost entirely by an increase in the number of polymorphs. With infected samples, however, changes in the average total cell count were caused by changes in the numbers both of polymorphs and of cells other than polymorphs. Blackburn (1966) found that, taking the infected and uninfected samples together, the rise in the average total cell count from one lactation to the next was due mainly to an increase in the number of polymorphs. More than 79 % of the samples examined, however, were uninfected samples (see Table 1) and this would tend to overshadow the effect of the infected samples on the proportions of the different types of cells present.

In any one lactation, the samples from which coagulase-negative staphylococci were isolated showed higher average total cell counts than did samples which were free from staphylococci and streptococci. This finding might be taken as evidence that these organisms can cause subclinical mastitis. Much higher average total cell counts were obtained for samples from which coagulase-positive staphylococci were isolated, the count reaching 4·94 million/ml in the 5th lactation. This was preceded by a fall from 3·44 million/ml in the 3rd lactation to 2·09 in the 4th lactation. The question arises as to whether the lower cell count in the 4th lactation was due to the disposal from the herd of cows with infected quarters. Seven cows were disposed of after the 3rd lactation and one of them was infected with coagulase-positive staphylococci in 3 of the 4 quarters of the udder. This cow contributed 20 of the 139 samples from animals in the 3rd lactation, and if these 20 samples had been excluded the average total cell count for the 3rd lactation would have been 2·81 instead of 3·44 million/ml. Although samples from this animal had a marked effect on the cell counts in the 3rd lactation, its removal from the herd could not entirely have caused the fall in cell count that occurred in the 4th lactation. It would appear from the results of this experiment that there was an increasing reaction of the udder to coagulase-positive staphylococci up to the 3rd lactation, less reaction in the 4th lactation, and the greatest reaction in the 5th lactation.

The average total cell count of samples from which streptococci were isolated reached a peak of 4·62 million/ml in the 2nd lactation. As no cows were disposed of from the herd after the 2nd lactation it would appear from the results of this experiment that the most marked reaction to streptococci occurred in the 2nd lactation.

The number of samples showing coliform organisms was too small to give a lactation-to-lactation average cell count, and the average total cell count of 0·68 million/ml was made up of a range of cell counts of less than 0·01 to 8·35 million/ml.

The percentage of the samples which were infected increased from lactation to lactation, except for samples infected with coagulase-positive staphylococci, in which instance the percentage of samples infected in the 6th lactation was slightly less than in the 5th lactation, and in the 7th lactation slightly less than in the 6th lactation. Almost twice as many samples were infected with streptococci in the 7th lactation as in the 6th lactation.

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Iron and vanadium requirements of lactic acid streptococci

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Lactic acid streptococci have an absolute requirement for magnesium and potassium and failed to grow in milk deionized with IRC 50 resin (sodium form) (Reiter unpublished; see also Fisons, 1958; Reiter, 1956). These findings were confirmed in preliminary experiments using the defined medium of Reiter & Oram (1962). Requirements for other metal ions have now been investigated. Two strains of *Streptococcus lactis* (ML_3 and C_{10}) and one of *Streptococcus cremoris* (HP) were used. They were maintained as milk cultures, and before use subcultured twice in the synthetic medium containing Ca, Mg, Fe, Zn, Mn, Co, Cu and Mo (cf. Reiter & Oram, 1962) and then finally in the synthetic medium containing only Mg and K (20 ppm.), and Fe (0·01 ppm.).

All glassware used in these experiments was treated with chromic acid, washed with tap water and glass-distilled water and finally autoclaved in deionized water. The culture medium was extracted with 8-hydroxyquinoline at pH 5·0 and again at pH 6·6 as described by Nicholas (1952). Spectroscopically pure metal salts (Johnson, Matthey & Co. Ltd, London) were used throughout. The assays were done in optically matched 150 × 18 mm Pyrex tubes and growth was measured as percentage transmission at 580 nm.

Fig. 1 shows the growth response of *Str. cremoris* HP and *Str. lactis* C_{10} to iron and vanadium. HP shows a greater requirement than C_{10} for both metals. It was difficult to obtain regularly consistent results with ML_3 in the control tubes because growth often occurred in the absence of added iron. The minimum requirement of this strain appeared, therefore, to be below 0·0002 ppm. Fe, the threshold of residual iron after the treatment of the medium with 8-hydroxyquinoline. A satisfactory growth response to graded supplements of iron and vanadium was, however, obtained after the addition of 4 mg α,α'-dipyridyl to the culture medium (Fig. 2).

Higher concentrations of iron than of vanadium were required by ML_3 in the presence of α,α'-dipyridyl because iron is more strongly sequestered by the chelating agent. A growth response to iron or vanadium was also observed with HP and C_{10} when assayed under these conditions.

With all cultures molybdenum failed to replace iron or vanadium; similarly no growth was obtained with cobalt, zinc and copper in the range of 0·001–1 ppm., or with manganese up to 10 ppm. when added in place of iron or vanadium.

Thus, the requirements of these organisms for iron can be satisfied alternatively by vanadium but not by molybdenum, as observed for *Azotobacter vinelandii* and *Clostridium pasteurianum* (Bortels, 1930; Nicholas, 1958). These workers found that the omission of molybdenum from the growth medium depressed nitrogen fixation

to a greater extent than did the omission of iron. Nitrogen fixation was mainly dependent on molybdenum irrespective of the valency state, whereas vanadium was found to be active only as V^5 or V^4 and not as V^3. A requirement for vanadium in the alga *Scenedesmus obliquus* has also been demonstrated (Arnon & Wessel, 1953) and it

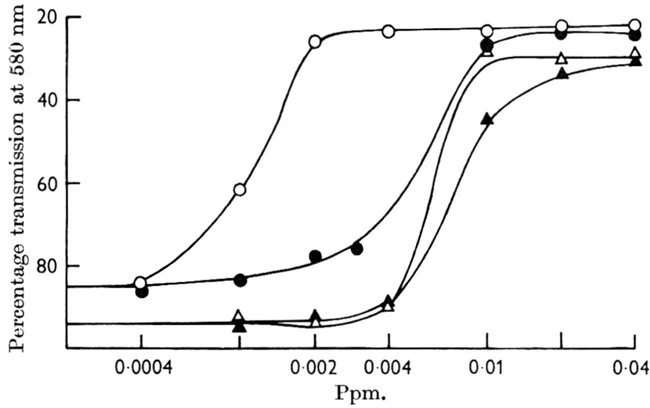

Fig. 1. Growth response of *Str. lactis* (C_{10}) and *Str. cremoris* (HP) to Fe and V in oxine-treated medium. △—△, HP + $FeSO_4.7H_2O$; ▲—▲, HP + NH_4VO_3; ○—○, C_{10} + $FeSO_4.7H_2O$; ●—●, C_{10} + NH_4VO_3.

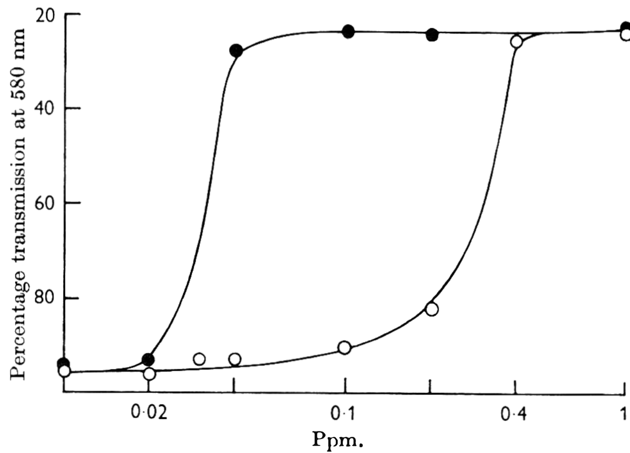

Fig. 2. Growth response of *Str. lactis* (ML_3) to Fe and V in oxine-treated medium containing 0·1 mg % α,α′-dipyridyl. ○—○, $FeSO_4.7H_2O$; ●—●, NH_4VO_3.

has been claimed that vanadium is essential for the growth of *Aspergillus niger* (Bertrand, 1942). To our knowledge these are the only micro-organisms in which vanadium has been reported to act as an essential trace metal, although it is sometimes included in synthetic media for the cultivation of bacteria. The accumulation of vanadium in the blood cells of tunicates (Rummel *et al.* 1967) suggests also a possible role of the metal in the metabolism of these organisms.

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The effects of variations in pH, of the removal of calcium and of the addition of sulphur-bond inhibitors on the rate of setting of renneted milk

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SUMMARY. The rate of increase of the complex rigidity modulus of renneted skim-milk was measured, both in a modified form of the torsiometer previously described and in the thrombelastograph, an instrument designed for the study of blood coagulation. Milk diluted with water was also tested.

The effect of holding the milk is to slow down the process of coagulation without affecting the 'coagulation time' (i.e. its starting time). In undiluted milk a fall in pH from 6·66 to 6·18 does not affect maximum firmness but a later softening of the curd is accentuated in diluted and undiluted milk. Again, the coagulation time is not affected but the rate of firming up is reduced. Potassium oxalate, used to reduce the calcium content, shows similar effects.

N-Ethyl maleimide, a reagent specific for sulphydryl groups, had no effect on undiluted milk; but with dilute milk, at a pH of about 6·0, there was a decrease in maximum firmness and there were also differences in the later softening of the curd.

The significance of these effects on bonds formed during postenzymic coagulation is discussed.

The trend towards the use of continuous processes (cf. Mann, 1965) in certain sections of the dairy industry must involve new techniques of many kinds. This is especially true for continuous cheese-making. The rates of the processes involved in the different stages of curd formation are much more critical than was the case with the classical methods, in which the timing of the processes could be controlled by the subjective assessments of the expert cheese-maker. The new methods require an understanding of the quantitative effects of all the variable and controllable factors on the rate of production of curd. These are best studied by direct measurements of the increase in the 'firmness' (complex modulus) of the curd during setting, both in the factory and in the laboratory.

The aim of the present work was to measure, with as much accuracy as was possible, changes in rate of setting and in subsequent consistency of curd under laboratory conditions with controlled variation in pH, and in contents of calcium and of *N*-ethyl maleimide (NEME), and also to develop some ideas concerning the nature of the

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bonding induced by the action of rennet on milk and on the syneretic processes which follow.

SUMMARY OF EARLIER WORK

In earlier papers, Scott Blair & Burnett (1957, 1958, 1959) described an apparatus for studying the rate of setting of milk curd in which air pressures were applied to one end of a sample of curd in a wide U-tube and the displacements at the other end were measured. In this way 'static' elastic moduli and ordinary viscosities were calculated but the method was not as satisfactory as the dynamic method used in the present work. Fat-free dried milk was generally used and the effects of such factors as pasteurization, varying calcium contents, milk and rennet concentrations, and low-temperature pretreatment were studied. Equations relating the viscosities and rigidity moduli to time were proposed but these were not as satisfactory as that used in the present work (see Scott Blair & Burnett, 1963*a*). The studies of the effects of changes in calcium content were confined to quite large quantities of calcium. Also, the effects of very small additions of calcium on the complex modulus were studied by means of a device consisting of a ball moving in the coagulum (Tuszyński, 1967).

EXPERIMENTAL

Two instruments were used for following the process of milk coagulation. The first was a double torsiometer, similar to that described by Burnett & Scott Blair (1963). An earlier form of torsiometer was described by Scott Blair, Burnett & Lilley (1958). In the laboratory model, a stainless steel cylinder (diam. 8 cm, height 8 cm) is very slowly oscillated through a small angle (15°), the cylinder being immersed in 3 l of renneted milk in a beaker, with temperature held at 32 °C. At the top of the cylinder is a soft, coiled spring, the distortion of which is measured on each swing. By accelerating the head, not only the amount but also the average rate of strain is kept constant. Two such instruments are mounted together so that, for example, one may be used for an experiment and the other for a control, simultaneously. The readings on the dials which record the torque on the springs thus give a direct measure of the average (complex) modulus of the setting gel during the oscillation.

In the commercial model of the torsiometer described by Plint, Burnett & Scott Blair (1967), the oscillations are sinusoidal. In the laboratory instrument, they are linear; but, by running an experiment with identical milks in both the beakers and with one cylinder operating at twice the frequency of the other, it was shown that there was no significant frequency dependence. This means that the motion may be treated as if sinusoidal (see below).

The second instrument to be used was the thrombelastograph (Hartert, 1952; Hartert & Schaeder, 1962). This instrument was originally designed for studying blood coagulation. It combines 3 torsional units, each of which takes 0·3 ml of liquid. Again, a stainless steel cylinder is suspended in a cup and the torque on a torsion wire from which the cylinder is suspended is recorded. But with the thrombelastograph it is the cup that is oscillated through a small angle. The stress is again measured directly from the torque on the wire but, in this case, the strain depends on the difference in the angle of torque from that of the applied twist on the cup. The motion

is linear but there is a 1-sec pause at the end of each swing. A beam of light falls on a mirror on the torsion wire and part of this is so reflected as to be visible on a screen (3 small spots of light of different colours are seen). But also the beams are projected onto a moving photosensitive paper and these scan up and down the paper, making a dark line during the 1-sec resting periods. The amplitude between the 2 dark lines (a mm) is related to G by the simple formula: $G \propto a/(100-a)$, since the total width of the chart is 100 mm.

If the annular space between the cylinders were full of an undeformable solid, a would be 100 and G would be infinite. If full of air, a would be zero and G would be zero. Since G is in arbitrary units (as it is also with the torsiometer) it is convenient to arrange that when $a = 50$, G should be 100, hence the equation is written: $G = 100a/(100-a)$. A table is provided with the instrument showing the values of G corresponding to values of a from 1 to 99, but for milk, where a is generally rather small, accurate graphs have been drawn to give more precise values for G over the range of a from about 1 to 20.

Unfortunately, it was not possible to change the temperature control from 37 °C on the thrombelastograph, so that an allowance must be made in comparing the results directly with those of the torsiometer at 32 °C.

It has already been shown (Scott Blair & Burnett, 1963a) that a simple equation relates the increasing value of G to the time, provided that the time zero is taken not from the moment of addition of rennet (t_r) but from the time when the milk just ceases, at fairly high rates of flow, to behave as a true (constant viscosity) fluid. It is impossible to observe this change with the torsiometer and difficult (but not impossible) with the thrombelastograms even when these are magnified fivefold, as has been usual in our experiments with milk.

The time zero t_c is best determined in a separate experiment, in which a column of milk falls in a gradient tube below which is attached a fine vertical capillary, the milk forming a pool in the containing vessel (see Scott Blair & Burnett, 1963b). The height of the falling column is recorded every 6 sec using a bell metronome and is plotted against time on semi-logarithmic paper. The point of time at which the curve breaks away from the straight line gives t_c. Unfortunately, only a limited number of these tests can be done, since the viscometer deals with only one sample while the thrombelastograph takes 3.

The equation which has been found to hold remarkably well for renneted milk up to the condition suitable for cutting (and also for blood in the earlier stages of coagulation) is given by Scott Blair & Burnett (1963a):

$$G = G_\infty e^{-\tau/t},$$

where G_∞ is a theoretical maximum modulus, τ is the time taken to reach $1/e$-th of this value and t is the time, taking t_c as the zero. From this it follows that plots of log G against $1/t$ should be linear and special graph sheets are available with logarithmic against reciprocal scaling.

We are not the first to use the thrombelastograph for the study of milk coagulation; but, so far as we know, no attempts have been made to establish equations relating G to time. Quite arbitrary points are generally taken to define the shape of thrombelastograms. Jacquet & Marçais (1964) point out that the Greek word

θρόμβος applies as well to milk as to blood and they show thrombelastograms illustrating the effects of additions of $CaCl_2$ and $MgCl_2$. They point out that the use of very small samples, while most valuable for work on blood, is of no advantage for milk. The thrombelastograph does have the advantage, however, that 3 experiments can be started at about the same time (e.g. duplicate experiments and a control), the instrument can be left to run for as long as is desired, and the thrombelastograms, enlarged if necessary, can be analysed at leisure. Other workers in France (Tarodo de la Fuente & Frentz, 1966) have studied renneted dried milk with a thrombelastograph. They use a syphon-tube device to find the starting-time of coagulation but use Hartert's arbitrary parameters and do not propose any coagulation equation. They are mainly concerned with the effects of varying temperature, of pasteurization and of additions of calcium. They also noted, as we did, that, after the same interval of time, curd from stored milk is much softer than that from fresh milk.

In normal blood work, the amplitude in the thrombelastogram is measured to allow for the width of the dark line produced during the 1-sec stationary periods. But even with blood, and most certainly with milk, there is some stress relaxation during these periods and this results in a widening of the line (Hartert, 1948). Since the true values of a would be best defined *before* the relaxation, it is better to measure the total width of the graph and to subtract the 'optical width', i.e. that of the zero line before any divergence occurs. On the fivefold-magnified charts, this measures just under 3 mm. Probably the correct value on the original charts is 0·5 mm. The width of these 'bands' in excess of the 'optical width', being a measure of relaxation, also indicates the amount of flow during the rest period.* In this sense, the thrombelastograph can measure a viscosity as well as the generally preponderant elastic modulus. (There is some confusion in the literature of thrombelastography in the use of the term 'elasticity'. It is best always to refer to 'elastic modulus'. As an example, rubber is more 'elastic' than concrete but has a far lower elastic modulus.)

The significance of the complex modulus

So far, we have used the symbol G to represent what is known as the 'complex modulus'. For sinusoidal straining, part of the modulus (written G') is concerned with energy which is temporarily stored during the oscillation and recovered on the return swing. This is called the 'real' part of the modulus. But there is also energy which is lost as heat and the associated modulus is unfortunately known as the 'imaginary' part (iG''). The complex modulus is written with an asterisk, so that we have the equation: $G^* = G' + iG''$.

Since, in this work, it has not been possible to measure phase angles and so to determine G' and G'', we have in general omitted the asterisk and used G for the complex modulus throughout. As the milk gradually sets, so G' gains at the expense of G''. Even with blood, for which it is claimed that the thrombelastograph does not measure 'viscosity', this is true only in a certain sense. Any deformations for which the required energy is not temporarily stored but is dissipated as heat may be expressed in terms of a 'viscosity' which is obtained by dividing the imaginary part

* An attempt was made to measure the width of these bands, but the errors of measurement are relatively large. All that can be said is that, in the earlier stages of setting, the viscosity falls with rising stress but in the later stages, when the stress is nearly constant, the viscosity tends to rise with time.

of the complex modulus by the frequency. Indeed the whole equation may be rewritten in terms of a complex viscosity, of which the imaginary part is elastic. The introduction of the unfortunate term 'imaginary' indicates no more than the presence of i (defined as $\sqrt{-1}$), which is introduced to represent the condition when stress and strain are precisely out of phase. It will be observed that, in a sinusoidal oscillation, at the end of the swing, strain is maximal and rate of strain is zero, whereas, in the middle of the swing, strain is zero and rate of strain is maximal. The elastic modulus is given by the ratio of stress to strain and the viscosity by that of stress to rate of strain. Turning through 180° produces no change in the significance of a point on a sinusoidal wave diagram. Turning through an angle of 90° is an operation which when performed twice produces no change. Since '1' is the accepted operational symbol for 'no change in magnitude', we have $i \times i = 1$, so that turning through an angle of 90° is best represented by i.

All this generally applies to a strictly sinusoidal motion. Other motions, such as those of the torsiometer or the thrombelastograph, would normally be expressed in terms of a Fourier series. However, the later terms of this series allow for the effects of changing frequency on the modulus. They may therefore be ignored in the case of milk, since, as has been shown, the modulus is independent of frequency over quite a wide range.

RESULTS

The torsiometers were designed for use only up to a little beyond the stage of coagulation at which curd is normally cut for cheese-making; when left for much longer periods, the values of G would go off the scale. The thrombelastograph, on the

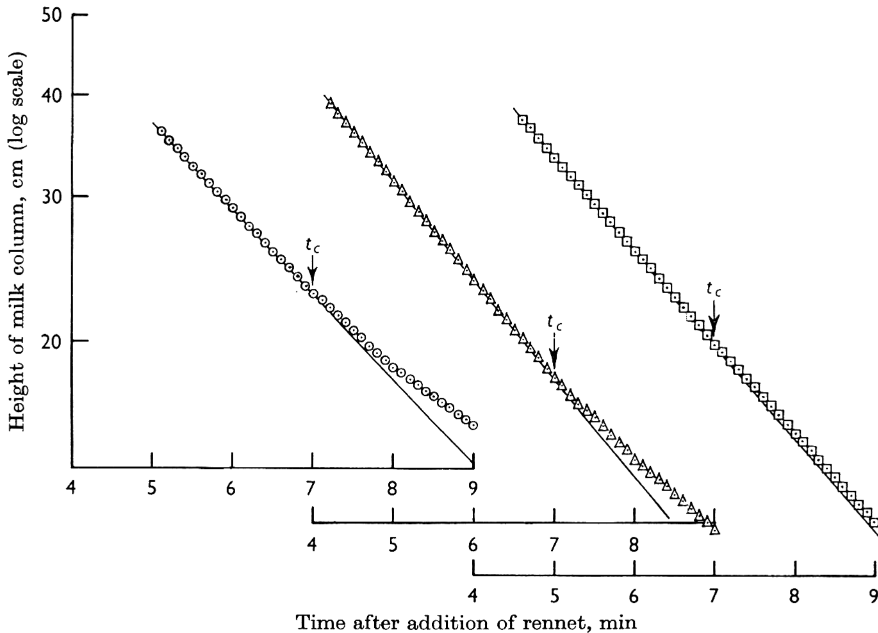

Fig. 1. Influence of holding time and additions of NEME on the value of t_c. ⊙, 17 h after adjusting pH to 6·20 control; △, 18 h after adjusting pH to 6·20, 4 mg % NEME; ⊡, 19 h after adjusting pH to 6·20 control.

other hand, was made for testing the much firmer (human) blood clots, and most milk samples ranged over only about half of the chart or even less. For these reasons, experiments were done not only on undiluted fat-free milk but also on samples diluted with one part of tap water to 2 parts of milk (1 : 2) and also 1 part of tap water to 5 parts of milk (1 : 5). The milk used came from the same herd of Friesians throughout. A typical casein analysis gave 2·57 % and it is thought that values did not differ

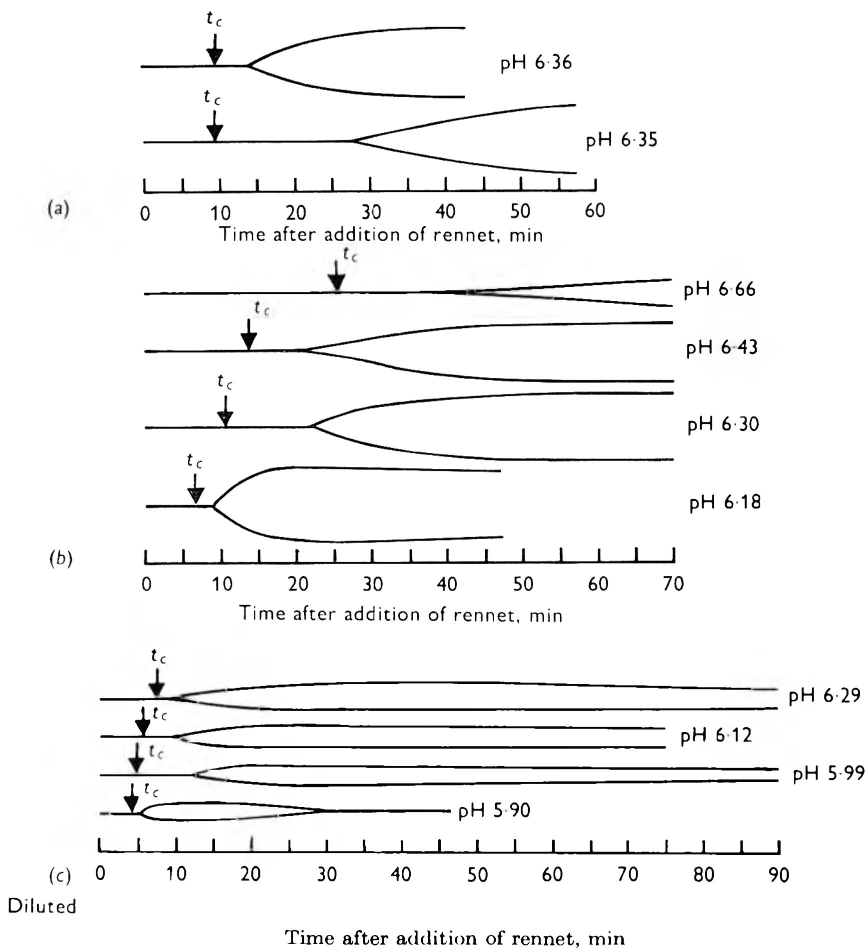

Fig. 2. Thrombelastograms showing effects of changes in holding time, pH and dilution of milk.

much from this figure during the course of the experiments. The first effect to be studied was that produced by holding the milk even when the pH did not change appreciably during the holding. It was found that holding for 4 h at temperatures between 6 and 17 °C had no effect on the value of t_c (see Figs. 1 and 2a*) but that the subsequent coagulation was slowed down. The second variable to be studied was the pH. Figure 3 shows a number of torsiometric curves plotted on logarithmic–reciprocal paper. Although these are straight lines up to G values well above those suitable for

* In the thrombelastograms, the time at which rigidity started will be seen to vary. This depended on the pH but also on the holding time of the milk.

cheese-making, it will be seen (Fig. 4) that the equation sometimes fails to hold at still higher values of G, as measured by the thrombelastogram. An experimental value of G_{max}, which is less than the theoretical G_{∞}, can be measured from thromb-

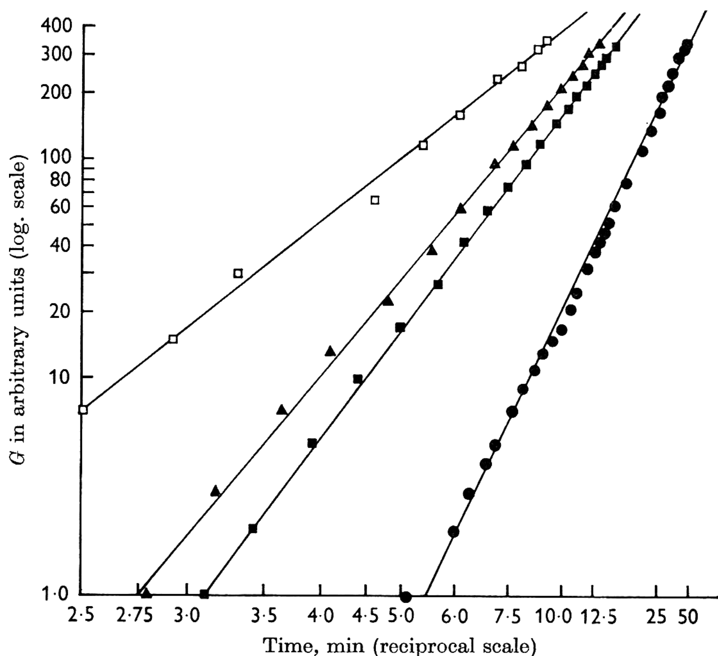

Fig. 3. Torsiometer setting curves showing effects of changes in pH of skim-milk undiluted at pH: 6·65, ●; 6·35, ■; 6·28, ▲; 6·12, □.

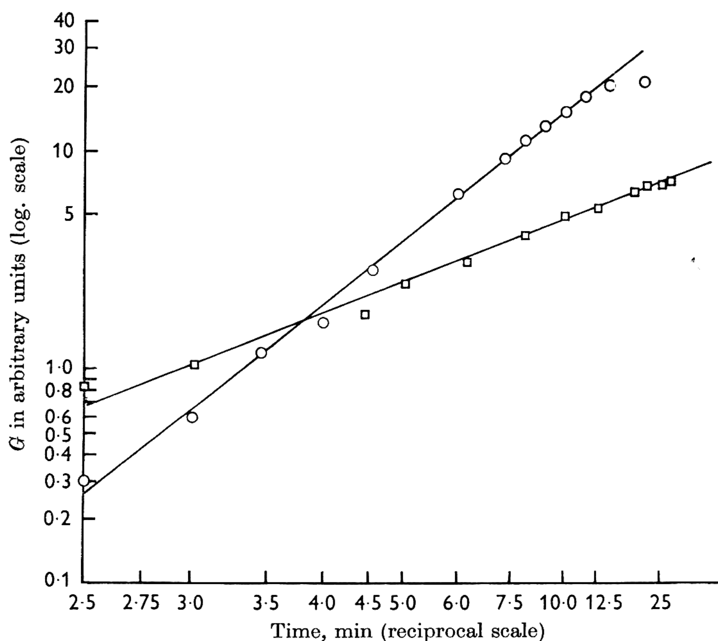

Fig. 4. Thrombelastogram setting curves for undiluted and diluted skim-milk samples. ○, Undiluted pH 6·28; □, diluted pH 6·26.

elastograms. At a still later stage, the gel sometimes softens. It is possible that the falling away from the curve of the equation is caused by an interaction of the firming and softening processes. We are somewhat doubtful about this hypothesis, however, since it does not appear to explain a very similar phenomenon in the coagulation of blood. The cause of the softening of milk gels is not known for certain but, by analogy with blood, it may well be a type of 'retraction' or syneresis on a micro-scale. Most of the softening curves are of a simple exponential type (see Fig. 5).

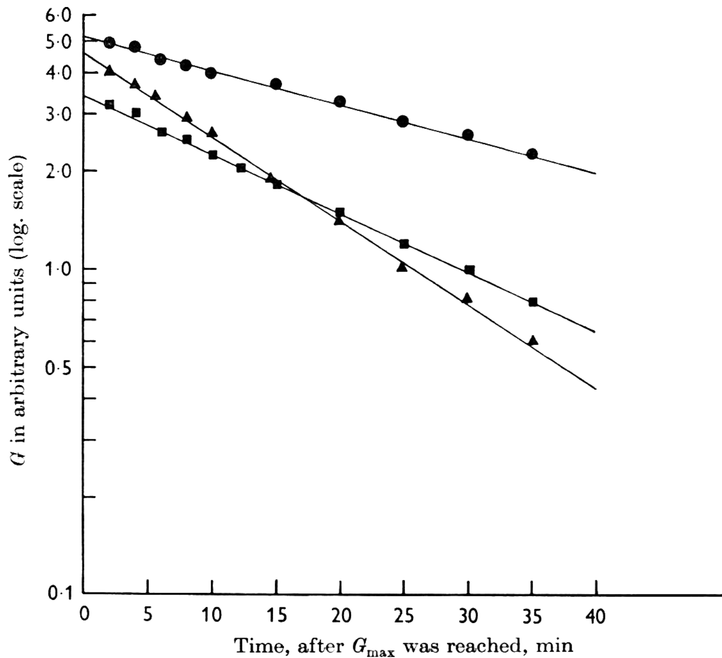

Fig. 5. Thrombelastogram softening curves for diluted skim-milk with and without addition of *p*-chloromercuribenzoic acid at different pH values. ●, Control pH 5·98; ▲, 30 mg % *p*-chloromercuribenzoic acid added, pH 5·96; ■, control pH 5·88.

It will be seen from Fig. 2*b* that, for the undiluted milk, a fall in pH from 6·66 to 6·18 does not affect G_{max}. The parameters τ and t_c and the interval between t_c and the onset of rigidity are all reduced progressively and softening is induced. A fall in pH reduces G_{max} and greatly accelerates the softening; and the magnified thrombelastograms show increased relaxation. This is shown in Fig. 2*c*.

The third factor studied was the effect of reducing slightly the calcium content of the milk by addition of potassium oxalate equivalent to 7·2 mg % and 14·5 mg % calcium. With undiluted milk, this has no effect on the value of t_c but τ is increased. G_{max} is only slightly affected at concentrations of or below 7·2 mg % but falls steeply at higher concentrations. These results are shown in Fig. 6. Experiments were done over a pH range of 6·44–6·00. The effect of pH was as expected and independent of the action of the oxalate, nor was the effect of oxalate directly influenced by pH.

The fourth and last effect to be studied was the action of the compounds *p*-chloromercuribenzoic acid and NEME, believed to be specific reagents for sulphydryl (—SH) groups. The former is insoluble in water and is dissolved in N/10 NaOH, whereas the

latter is water-soluble. Since the effect of these 2 substances on the setting of milk appears to be the same, the latter was, in general, used.

Using undiluted milk in the pH range from 6·36 to 6·02 and NEME concentrations up to 30 mg %, no marked differences either in G_{max} values or in softening of the curd could be shown. Slight differences under 4 % are due rather to the inaccuracy of the methods involved than to the processes occurring during coagulation.

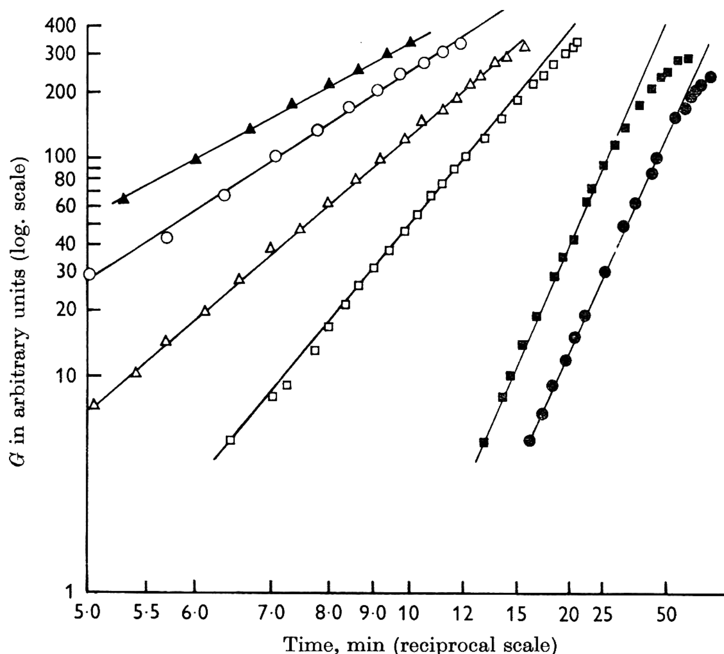

Fig. 6. Torsiometer setting curves showing influence of additions of potassium oxalate at different pH values. ▲, pH 6·16 control; △, pH 6·13, 7·2 mg % Ca removed; ■, pH 6·11, 14·5 mg % Ca removed; ○, pH 6·20 control; □, pH 6·21, 7·2 mg % Ca removed; ●, pH 6·20, 14·5 mg % Ca removed.

Using 5 parts of milk diluted with 1 part of water at pH values about 6·0 gives a decrease of G_{max} reaching about 10–14 % with increasing amounts of the blocking agent up to 11 mg %; but most of the decrease of G_{max} had been observed already by the addition of from about 2 to 4 mg % of the NEME. The further decrease with increasing amounts of the NEME was very small. The same ratio of decrease of G_{max} was observed with milk diluted 1:2 over a pH range from 6·30 to 5·97 by the addition of 3·8 mg % of the NEME. Differences in softening of the curd (shown in Fig. 7) were observed at pH values from 6·02 to 5·94, depending highly on the concentration of the NEME. Samples of milk diluted 1:2 treated with NEME up to concentration of about 2 mg % showed an increase in the rate of softening. At higher concentrations of the NEME up to 14 mg % the rate of softening falls below that of the untreated milk. With milk diluted 1:5 the concentration of NEME needed for increasing the rate of softening was higher (about 4·8 mg %).

A slow increase of the rate of setting (decrease of τ) in the early stages of the coagulation could be shown by the addition of the NEME to diluted milk, especially at low pH values (about 6·00). These results are given in Fig. 8.

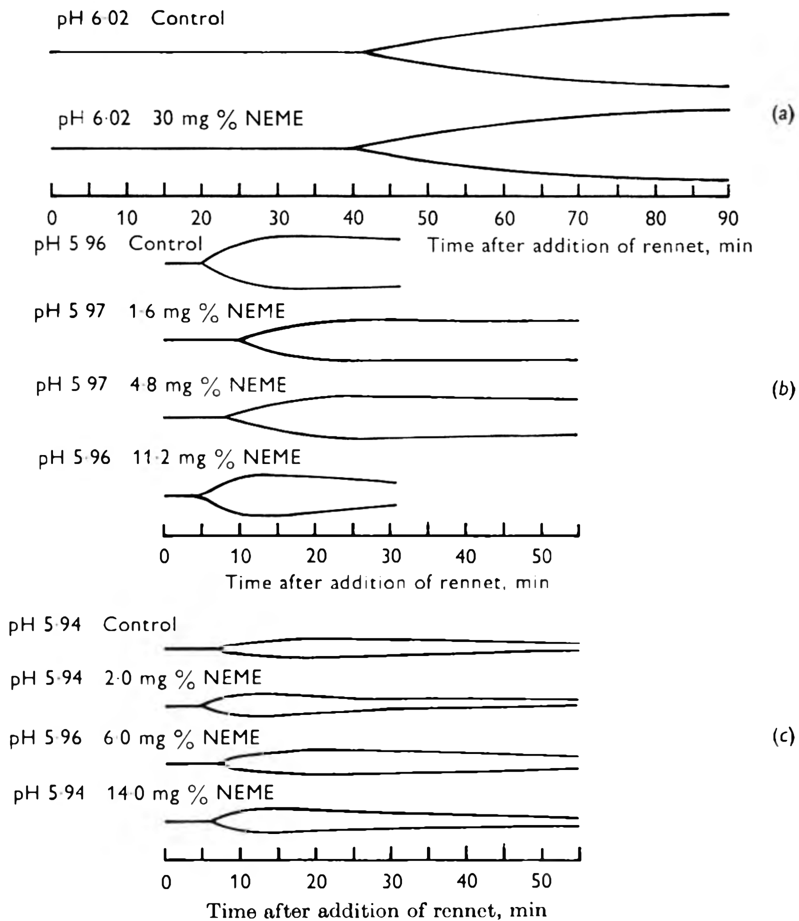

Fig. 7. Thrombelastograms showing influence of additions of NEME with milk (*a*) undiluted; (*b*) diluted (1:5) and (*c*) diluted (1:2).

DISCUSSION

The influence of holding time at low temperatures on the coagulation of milk has been studied by many authors (Schwarz & Mumm, 1948; Fricker, 1958) and the decrease of calcium content in the serum of milk during cold storage seems to be responsible for the extension of the coagulation time. Since it has been known that the term 'rennet coagulation' describes a chain of quite different reactions, it was important to find out which parts of the whole process were influenced by holding the milk at lower temperatures. If the time of addition of rennet is taken as 'zero' then 't_c' expresses the period needed for the increase of the viscosity of renneted milk. As has been previously shown (Foltman, 1959), at pH 6·3 the coagulation commences at the time of the end of the enzymic stage of rennet action. No influence on the enzymic stage of holding the milk at lower temperatures was found in our experiments, if it is correct to assume that 't_c' expresses the time of enzymic reaction. It is in good agreement with previous observations (von Hippel & Waugh, 1956) that the calcium content has no influence on the action of rennet on milk. As shown in Fig. 2*a*,

holding milk at lower temperatures causes (*a*) an extension of the time between t_c and the onset of rigidity, and (*b*) a decrease in the rate of solidification. This was also observed recently by others (Tarodo de la Fuente & Frentz, 1966). This probably means that the physico-chemical processes which follow the enzymic action of rennet are identical from the very beginning up to maximum rigidity, or, at least, they depend in the same way on the ageing of the milk. It is most probable that the rate of the whole process depends on the catalytic activity of calcium ions. It might also be due to the size changes of the micelles induced by altering the calcium equilibrium, which has been suggested as being a very slow process (Waugh & Noble, 1965). It is

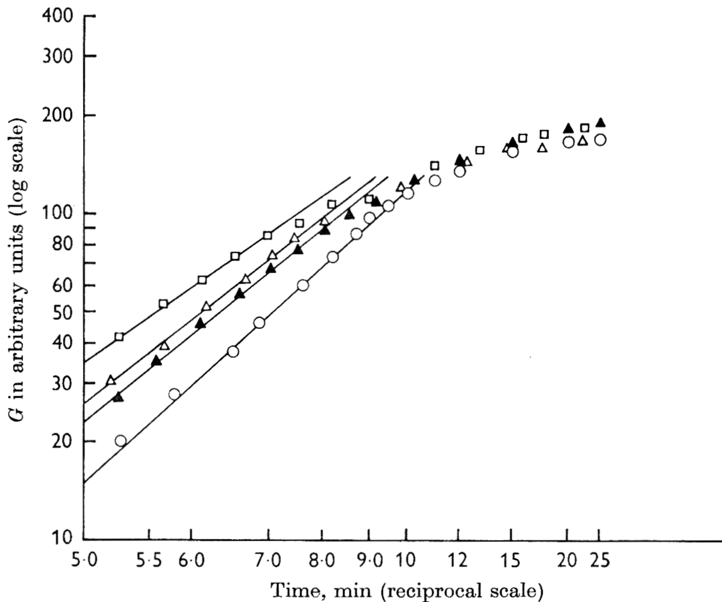

Fig. 8. Torsiometer setting curves showing the influence of additions of NEME on skim-milk diluted (1/2) at pH 5·94. ○, control; ▲, 2·0 mg % NEME; △, 6·0 mg % NEME; □, 14·0 mg % NEME.

known that lowering pH in milk causes changes in calcium equilibrium. It has been reported (Ritter, 1965) that lowering of pH to 6·2–6·3 causes only very small changes in calcium equilibrium. In the range from 6·3 to about 5·9, the colloidal calcium phosphate of the serum was solubilized and in lowering the pH from 5·9 to about 5·5, the casein-bound calcium went into solution. However, in recent experiments by one of us (W.T), it was found that the soluble calcium and phosphorus in milk serum increased progressively with lowering of pH to the values of 5·3–5·2. The amount of calcium in serum increased at a greater rate than the amount of phosphorus. They both increased more slowly at pH values below 5·4–5·3. At rather high pH values, near the natural pH of milk, the increase of soluble calcium in milk serum was also slow.

A strong dependence of the quantity of casein-bound calcium on the content of free ionic calcium and of the total amount was reported some years ago (Carr, 1953; Zittle, DellaMonica, Rudd & Custer, 1958). The investigation of the formation and structure of casein micelles (Noble & Waugh, 1965) and of the interaction of

bovine caseins with divalent cations (Chien Ho & Waugh, 1965) seems to suggest that at least 2 different calcium bonds can exist in casein micelles. Our experiments (see Fig. 2*b*) with undiluted milk seem to suggest that even on lowering the pH values to about 6·2, the final value of the complex modulus is only slightly affected. In conformity with the results obtained by Carr (1953) and by Zittle *et al.* (1958) the casein-bound calcium is involved in the equilibrium changes from the very beginning. The partial removal of calcium from casein occurs at the beginning of the reaction. This deals with that part called 'casein-bound calcium' (White & Davies, 1958), which is only to a very small extent, if at all, responsible for the value of the complex rigidity modulus.

The influence on the rate of coagulation is certainly due to the activity of ionic calcium in milk serum, which increases with the decrease of pH.

The dilution of milk with water causes changes both in the size of micelles and in the calcium equilibrium of the milk components (Hostettler & Imhof, 1951). The decrease of the value of the complex modulus by lowering pH from about 6·3 to about 5·9 seems to show that the part of the calcium which is more weakly bound to casein is removed from the micelles through dilution. This again could be in conformity with the results obtained by Carr (1953) and by Zittle *et al.* (1958). The progressive acidifying of the milk diluted 1 : 2 also causes the partial removal of that part of the casein-bound calcium which is responsible for creating bonds producing rigidity.

This suggestion is supported by the experiments with partial removal of calcium, through the addition of potassium oxalate to undiluted skim-milk. It was supposed that the concentration of added potassium oxalate is stoichiometrically related to the amount of calcium. It is also assumed that this is independent of the pH over the range investigated. If this is correct, it might suggest that small changes in calcium equilibrium in milk involve mostly that part of the casein-bound calcium which plays no role in forming bonds responsible for rigidity (see Fig. 6). Further removal of calcium causes changes in calcium equilibrium of the milk, in which the other part of the casein-bound calcium is involved. The latter takes part in producing rigidity and is responsible for the final value of the complex modulus.

It was reported by Christ (1956) that milk treated with 3·75 mg % NEME showed only slight syneresis after clotting. Since NEME is believed to be a specific reagent for —SH groups, this result suggested that —SH groups were involved in syneresis or coagulation. More recently, Hill (1964) reported that —SH groups are present in casein micelles but that they can only be detected in strongly disaggregating conditions. We have found no effect of NEME on undiluted milk at any stage of coagulation or of softening of the curd, and this result appears to rule out any involvement of —SH groups in the normal clotting of milk On the other hand, in samples of diluted milk, adding NEME affected all post-enzymic stages of the coagulation. These effects are not easy to interpret, since it is unlikely that the dilutions used caused sufficient disaggregation of the casein micelles to expose the —SH groups. Morever, the effects on the latest stages of coagulation seem to be of a rather complicated nature, as is seen in Figs. 7*b* and *c*. It seems to us that further progress on this problem may require studies on solutions of purified casein.

We are indebted to Miss H. R. Chapman for preparing the milk samples, to Mr N. Gruber for the photographic processing and special enlargements of the thrombelastograms and to Dr R. Aschaffenburg for helpful discussions on the chemical aspects of the work. We are also much indebted to Prof. H. Hartert and Messrs Fritz Hellige and Co. (Freiburg-i-B.) for the loan of the thrombelastograph.

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The folic acid activity of some milk foods for babies

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SUMMARY. Folic acid activity was determined for National Dried Milk and for 5 proprietary dried milk foods for babies, for a proprietary liquid milk baby food and for 8 brands of tinned evaporated milk. For comparison, values were determined for mature breast milk, for raw bulk cow's milk, for bottled pasteurized cow's milk and for goat's milk.

Human milk and raw and pasteurized cow's milk all had much the same folate activity, equivalent to about 54 μg folic acid/l. Values for goat's milk were much lower, around 6 μg/l.

Values for the reconstituted baby milks ranged from 9 to 65 μg/l, though only 2 of the products had markedly lower values than breast milk. The question is discussed whether the folic acid requirement in infants can be met by formulas based on cow's milk without having recourse to folic acid supplementation.

Folic acid may be of special importance to the bottle-fed infant, for whom preparations based on cow's milk constitute the main source of nutriment. Cow's milk and mature breast milk are about equally rich in folate, but the vitamin is readily lost during heat processing, and Matoth, Pinkas & Sroka (1965) found that dietary intake of folate was lower in artificially fed than in breast-fed infants and was inadequate for their needs, especially in infants being given reheated pasteurized or sterilized milk. Naiman & Oski (1964) gave a range 62–100 μg/l for cow's milk, and reported that preparations of a proprietary baby food contained substantially less, between 9 and 29 μg/l. They found wide variation in the folate content of reheated evaporated milk, which ranged from 8 to 86 μg/l. Ghitis (1966) found lower folate contents in 2 proprietary dried milk baby foods than in fresh cow's milk, and showed that the folate in a reconstituted milk powder was comparatively labile to heat.

The present paper reports the folic acid activity of National Dried Milk and of 5 proprietary dried milk foods for babies, of a proprietary liquid milk baby food, and of 8 brands of tinned evaporated milk. For comparison, values are given for mature breast milk, for bulk raw milk from the Institute herd of Friesian cows, for bottled pasteurized cow's milk as supplied by 3 large dairies in the Reading area, and for goat's milk.

EXPERIMENTAL

In all, 22 samples of human milk were obtained, from 10 mothers of babies aged between 7 days and 7 months. The samples (20–60 ml) were obtained by manual expression at the time of the baby's feeding, usually in the morning, and were stored at −30 °C until required for assay. Samples obtained in this casual manner were

perhaps not truly representative of all the milk secreted during 24 h, but for the purposes of the present study no more serious interference with the feeding routine was warranted.

The cow's milk was refrigerated, bulked, evening and morning milk from the Institute herd of Friesian cows. In all, 16 samples were taken at intervals during a period of about 12 months. In addition, pint bottles of pasteurized milk, as supplied by each of 3 Reading dairies, were obtained from roundsmen on 2 successive days. Bottled pasteurized milk was also obtained from the Institute dairy.

Goat's milk was obtained from British Saanen goats of the Institute herd. Individual samples of milk were obtained from 17 animals, at the same morning milking.

Seven baby foods were examined and are identified in this study by letters, as indicated: National Dried Milk, full cream, NDM; a proprietary dried full-cream milk food, MA; 2 proprietary half-cream milk foods, MB and MC; and a proprietary food containing added whey proteins, MD. In addition to these was included a proprietary 'filled' milk designed to simulate breast milk in gross chemical composition more closely than do infant formulas based directly on cow's milk. The product is marketed as a dry powder and also in liquid form. Samples of both were examined and are identified as ME and ME (liquid).

Tins or packets, one of each product, were purchased in Reading on 3 occasions at intervals of about 2 months; the National Dried Milk was obtained at child welfare clinics and the proprietary foods at chemists' shops. The dried milk powders were reconstituted by a uniform procedure, as follows: 15 g milk powder were weighed into a 250-ml amber-glass conical flask and dissolved, with stirring, in 180 ml distilled water that had been boiled and allowed to cool to 65 °C. The reconstituted milk was cooled to about 20 °C in a bath of cold water and finally diluted to 200 ml. ME (liquid) was reconstituted by dilution with an equal volume of the boiled water.

Two samples of each of 8 brands of tinned evaporated milk were examined, and are identified as products EM 1–EM 8. Products EM 1–EM 5 gave instructions on the tins for their use as baby foods. Before assay, the evaporated milks were diluted with 2 volumes of distilled water that had been boiled and cooled to 65 °C.

Measurement of folic acid activity. Folic acid activity was assayed microbiologically with *Lactobacillus casei*, by an adaptation of the procedure recommended by Herbert (1961) for the assay of folate in blood serum. The test extracts were prepared as follows. To a 2-ml sample of milk, in a 150 × 15 mm test tube, was added 1 ml of buffer solution, made by dissolving 1 g ascorbic acid in 100 ml (w/v) 1·42% Na_2HPO_4 solution and adding 4 N-NaOH to pH 7·8. The tubes prepared in this way were heated in a bath of boiling water for 2 min and cooled in cold water. To each was then added 1 ml of an extract of chicken pancreas, made by gently triturating 200 mg desiccated chicken pancreas (Difco Laboratories Inc., Detroit, U.S.A.) in 40 ml of ice-cold phosphate/ascorbate buffer and filtering through a plug of glass wool. The tubes, containing milk samples and added enzyme, were incubated for 2 h in a water bath at 45 °C. The contents were then acidified to pH 4·8 by addition of 0·1 N-HCl, diluted with water to 50 ml and filtered through Whatman no. 42 paper. The filtrates were readjusted to neutral pH and further diluted as needed for test.

All the milks were assayed at least twice, and the human milks 3 times. The between-assay standard deviation was about 10%.

RESULTS AND DISCUSSION

The values found for unheated human milk, cow's milk, goat's milk and pasteurized cow's milk are shown in Table 1. Human milk, raw cow's milk and the pasteurized market milk all had much the same folate activity, equivalent to about 53 μg folic acid/l. The values for goat's milk were much lower, and confirm the finding of Naiman & Oski (1964) that goat's milk is comparatively poor in folate. Tests were also done with the alternative assay organisms, *Streptococcus faecalis* and *Pediococcus cerevisiae*, which give 2 further patterns of response to the various naturally occurring congeners of folic acid. The results of these differential assays indicated that, in all 3 species, the milk folate is present predominantly as N^5-methyl tetrahydrofolate.

Table 1. *Folic acid activity* ($\mu g/l$) *in milk*

	Mean value	Range
Human milk (22 samples)	52	31–81
Cow's milk (bulked herd milk; 16 samples)	55	37–72
Pasteurized cow's milk (8 samples)	51	40–65
Goat's milk (17 samples)	6	2–11

Table 2. *Folic acid activity in milk foods for babies*

	Folic acid activity*	
Product	μg/g	μg/l after reconstitution
NDM	0·42, 0·37, 0·35	52, 46, 44†
MA	0·35, 0·47, 0·39	44, 59, 49†
MB	0·30, 0·32, 0·33	37, 40, 41†
MC	0·25, 0·24, 0·40	31, 30, 50†
MD	0·53, 0·57, 0·46	66, 71, 57†
ME	0·16, 0·20, 0·19	20, 25, 24†
ME (liquid)	—	12, 9, 10, 6‡

* Values are given for 3 samples of each milk powder, and for 4 samples of ME (liquid) (see p. 86).
† Calculated values for reconstituted milk containing 12·5 % (w/v) total solids.
‡ Values are for milk reconstituted by dilution with an equal volume of water.

Table 2 shows the folic acid activity found in the dried milk baby foods, expressed as μg/g and as μg/ml of reconstituted milk containing 12·5 % (w/v) total solids. Also listed in the table are values for ME (liquid), reconstituted by dilution with an equal volume of water. Expressed as percentages of the value of 52 μg/l found for breast milk, the averages for the different reconstituted milks were: NDM, 91 (65); MA, 98 (92); MB, 75 (97); MC, 71 (91); MD, 125 (154); ME, 44 (44), and ME (liquid), (17). The values in parentheses were calculated for milks reconstituted as for feeding month-old infants in accordance with the manufacturer's directions. The mean weights of milk powder delivered by the scoops provided with the different products ranged from 4·0 to 5·1 g. No scoop was provided with the tins of National Dried Milk, but the weight of one measure of the product was given as $\frac{1}{8}$ oz. (3·55 g).

The 2 brands of ME, and especially the ME (liquid), were comparatively poor in folate, as were also 2 of the 3 samples of MC, and the National Dried Milk reconstituted as directed on the tin. This latter low value reflects the comparatively high dilution recommended, to about 9 % (w/v) as compared with, for example, about 15 % (w/v) for MC. Comparison on the basis of 12·5 % total solids content is more

straightforward, and on this basis the National Dried Milk compares more favourably. Product MD contains additional whey proteins, with which much of the folate in milk is associated. It was not unexpected, therefore, that MD was comparatively rich in folate—richer indeed than breast milk or raw cow's milk.

No expiry date was marked on the tins of ME powder. The tins of ME (liquid) were dated, as were the tins or packets of all the other products, and all were assayed several months before this expiry date. The expiry date gave no clue to the date of

Table 3. *Folic acid activity in tinned evaporated milk*

	Folic acid activity*		
		μg/l after dilution with water	
Product	μg/l	A†	B‡
EM 1	107, 117	43, 47	36, 39
EM 2	164, 134	65, 53	55, 45
EM 3	96, 81	38, 32	32, 27
EM 4 (sweetened condensed)	164, 158	47, 45	19, 18§
EM 5	99, 95	40, 38	36, 35
EM 6	114, 104	46, 42	—
EM 7	123, 105	49, 42	—
EM 8	111, 111	44, 44	—

* Values are given for 2 samples of each product.
† Diluted to be equivalent in total solids content to fresh cow's milk.
‡ Diluted as recommended by the manufacturers for feeding a 10–11 lb baby.
§ In interpreting the instruction to dilute 3 teaspoonfuls of the milk with 4 fluid oz of water, the teaspoonful was taken as 5 ml—a generous measure.

manufacture, and it seems likely that there was wide variation in the recommended shelf life. Thus, on the tins of National Dried Milk, purchased at busy welfare clinics, the dates given were about 4 months ahead of the date of purchase and so these tins were probably more conservatively rated than the tins of product MD, for example, which were post-dated up to 21 months ahead of the date of purchase, or than the ME powder, for which no limitation on shelf life was recommended.

The folate content of ME (liquid) was little higher than that of goat's milk, the consumption of which as the sole diet is well known to cause megaloblastic anaemia. Naiman & Oski (1964) similarly reported low folate values for this product, averaging 18·8 μg/l as compared with the mean value of 89·5 μg/l which they found for market milk.

Table 3 shows the folate activity measured in 2 sample tins of each of 8 brands of tinned evaporated milk. After dilution with water to their equivalent volumes of fresh milk, the different products were broadly alike in folate potency, with mean contents that ranged from 35 μg/l for EM 3 to 59 μg/l for EM 2. It is interesting, and probably significant, that the highest values were found with product EM 2, which was the only one of the products advertised to contain added ascorbic acid. When diluted as recommended for feeding a 10–11 lb baby, the average values for the different milks, expressed as percentages of the value of 52 μg/l for breast milk, were as follows: EM 2, 97; EM 1, 72; EM 5, 68; EM 3, 58; and EM 4, 36. It is evident from the values in column A (Table 3) that this last low value derives from the apparently

excessive dilution recommended (3 teaspoonfuls of milk to 4 fluid oz of water) and not from any relative deficiency of folate in the product.

Matoth *et al.* (1965) found the blood folate concentration to be significantly lower in infants reared on reheated pasteurized or sterilized milk, or on various proprietary infant milk formulas, than in breast-fed infants. They concluded that dietary intake of the vitamin was lower in the artificially fed infants, and supported this conclusion with values averaging 14·6 μg/l for reconstituted dried milk and 11·9 μg/l for boiled pasteurized milk, as against 37·7 μg/l for fresh cow's milk. The present study shows that, excepting ME and ME (liquid), none of the processed milks was intrinsically poor in folate and that most were not markedly inferior to breast milk.

It must be borne in mind, however, that these results are indicative of the *potential* value of the milks as sources of folate, and that much of the vitamin may well be lost during the preparation of the milk for use. Reheating liquid milk is especially damaging to the folic acid. Burton, Ford, Franklin & Porter (1967) studied the effects of repeated heat treatments on the levels of some vitamins of the B-complex in milk. With folic acid, the effects were not simply additive—the first heat treatment appeared to potentiate the destructive effects of subsequent treatments. The explanation for this is that the loss of folic acid is related to the content of reduced ascorbic acid in the milk; the higher the initial level of ascorbic acid, the smaller is the loss of folic acid (Ford, 1967). Partial oxidation of the milk's ascorbic acid during the initial heating renders the folate more vulnerable to destruction during subsequent heating. Clearly, care is needed if the destruction of folic acid during the preparation of the feed is to be avoided, but the generally recommended procedure of dissolving the milk powder in warm, boiled water causes little or no loss. Perhaps less satisfactory is the common practice of preparing several feeds at one time and reheating them for use after a period of storage in a refrigerator.

Matoth *et al.* (1965) comment that the use of bottled pasteurized milk for infant feeding, and the practice of boiling it for increased safety, are widespread in the U.S.A. and in other countries. They showed, as did Ghitis (1966), that this boiling destroys most of the folate in the milk. In hospital milk kitchens stringent precautions are taken to guard against the occurrence of epidemics of diarrhoea and other milk-borne infections among newborn babies, and the preparation of the formulas generally involves more drastic heating than is necessary in the home. In many hospitals 'terminal sterilization' is practised. The reconstituted milk is dispensed into feeding bottles which are later heat-sterilized before being issued to the ward, where they may be heated yet again before use. It seems highly probable that, after all this, little of the original ascorbate or folate would remain. This loss might be of special importance in the nurture of premature babies, who are dependent on the hospital dietary for very much longer than are normal babies.

Ghitis (1966) recommended that folic acid should be added to processed milks intended for infant feeding, and suggested that a supplement of 50 μg folic acid/l reconstituted milk should afford ample protection against folate deficiency. This is consistent with an estimate from clinical experiments (Vélez, Ghitis, Pradilla & Vitale, 1963) that the daily folate requirement in infants is of the order 5–20 μg. Matoth *et al.* (1965) similarly put the requirement at 20 μg/day. Taking 20 μg to be the daily requirement, all the present milks amply provided this quantity, except

ME and ME (liquid), and product EM 4 diluted for use according to the maker's instructions. However, 20 μg/day allows little margin for safety and should be regarded as the minimal requirement. A daily intake of 800 ml breast milk (cf. Kon & Mawson, 1950), containing 52 μg folate/l, would supply 42 μg—or more than double this estimated minimal requirement. As so much uncertainty attends the estimate, it might be prudent to take 40 μg, as provided by breast milk, to be the daily requirement for optimum nutrition. The question then arises whether this could be supplied by formulas based on cow's milk, without having recourse to folic acid supplementation. Out of the 8 tinned evaporated milks only EM 2, to which ascorbic acid is added during manufacture, would provide this amount, as would 2 of the 6 milk powders. The deficit in the remaining products was generally small and could undoubtedly be reduced by improvements in the manufacturing process. The loss of folate, as of vitamin B_{12}, is a consequence of the oxidative destruction of the milk's ascorbic acid (Ford, 1967) and careful exclusion of oxygen from the milk during processing and subsequent storage may offer a means of stabilizing all 3 of the vitamins in liquid milk products. Alternatively, as in the manufacture of product EM 2, supplementation with ascorbic acid might be more practicable, as it would be also in the spray drying of milk, where the exclusion of oxygen is not feasible.

Given that the natural folate in milk can be conserved during manufacture, then careful preparation of the food in the home, for immediate use, is unlikely to cause significant loss. The situation is clearly different with milk used in hospitals, and a case can be made out for supplementation with folic acid, which is probably less labile than the natural folate of milk. But against this it may be argued that unsweetened evaporated milk is sterile as also, for practical purposes, are dried-milk baby foods. If these products are reconstituted with sterile water and dispensed into sterile bottles, by trained nurses working under strictly hygienic conditions, there should be no need for further drastic heating. 'Terminal sterilization' seems to be designed mainly to cover the possibility of faulty preparation.

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Note added in proof. We are informed by the manufacturers of the milk foods here designated ME and ME (liquid) that they are actively considering the possibility of supplementing the products with folic acid.

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A new approach to the measurement of the quantitative effects of inherent and environmental factors on the composition of the milk of individual cows and of herds, with particular reference to lactose content

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SUMMARY. (1) A tentative scheme is proposed for the quantitative estimation of the effects of various factors on one major milk constituent, lactose, from analyses of the milk of individual cows within herds. The potential lactose content in the milk of individual cows is predicted from the observed potassium-to-lactose ratio in the milk, and the difference between the predicted potential and the actual lactose content is partitioned into fractions that are attributed to effects due to age, inter-quarter difference and changes with stage of lactation. The scheme was applied to 2 commercial herds, one (herd A) producing milk of low and the other (herd B) milk of normal SNF content.

(2) Lactation mean values for fat, SNF and lactose contents of the milk of individual cows showed a wide range of the same order in each of the 2 herds. Of the mean difference in SNF percentage of 0·30 between the herds, 0·14, or 47 %, was due to a difference in lactose content.

(3) The predicted potential milk lactose content of the cows in the 2 herds ranged from 5·04 to 5·66 (g/100 g milk water). Herd mean values were 5·394 for herd A and 5·244 for herd B.

(4) Herd mean values for the effect on the lactose content (g/100 g milk water) of age, of changes with stage of lactation and of inter-quarter difference were −0·127, −0·080, and 0·073 respectively in herd A, and −0·197, −0·058 and −0·078, respectively, in herd B.

(5) The relative importance of the various factors in accounting for differences between the herds in the lactose content of their bulk milk were: predicted potential lactose content 61 %, effect of age 28 %, effect of changes with stage of lactation 9 % and effect of inter-quarter difference 2 %.

(6) The errors attached to the estimation of potential milk lactose content and the effects of age, of changes with stage of lactation and of inter-quarter difference are discussed.

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The main factors that influence the chemical composition of milk—the breed and individuality of animals, stage of lactation, age or lactation number, infection of the udder and level and type of feeding—are well established, but the importance of each of these factors in determining the differences between commercial herds in the composition of their bulk milk is still uncertain. The quantitative effects on milk composition of these different factors have been determined singly under defined, experimental conditions (see Rook, 1961) and this evidence suggests that only genetic potential, age (or lactation number) and level and type of feeding could make an important contribution to the observed differences between herds in the composition of their bulk milk. Changes in milk composition due to stage of lactation are unlikely to contribute to between-herd differences in milk composition, since they are due to inevitable, physiological changes that are common to all cows, and, because of the reduced yield of milk from infected quarters, udder infections would not be expected to influence materially the composition of bulk milk. Rowland, Neave, Dodd & Oliver (1959) and O'Donovan, Dodd & Neave (1960) determined, from data collected in one herd, the effect of udder infection on the fat and solids-not-fat (SNF) content of the milk of infected quarters and of the bulk milk of infected cows. On the basis of these results, they calculated that even if 50 % of all cows in a herd had 2 quarters of the udder infected for 4–5 months in each lactation, herd average percentages for fat and SNF would each be depressed by not more than 0·05.

Investigations with commercial herds in which estimates of heritability of inter-herd variation in milk composition were made, have indicated that about 50 % of the differences between herds in fat content (Robertson & Rendel, 1954; Wiener, 1960; Brumby, 1961), about 25 % of the differences in SNF content and about 50 % of the differences in protein content (Brumby, 1961) are due to differences in genetic potential. The observations of Foot, Line & Rowland (1961) (see also Foot, 1964), who compared the SNF content of the milk of heifers brought from a number of commercial herds and kept as a single herd with that of contemporary heifers remaining on the farms, are in broad agreement with the above estimate for SNF content. Whilst there was some evidence of differences between herds in genetic potential, between-herd differences in SNF content were due mainly to the effects of farm environmental factors, of which the level of herd feeding appeared to be the most important.

From some farm surveys, the conclusion has been reached that the level of feeding on the farm and the incidence of mastitis are the main factors that determine the differences between herds in SNF content (Burr, 1939; Edwards, 1958) and that genetic potential, age and stage of lactation are relatively unimportant (Edwards, 1958). Others, however, have attached a greater importance to differences in genetic potential (McLean, 1951; Provan, 1956; Munro & Bailey, 1958; Holland, 1960), and in an investigation of herds in the West Midlands producing milk low in SNF, Griffiths & Featherstone (1957) failed to obtain an improvement in SNF content in response to improved feeding and they concluded that, at least for the herds studied, the primary cause of low SNF content was a poor genetic potential. In a later study with 10 herds showing a wide range of SNF content, Alexander & Leech (1960), using an analysis of variance technique, found that 70 % of inter-herd differences in SNF content was related to differences in age, stage of lactation, cell count and breed, and

they were unable to demonstrate an association between herd feeding level and SNF content.

Whilst it is reasonable that the quantitative importance of different factors should vary to some extent from investigation to investigation, the marked lack of conformity between results is surprising and must raise doubts as to the validity of some of the information collected. Discrepancies may have arisen from an inadequate measurement of certain of the factors studied. The accurate measurement under field conditions of plane of nutrition, and of incidence of udder infection in particular, would have required far greater technical facilities than those described in the reports. Alternatively, it is possible that the experimental methods themselves are at fault and that even with much more accurate measurement of the various factors they might still have failed to demonstrate a more consistent pattern. Technical considerations apart, there is an important criticism of the value of these approaches. None can readily be used for the measurement of the quantitative importance of the effect of each of the factors on milk composition in an individual animal or in an individual herd.

In view of the lack of progress made with the above methods, an alternative approach has been sought in which the effects of various factors on milk composition could be determined directly from analyses of the milk itself. A tentative scheme for one major milk constituent, lactose, has been worked out, and it has been examined by applying it to 2 commercial herds, one producing milk of low and the other of normal SNF content.

EXPERIMENTAL

A scheme for the separation of the effects of various factors on milk lactose content

The lactose content of the milk of individual animals appears to be at a maximum in the milk from uninfected quarters in the mid-part of the first lactation (Rook & Wood, 1959; Walsh & Rook, 1964; Rook & Campling, 1965). For the purposes of the proposed scheme, this value will be taken as an index of the potential milk lactose content, and when the lactose content of milk actually secreted is less than this value, the loss in lactose content will be assumed to have arisen from a change in composition due to stage of lactation, age or infection of the udder. Effects of feeding will be ignored. There is good experimental evidence that milk lactose content is depressed in early and late lactation, by age and by infection of the udder: variations in level or type of feeding, with the exception of marked underfeeding, have been shown to be without effect on milk lactose content (see Rook, 1961).

The scheme formulated requires the measurement of the lactose content of the milk secreted by an individual animal during the course of an investigation and of the potential lactose content as defined above, and the quantitative allocation of the difference between these 2 values to effects ascribed to changes with stage of lactation, age and infection of the udder. In commercial herds, a direct measurement of potential lactose content would not be possible for a majority of animals, because of their stage of lactation, age or the presence of udder infection, and an indirect approach is required. The methods proposed for the prediction of potential lactose content and for the quantitative assessment of the reduction in lactose content due to the various environmental factors are set out below.

Prediction of potential milk lactose content. In an earlier paper (Walsh & Rook, 1964), an investigation was made of variations in the lactose and potassium concentrations in the milk of Friesian heifers free from infections of the udder and in mid-lactation. Approximately constant values were obtained for the milk of individual animals and an inverse relationship between the lactose and potassium contents of the milk of different animals was demonstrated. The following highly significant ($P < 0{\cdot}001$) relationship was found:

$$L = 7{\cdot}4242 - 0{\cdot}01095K, \qquad (1)$$

where L is the lactose content (g/100 g milk water) and K is the potassium content (mg/100 g milk water). The lactose and potassium contents of milk had been shown previously to decrease in early and late lactation, with age and with infection of the udder, but the potassium-to-lactose ratio appeared to be largely independent of these changes and to be characteristic of the individual animal. In the paper, the potassium-to-lactose ratio was shown to be independent of age in a single animal only, but additional evidence has since been obtained (J. P. Walsh, unpublished). The effects of level or type of feeding on the potassium-to-lactose ratio were not directly examined, but in separate experiments, lactose (see Rook, 1961) and potassium (Meyer, 1964; Dawes, 1965; van Koetsveld, 1965) contents have been shown to be little affected by diet. Also, by comparing the members of monozygotic twin pairs and dams with daughters the ratio was shown to have a high heritability. In commercial herds of Friesian cattle, in which the performance of the heifers is found to conform with the relationship, the potassium-to-lactose ratio would appear to offer a possible basis for the prediction of the milk lactose content of infection-free quarters in the mid-part of the first lactation. Potential lactose content (g/100 g milk water) will, therefore, be predicted using the following formula derived from the equation (1):

$$\text{potential lactose content} = \frac{7{\cdot}4242}{1 + 0{\cdot}01095R}$$

where R is the ratio of potassium (mg/100 g milk water) to lactose (g/100 g milk water). Values are expressed on a water basis to exclude the effects of variation in the concentration in milk of other constituents.

The quantitative allocation of the difference between the potential lactose content and the actual lactose content to effects due to changes with stage of lactation, age and infection of the udder. The direct measurement of actual lactose content and the prediction of potential lactose content may be based on analyses of the bulked milk from all 4 quarters of the udder. The procedure proposed for the assessment of the effects of changes with stage of lactation, age and infection of the udder require the analysis of milk from the separate quarters. The quarter of the udder showing the highest lactose content (g/100 g milk water) in the mid-part of the current lactation will be referred to as the 'best' quarter.

Effect of age. This will be measured as the difference between the potential lactose content (g/100 milk water) and the lactose content (g/100 g milk water) of milk secreted in mid-lactation by the best quarter. When infections are present in all 4 quarters, the observed difference will result partly from current infections and will be

a measurement of all previous and present causes of loss of lactose content in the best quarter.

Effect of changes with stage of lactation. This will be measured as the difference between the lactose content (g/100 g milk water) of milk secreted in mid-lactation by the best quarter and the weighted lactation mean value for the lactose content (g/100 g milk water) of the milk from the same quarter.

Effect of infection of the udder. This will be measured as the difference between the weighted lactation mean value for the lactose content (g/100 g milk water) of the milk of the best quarter and the weighted lactation mean value for the lactose content (g/100 g milk water) of the milk from all udder quarters, and will be referred to subsequently as the inter-quarter difference.

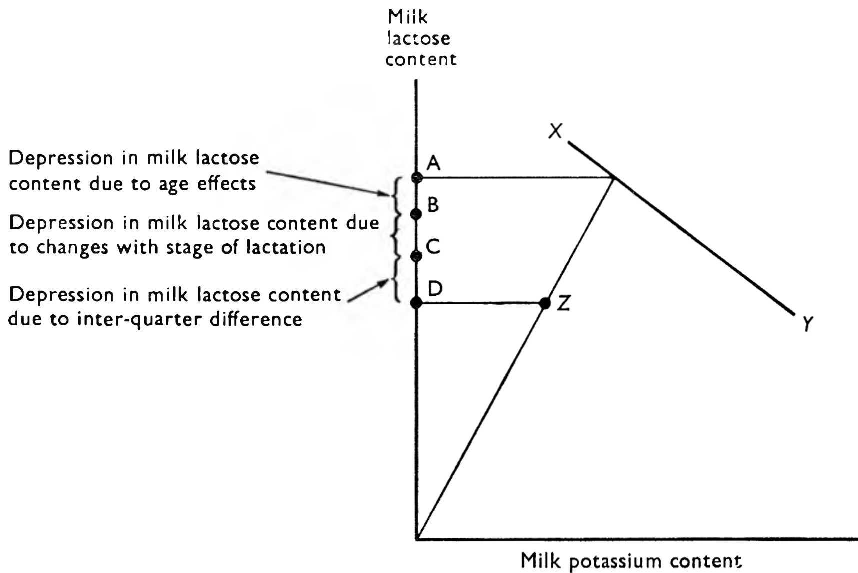

Fig. 1. Diagrammatic representation of the scheme for the prediction of potential lactose content and the allocation of the difference between predicted potential and actual lactose content to effects due to changes with stage of lactation, age and inter-quarter difference. X, Y is the regression line defining the inverse relationship between the lactose and potassium contents of the milk of Friesian heifers free from infections of the udder and in mid-lactation. Z is defined by the observed lactation mean values for lactose and potassium contents of the bulk milk of the udder. The lactose value 'A', the predicted potential lactose content, is given by the intercept with the line X, Y of a line passing through the origin and point Z. The lactose value 'B' is the mean lactose content of milk secreted by the 'best' quarter in mid-lactation. The lactose value 'C' is the lactation mean value for the lactose content of the 'best' quarter. The lactose value 'D' is the lactation mean value for the lactose content of the bulk milk of the udder.

A diagrammatic representation of the scheme for the prediction of potential lactose content and the quantitative allocation of the difference between the potential and actual lactose content to effects due to changes with stage of lactation, age and inter-quarter difference is given in Fig. 1.

The herds

Details of the herds and of their management are as follows:

Herd A. A pedigree Friesian herd of 21 cows. Cow replacements were bred and reared on the farm and for some years exclusive use had been made of the Artificial Insemination Service of the Reading Cattle Breeding Centre. The herd was mainly autumn-calving.

The cows were machine-milked in a cowhouse using Gascoigne double-stretch liners and at a pulsation rate of 48 cycles/min, a pulsation ratio of 45:55 and a pipeline vacuum of 15 inHg.

For a number of years the SNF percentage in the bulk milk produced by the herd was about average for the breed, ranging from 8·60 to 8·72.

Herd B. A pedigree Friesian herd of 28 cows. Cow replacements were bred and reared on the farm. Although the Artificial Insemination Service of the Reading Cattle Breeding Centre was being used exclusively at the time of the investigation, half of the cows in the herd had been sired by one bull. The herd was mainly autumn-calving.

The cows were machine-milked in a bail using Alfa-Laval hooded liners at a pulsation rate of 51 cycles/min, a pulsation ratio of 65:35 and a pipeline vacuum of 21 inHg.

In the 4 years before this investigation the SNF percentage in the bulk milk of the herd was consistently substandard, ranging from 8·32 to 8·48.

Sampling and methods of analysis

Milking and milk-sampling. At predetermined intervals throughout a single lactation, animals were milked at consecutive evening and morning milkings with a machine designed for the separate collection of milk from the quarters of the udder. The brand of liners, the pulsators, pulsation rate and ratio were as used by the farmers on other occasions. At each milking, the milk from each udder quarter of each cow was weighed, a sample taken, and a weighted composite sample of the evening and morning milk for each quarter was prepared for chemical analysis. Immediately before the evening milking, 10 ml of foremilk were taken from each quarter for bacteriological examination.

Milking for experimental purposes was done at 14-day intervals between the 5th and the 112th day of lactation, and during the last 60 days. In the intervening period the frequency of testing was reduced to once every 28 days. From an analysis of existing data this frequency of testing was found to give about the same order of standard error attached to the mean lactose contents calculated for each of the 3 periods.

Bacteriological methods. The foremilk sample, taken after careful disinfection of the teat orifice with alcohol (70 %, v/v), was studied by plating about 0·5 ml fresh milk on aesculin blood agar, incubating for 2 days at 37 °C and then recording the numbers and types of all colonies. A coagulase-negative test was the basis for distinguishing micrococcal from staphyloccocal colonies. A Whiteside, test, using the method of Murphy & Hanson (1941), and a cell count, using the method of Breed (1929), were done on all samples.

An udder quarter was classified as infected if at any stage in the lactation patho-

genic bacteria were found on more than 2 consecutive sampling occasions and indirect tests (Whiteside and cell counts) were positive. In certain cases, also, a positive Whiteside or cell count result in the absence of pathogens or in the presence of micrococci or non-haemolytic corynebacteria was regarded as indicating infection if comparable cell counts were not obtained for other quarters: it should not be inferred that pathogens had not been the cause of infection, as the infection could have arisen and received therapy between the monthly samplings, or disappeared spontaneously.

Chemical methods. Milk samples were analysed for fat by the Gerber method (British Standards Institution, 1955) and for total solids gravimetrically (British Standards Institution, 1951). Lactose was determined by a modification of the method of Hinton & Macara (1927) and potassium by flame photometry of a suitably diluted trichloroacetic acid filtrate of milk (1 vol. of milk: 4 vol. of 10% (w/v) aqueous solution of trichloroacetic acid), using a Unicam S.P. 900 flame photometer.

RESULTS

The name, the last date of calving, the number of days in lactation, the lactation number, the frequency of testing, the mean daily yield and the weighted lactation averages for fat, SNF and lactose contents for each of the cows in each of the 2 herds are given in Tables 1 and 2. The herd mean values for daily milk yield, and fat, SNF

Table 1. *Details for the cows in herd A*

Cow	Calving date	Length of lactation, days	Lactation no.	No. of tests	Weighted lactation mean values* for the contents in milk of: Fat, %	SNF, %	Lactose, %	Mean daily yield, lb
Beauty 2	24. ix. 62	318	1	15	3·98	9·12	4·86	23·9
Damsel 3	1. xii. 62	294	3	15	3·84	8·86	4·58	37·6
Darkie	1. x. 62	304	1	15	3·79	8·68	4·70	22·9
Dimple 4	13. ix. 62	315	2	15	4·36	9·23	4·56	30·9
Dimple 5	13. ix. 62	287	1	14	4·48	9·15	4·61	14·9
Dimple 6	8. ix. 62	315	1	15	3·94	9·02	4·66	25·3
Doris 2	18. xi. 62	326	3	16	3·96	8·35	4·55	50·5
Dreamie 2	6. xi. 62	302	5	14	3·94	8·76	4·41	50·2
Dreamie 4	23. xii. 62	305	2	16	3·92	8·70	4·58	43·4
Dreamie 6	24. ix. 62	317	1	15	3·83	9·05	4·78	21·8
Ida	20. xi. 62	317	1	15	3·75	8·86	4·99	32·8
T. Maude	1. i. 63	401	10	18	3·32	7·82	4·25	55·7
C. Maude	17. xi. 62	292	4	14	3·87	8·03	4·18	54·2
Maude 2	28. x. 62	332	2	18	3·97	8·32	4·32	41·5
Maude 3	16. xii. 62	259	1	13	4·30	8·90	4·54	29·5
Maude 4	13. ix. 62	287	1	14	3·29	8·21	4·42	23·4
Sheila 2	13. ix. 62	315	1	15	3·89	8·85	4·70	23·3
Stella	17. ii. 63	353	8	16	3·99	8·52	4·30	54·6
Stella 2	29. viii. 62	344	2	16	3·90	8·92	4·54	39·6
Sulky	23. viii. 62	368	4	19	3·83	8·18	4·11	42·8
Sweetie	1. x. 62	311	2	15	3·56	8·64	4·59	44·2
Mean value		317·2	2·7	15·4	3·854	8·570	4·478	36·93
Range					3·29–4·48 (1·19)	7·82–9·15 (1·33)	4·11–4·99 (0·88)	14·9–55·7 (40·8)

* Weighted according to milk yield and omitting records for fat, SNF and lactose for the first 4 days in lactation.

and lactose contents were all considerably higher for herd A than for herd B. Of the mean difference in SNF percentage of 0·30 between the herds, 0·14, or 47 %, was due to a difference in lactose content. A narrower range of mean daily milk yield was obtained for cows in herd B than in herd A but the lactation mean values for fat, SNF and lactose content showed a wide range of the same order in each of the 2 herds.

Table 2. *Details for the cows in herd B*

Cow	Calving date	Length of lactation, days	Lactation no.	No. of tests	Weighted lactation mean values* for the contents in milk of: Fat, %	SNF, %	Lactose, %	Mean daily yield, lb
Blackbird 1	14. x. 62	362	8	19	3·16	8·20	4·23	35·6
Blackbird 3	5. vii. 62	273	2	13	3·10	8·14	4·26	30·2
Blackie	12. xii. 62	317	10	15	3·04	7·83	4·03	34·2
Charm	11. viii. 62	237	2	13	2·68	7·50	4·10	29·9
Clover	27. x. 62	279	6	15	3·90	8·10	4·11	31·0
Clover 2	10. x. 62	282	4	14	3·72	8·60	4·61	39·7
Collona	8. i. 63	290	1	16	3·57	8·78	4·71	24·8
Colonist	6. xi. 62	277	4	14	3·42	8·07	4·18	32·1
Contented	12. vii. 62	295	1	17	3·83	8·70	4·66	21·5
Deirdre 1	14. vii. 62	307	3	16	3·52	8·19	4·27	30·7
Dot 2	31. x. 62	275	3	15	3·25	8·05	4·36	28·1
Favourite 2	26. vii. 62	337	1	16	3·68	8·47	4·41	20·2
Foundation 2	7. vii. 62	252	3	14	3·20	8·06	3·96	20·9
Graceful	25. v. 62	245	7	14	3·50	8·47	4·42	36·2
Jess	28. x. 62	306	6	16	3·39	8·30	4·37	32·7
Jess 1	7. vii. 62	294	2	16	3·35	8·38	4·46	32·5
Marigold	26. xii. 62	282	3	16	3·74	8·43	4·44	26·3
Marigold 2	18. vi. 62	287	1	14	3·26	8·85	4·76	22·4
Martha	15. vii. 62	313	1	17	3·80	8·96	4·66	25·3
Rhoda	8. vii. 62	237	3	13	3·37	8·29	4·26	27·3
Rhoda 2	21. vii. 62	258	2	15	3·60	8·17	4·31	30·4
Rhoda 3	26. vii. 62	274	2	16	3·75	8·72	4·44	23·6
Rita	18. xii. 62	275	6	13	3·85	8·74	4·39	28·4
Rita 5	7. vii. 62	315	1	15	3·16	7·96	4·38	27·7
Ruth 2	1. xi. 62	323	1	16	3·05	8·18	4·51	32·4
Snowdrop 2	10. x. 62	282	3	14	3·23	7·95	4·05	32·2
Suzette 1	28. v. 62	224	4	11	3·32	7·89	4·04	29·2
Suzette 2	20. vii. 62	168	2	10	3·25	7·95	4·05	36·9
Mean value		280·9	3·3	14·8	3·409	8·273	4·338	29·28
Range					2·68–3·90 (1·22)	7·50–8·96 (1·46)	3·96–4·76 (0·88)	20·2–39·7 (19·5)

* Weighted according to milk yield and omitting records for the first 4 days in lactation.

The separate effects of the various factors on milk lactose content

Predicted potential lactose content. For each cow in each herd a mean value for potassium content (mg/100 g milk water) and for lactose content (g/100 g milk water) was calculated for a 98-day period beginning with the 11th day of lactation (referred to hereafter as the 'mid-lactation' period) from the values for all udder quarters. This period, though chosen arbitrarily, was sufficiently long to permit, with the frequency of testing adopted, an accurate estimate of mean values, and yet it did not extend into those periods of lactation when slight changes in the ratio of lactose to potassium content in milk occur.

The relationship between the potassium and lactose contents found for the milk of infection-free quarters of the udders of heifers in the 2 herds conformed with that found previously (Walsh & Rook, 1964) for a majority of other Friesian heifers (Fig. 2) and the potential milk lactose content of each animal in the herds was therefore predicted from the formula (see p. 94) and the results are given in Tables 3 and 4. The range of values was from 5·18 to 5·66 in herd A and from 5·04 to 5·50 in herd B. The difference between the herd mean values for predicted potential lactose content was 0·150 g/100 g milk water.

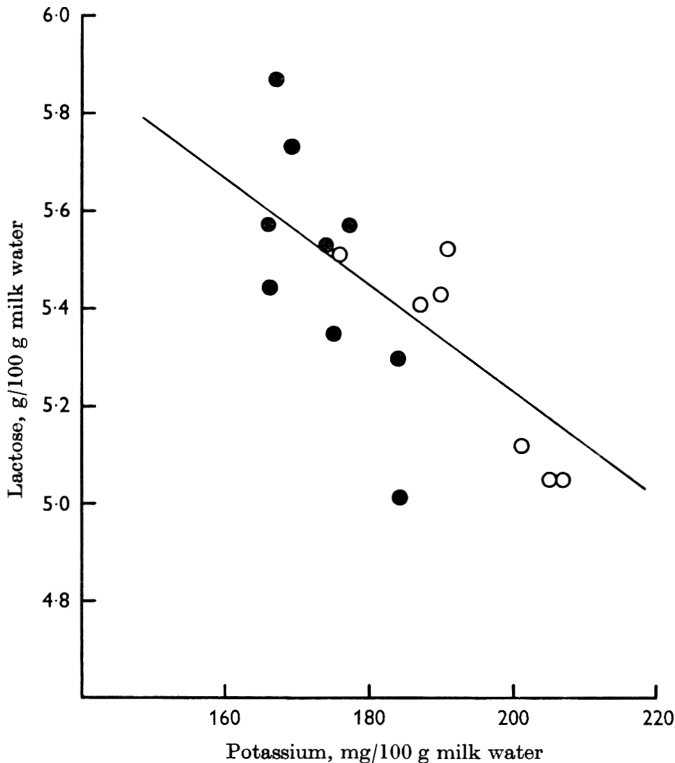

Fig. 2. The relationship between the lactose and potassium contents of the milk for 9 heifers in herd A, ●, and for 7 heifers in herd B, ○. Values are for milk obtained from infection-free quarters of the udder when the cows were in mid-lactation. The line is $Y = 7{\cdot}4242 - 0{\cdot}01095X$ (see Walsh & Rook, 1964).

Effect of age. The results calculated as described previously from analyses made over the mid-lactation period as defined above are given in Tables 3 and 4. Since the potential lactose content is predicted from a relationship determined from average values for all udder quarters, the use of the best-quarter value in the measurement of the effect of age will give a slight under-estimate. In heifers, the 'age' effect would, therefore, be expected to vary about a slightly positive mean value. For the frequency of testing adopted it was calculated that the probability of an over-estimate of a positive age effect or an under-estimate of a negative age effect of more than 0·07 was less than 0·05. For the effect of age to be significant ($P < 0{\cdot}05$) in an

individual animal it would have to be greater than $\pm 0{\cdot}20$. No bias, however, would be introduced between herds.

Within each herd there was a considerable range of values. Though the size of the effect tended to increase with lactation number (Table 5), considerable variation within each lactation number group was found. Herd mean values for the effect of age on lactose content, calculated from the values for individual cows weighted

Table 3. *The predicted potential lactose content and the effect of* (*a*) *age,* (*b*) *changes with stage of lactation and* (*c*) *inter-quarter difference and udder infection on the lactose content in the milk of each cow and for the herd bulk milk of herd A*

Cow	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of				No. of infected quarters
				(*c*)		
		(*a*) Age	(*b*) Changes with stage of lactation	Inter-quarter difference	Udder infection	
Beauty	5·61	+0·15	−0·15	−0·02	—	—
Damsel 3	5·37	+0·04	−0·14	−0·04	−0·02	1
Darkie	5·50	+0·12	−0·23	−0·03	—	—
Dimple 4	5·47	−0·11	−0·04	−0·05	−0·02	2
Dimple 5	5·53	−0·01	−0·08	−0·10	−0·06	2
Dimple 6	5·46	−0·10	±0·00	−0·02	—	—
Doris 2	5·58	−0·01	−0·24	−0·15	−0·13	1
Dreamie 2	5·51	−0·40	+0·08	−0·14	−0·14	3
Dreamie 4	5·45	−0·08	−0·02	−0·11	−0·07	2
Dreamie 6	5·60	+0·04	−0·08	−0·08	—	—
Ida	5·66	+0·28	−0·19	−0·04	—	—
T. Maude	5·25	−0·37	−0·07	−0·03	+0·01	2
C. Maude	5·26	−0·41	−0·09	−0·02	−0·01	1
Maude 2	5·19	−0·17	−0·05	−0·04	−0·02	1
Maude 3	5·38	+0·05	−0·14	−0·06	—	—
Maude 4	5·29	−0·26	+0·01	−0·05	−0·03	1
Sheila 2	5·56	−0·06	−0·05	−0·06	—	—
Stella	5·32	−0·05	−0·20	−0·16	+0·02	2
Stella 2	5·40	−0·10	−0·05	−0·04	0·00	2
Sulky	5·18	−0·35	−0·01	−0·16	−0·16	3
Sweetie	5·30	−0·07	+0·03	−0·04	−0·04	3
Herd mean value*	5·394	−0·127	−0·080	−0·073	−0·037	

* Weighted according to the yield of milk water of individual cows.

according to lactation yields of water, were $-0{\cdot}127$ for herd A and $-0{\cdot}197$ for herd B. In a majority of cows the best quarters were free from infection as judged by bacteriological tests, but 5 of the animals in herd B had infections in all quarters. Part of the age effect in these cows would thus be caused by present infection and this would contribute to the observed mean difference between the herds in the effect of age of 0·070 (Tables 3 and 4).

Effect of changes with stage of lactation. The results are given in Tables 3 and 4. With but few exceptions the effects were negative. The only marked positive effect, of $+0{\cdot}120$, was obtained for Snowdrop 2, one of the cows in which the best quarter was infected. Both the range of values in the 2 herds and the herd weighted mean values, of $-0{\cdot}080$ in herd A and $-0{\cdot}058$ in herd B, were similar.

Table 4. *The predicted potential lactose content and the effect of (a) age, (b) changes with stage of lactation and (c) inter-quarter difference and udder infection on the lactose content in the milk of each cow and for the herd bulk milk of herd B*

	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of				
			(b)	(c)		
Cow		(a) Age	Changes with stage of lactation	Inter-quarter difference	Udder infection	No. of infected quarters
Blackbird 1	5·29	−0·43	+0·03	−0·11	−0·11	4
Blackbird 3	5·08	−0·24	−0·01	−0·03	−0·02	1
Blackie	5·04	−0·33	−0·06	−0·13	−0·13	4
Charm	5·09	−0·32	−0·06	−0·14	−0·07	1
Clover	5·23	−0·47	−0·06	−0·03	−0·03	4
Clover 2	5·49	−0·01	−0·12	−0·10	−0·05	2
Collona	5·50	±0·00	−0·10	−0·03	+0·01	3
Colonist	5·48	−0·32	−0·22	−0·22	−0·22	3
Contented	5·39	+0·02	−0·06	−0·02	+0·01	2
Deirdre 1	5·06	−0·19	+0·01	−0·04	−0·01	1
Dot 2	5·22	−0·21	−0·06	−0·03	+0·07	2
Favourite 2	5·14	−0·08	−0·01	−0·03	−0·02	1
Foundation 2	5·22	−0·44	−0·16	−0·15	−0·15	3
Graceful	5·35	−0·18	−0·06	−0·10	−0·08	2
Jess	5·27	−0·06	−0·16	−0·10	−0·10	3
Jess 1	5·16	−0·09	+0·03	−0·04	—	—
Marigold	5·20	−0·03	−0·07	−0·04	−0·01	1
Marigold 2	5·38	+0·13	−0·02	−0·08	−0·08	3
Martha	5·36	+0·11	−0·06	−0·07	−0·04	1
Rhoda	5·23	−0·27	−0·12	−0·02	−0·01	2
Rhoda 2	5·23	−0·13	−0·08	−0·14	−0·14	3
Rhoda 3	5·20	−0·04	−0·05	−0·04	—	—
Rita	5·37	−0·16	−0·12	−0·06	−0·06	3
Rita 5	5·11	−0·06	−0·01	−0·11	−0·11	3
Ruth 2	5·19	−0·07	−0·02	−0·02	−0·01	2
Snowdrop 2	5·32	−0·79	+0·12	−0·09	−0·15	4
Suzette 1	5·12	−0·37	−0·15	−0·05	−0·05	4
Suzette 3	5·08	−0·33	−0·09	−0·10	−0·04	1
Herd mean value*	5·244	−0·197	−0·058	−0·078	−0·060	—

* Weighted according to the yield of milk water of individual cows.

Table 5. *Lactation number and the effect of age on milk lactose content*

Lactation no.	No. of cows	Mean effect of age on lactose content, g/100 g milk water
1	16	+0·017
2	11	−0·152
3	8	−0·236
4	5	−0·291
5	1	−0·398
6	3	−0·233
7	1	−0·177
8	2	−0·238
10	2	−0·351

Inter-quarter difference. There was a considerable variation in the effect of inter-quarter difference from cow to cow, values ranging from −0·015 to −0·223 (Tables 3 and 4). Best quarter values will be distinguished from the values for other quarters partly as a result of experimental error, and this will cause an over-estimate of inter-quarter difference of the same size as the under-estimate of the effect of age.

Table 6. *Details of the recorded infections in the 2 herds*

Pathogen	No. of infections	
	Herd A	Herd B
Staphylococcus aureus	9	34
Micrococcus sp.	1	12
Streptococcus agalactiae	2	0
Str. dysgalactiae	3	3
Str. uberis	0	4
Mixed infections	3	5
No pathogen found	8	5
Total	26	63

Table 7. *The relationship between the number of infected udder quarters and the mean effect of infection on lactose content*

No. of infected quarters	No. of cows	Mean effect of infection on the milk lactose content, g/100 g milk water
1	12	−0·035
2	12	−0·019
3	11	−0·109
4	5	−0·094*

* Comparison made with the best quarter.

Since frequent bacteriological tests were made on all quarters, it was possible to calculate the effect of infection directly as the difference between the weighted lactation mean value for all quarters of the udder and the corresponding weighted mean for non-infected quarters. The results are given in Tables 3 and 4. The incidence of infection in the 2 herds and the organisms giving rise to them are shown in Table 6. In animals with infections in all quarters, the lactation mean value for the best quarter had to be used as the value for the infection-free quarter, and the effect of the infection was consequently under-estimated. The inter-quarter difference tended to over-estimate the effect of infection on milk lactose content by an average of 0·027 g/100 g milk water, and, as would be expected, this tendency was most marked in infection-free animals. Since the proportion of infection-free animals was higher in herd A than in herd B, the inter-quarter difference technique over-estimated the effect of infection to a slightly greater extent in herd A.

The loss in milk lactose content associated with infections in individual cows did increase on average with the number of quarters infected (Table 7), but since in quarters classified as infected the duration and severity of infections would vary considerably, no very close association was to be expected.

Quantitative importance of the various factors in accounting for between-herd differences in lactose and SNF contents

The herd mean values for SNF and lactose contents and the estimated values for herd potential and for the effects of age, changes with stage of lactation and inter-quarter difference are summarized in Table 8. The major factor contributing to the between-herd difference in lactose percentage of 0·203 was the difference between the herds in predicted potential of 0·150. The effect of age was more pronounced in herd B than in herd A, accounting for a further difference of 0·070, but the effect of changes with stage of lactation gave a small difference of 0·022 in the reverse direction. The effect of inter-quarter difference was negligible.

Table 8. *Quantitative importance of the effects on milk lactose content of predicted potential, age, changes with stage of lactation and inter-quarter difference in determining differences between the 2 herds in the milk lactose and SNF contents*

	Herd A	Herd B	Difference between herds A and B	Relative importance in determining inter-herd differences, %
SNF content (g/100 g milk water) of milk produced	9·786	9·367	0·419	—
Actual lactose content (g/100 g milk water) of milk produced	5·114	4·911	0·203	—
Potential lactose content (g/100 g milk water)	5·394	5·244	0·150	61
Effect of age on lactose content (g/100 g milk water)	−0·127	−0·197	0·070	28
Effect of changes with stage of lactation on lactose content (g/100 g milk water)	−0·080	−0·058	−0·022	9
Effect of inter-quarter difference on lactose content (g/100 g milk water)	−0·073	−0·078	0·005	2

DISCUSSION

According to the scheme described here, differences between herds in the lactose content of their bulk milk are ascribed to 4 factors, namely, differences in predicted potential lactose content and differences in the effects of age, changes with stage of lactation and inter-quarter difference. The assumption is made that the techniques described achieve a correct partition of the observed differences between the different factors. Of the 4 factors considered, the measurement of the effect of changes with stage of lactation is most certain. In animals in which the best quarter is free of infection the estimate is subject only to errors due to analysis or frequency of sampling. Also, there is little doubt that infections are a major, though not necessarily the only, source of inter-quarter difference. The inter-quarter difference technique will over-estimate the effect of infection or other environmental causes of differences between quarters in lactose content because of possible small biological differences that may occur between healthy quarters, and because of analytical and sampling errors attached to estimates of values for individual quarters, and this will result in a similar under-estimate of the effect of age. As discussed earlier, however, the error will be small and should not lead to bias from herd to herd.

The accuracy of prediction of potential milk lactose content, and therefore also of the effect of age, which is calculated as the difference between the potential value and the mid-lactation value for the best quarter, is least certain. It will be determined by the extent to which the following assumptions are correct: (i) that variation about the regression of lactose on potassium in infection-free heifers in mid-lactation (Walsh & Rook, 1964) is due to environmental factors; (ii) that the ratio of potassium to lactose in the milk of individual cows in mid-lactation is a constant: (iii) that animals in herds to which the method is applied conform with the general relationship between lactose and potassium contents established earlier (Walsh & Rook, 1964).

Table 9. *The relationship between the number of heifers in a herd and the allowable mean deviation of the actual milk lactose content of infection-free quarters in mid-lactation from the potential lactose content predicted from the equation Y (lactose content, g/100 g milk water) = $7{\cdot}4242 - 0{\cdot}01095X$ (potassium content, mg/100 g milk water) for animals to be classified with 95 % confidence as belonging to the same population as that used to establish the regression*

No. of heifers in the herd ...	1	2	3	5	8	11	14
Allowable mean deviation, lactose, g/100 g milk water	0·204	0·146	0·120	0·094	0·075	0·065	0·059

The 95 % confidence limits for the measurement of potassium and lactose contents as calculated from separate determinations made at intervals throughout mid-lactation were ± 7 mg/100 g and ± 0·07 g/100 g, respectively. The resulting errors in the predicted lactose content would be ± 0·057 and ± 0·019 g/100 g milk water. Such within-animal errors would account for a part of the observed variation about the regression of lactose on potassium, and the remaining variation must arise from environmental factors or physiological or genetic differences between animals. One non-genetic source of variation already present at the beginning of the heifer lactation is that due to damage to udder tissue at parturition as indicated by the occasional recovery of red blood cells in the milk of freshly calved heifers; others may exist. The possibility of genetically determined differences in the regression of lactose on potassium is, however, indicated by the failure of a small proportion of the Friesian heifers examined so far, and those from one commercial herd in particular (Walsh & Rook, 1964), to conform with the empirical relationship established for a majority of the animals. A simple check can be made, however, for each herd on the basis of the observed agreement of the values determined in infection-free heifers in the mid-lactation period with predicted potential values. The 95 % confidence limits for the mean value for heifers for a single herd have been calculated for different numbers of heifers and are given in Table 9. These values were calculated for a mean potassium value of 185 mg/100 g milk water. For mean potassium values towards the extremes of the range of potassium values, the figure should be increased by 0·005.

If it is assumed that there is no environmental component of the variation about the regression line, it may be calculated that the lactose content of the milk of heifers in mid-lactation would be estimated to within 0·070 percentage units in 50 % of animals and to within 0·133 percentage units in 80 % of animals. The method of prediction, however, eliminates variation due to environmental or physiological causes and the

actual error should be less, and may be small in relation to the range of potential in lactose content in heifers of about 5·0–5·7 g/100 g milk water. Moreover, estimates of herd potential should be subject to little bias provided animals within the herd conform to the empirical relationship established earlier (Walsh & Rook, 1964) since they are measured as the average for the individual animals in the herd.

Though the basis of the prediction of potential lactose content obviously requires a more detailed examination and evaluation than has yet been possible, the scheme that we have formulated is clearly a technical possibility. Moreover, measurement of the effect of the various factors on milk lactose content is based on analyses of the milk of individual animals. The method can therefore be applied directly to individual animals, or to individual herds, without the need to make comparisons with other herds, and this represents a considerable advance over earlier techniques, which are essentially comparative. A wider exploration of the scheme, and of its ability to identify the causes of differences between herds in milk lactose content, appears to be warranted.

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The measurement of the effects of inherent and environmental factors on the lactose content of the milk of individual cows and of the herd bulk milk in a number of commercial herds

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SUMMARY. (1) In a previous paper (Walsh, Rook & Dodd, 1968) a scheme was devised in which the potential milk lactose content for individual cows was predicted from the observed potassium-to-lactose ratio in the milk, and the difference between the predicted potential and the actual milk lactose contents was partitioned into effects due to age, changes with stage of lactation and inter-quarter difference. The scheme was based on repeated analyses for potassium and lactose of milk from the separate quarters of the udder of each animal at intervals throughout a single lactation, and was applied to 2 commercial herds. A simplified scheme, in which analyses are confined to milk secreted in mid-lactation and in which the measurement of the effect of changes with stage of lactation is omitted, has now been applied to a further 6 commercial herds. The results are reported here, together with those for the mid-lactation period for the 2 herds studied previously.

(2) The potassium and lactose contents of the milk of uninfected quarters of the heifers in 4 of the 6 herds and of the second-lactation animals in the 5th herd, which had no heifers, were found to conform with the relationship established previously (Walsh & Rook, 1964) on which the prediction of potential lactose content is based. The values for heifers in the 6th herd did not conform, and the results for this herd are therefore not reported.

(3) The range of herd mean values for SNF content was 8·17–8·62 % and for lactose content 4·33–4·72 %. The overall range for all herds for the predicted potential lactose content of the milk of individual cows was 5·06–5·71 (g/100 g milk water). Herd mean values for predicted potential lactose content were, however, generally similar, ranging from 5·240 to 5·486 (g/100 g milk water).

(4) The range of values for individual cows and the herd mean values for the effects of age and inter-quarter difference showed important differences from herd to herd. Herd mean values for the effect of age varied from −0·028 to −0·266 (g/100 g milk water), and there was a similar range, of −0·074 to −0·251 (g/100 g milk water), for the effect of inter-quarter difference. In herds where the effect of age was high, the effect of inter-quarter difference also was high.

(5) The effects of inter-quarter difference and of udder infections were similar.

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The loss in milk lactose content was about twice as high in quarters infected with streptococci as in quarters infected with staphylococci.

(6) Estimates of herd values for potential lactose content and for the combined effects of age and inter-quarter difference based on analyses of herd bulk milk agreed with corresponding estimates based on analyses of the milk of individual animals within the herd.

(7) The importance of variation in milk lactose content as a source of variation in SNF content and the relative importance of predicted potential lactose content and the effects of age and inter-quarter difference as a source of variation in milk lactose content are discussed.

In the preceding paper (Walsh *et al.* 1968) a tentative scheme was put forward for the separation of the effects of predicted potential, age, changes with stage of lactation and inter-quarter difference (reflecting mainly the effects of udder infections) on the lactose content of the milk of individual cows. The scheme is based on repeated analyses for lactose and potassium of milk from the separate quarters of the udder of each animal at intervals throughout a single lactation and could not readily be applied to a large number of herds. Since the effect of changes with stage of lactation is due to inevitable physiological changes that are common to all cows, they are unlikely to be an important source of between-herd differences in the lactose content of herd bulk milk. A simplified scheme has therefore been considered in which analyses are confined to milk secreted in the mid-lactation period and in which measurement of the effect of changes with stage of lactation is omitted. It has been applied to 6 commercial herds of Friesian cows and the results are reported here together with the results for the mid-lactation period for the 2 herds studied in the previous paper (Walsh *et al.* 1968). As a further simplification, analyses were made of the herd bulk milk from 5 of the herds and used to predict the herd potential and the loss in lactose content due to the combined effects of age, inter-quarter variations and changes with stage of lactation in a manner analogous to that developed for individual cows.

EXPERIMENTAL

The herds

Details of 2 of the herds (A and B) have been given previously (Walsh *et al.* 1968). The 6 additional herds (C, D, E, F, G and H) studied were located within a 30-mile radius of Reading. Three of the herds (C, D and F) consisted entirely of pure-bred Friesian animals but the other 3 herds had a small number of animals of other breeds which were excluded from the study. Herds were selected to give a wide range of solids-not-fat (SNF) contents in the herd bulk milk, but the selection was conditioned by the need to ensure the willingness of farmers to co-operate and that sufficient information on a herd was available to indicate that Friesian animals were pure-bred.

Five of the herds (C, D, E, F and G) were milked in cowhouses with bucket machines and the 6th, herd H, in a milking parlour with a direct-to-can milking machine. The pulsation rates, ratios and pipeline vacuums in use were those recommended by the manufacturers of the equipment, with the exception of herd D, in

which a pulsation rate of 148/min was in use, as compared with a recommended rate of about 60.

Cow replacements were bred and reared on the farm in all herds. Herds F and H had their own farm bulls but the other herds used exclusively the Artificial Insemination service of the Reading Cattle Breeding Centre. A policy of autumn calving was invariably followed and the majority of cows tested calved in the last months of 1963 or the first months of 1964.

All herds were well fed and no obvious differences between herds were recorded. During the period of the investigation, mainly late autumn and winter, the diets consisted largely of roughage foods for maintenance and of concentrated foods rationed according to milk yield.

In all herds, when mastitis was detected by the milker, antibiotic therapy was given.

Sampling and methods of analysis

In the additional 6 herds (C–H) studied in this investigation, samples of the milk were obtained at consecutive evening and morning milkings on 3 occasions at 28-day intervals, the first occasion coming between the 28th and the 56th days of lactation. A sample of the herd bulk milk or, when the herd included cattle of other breeds, of the bulk milk of the Friesian animals, was also taken at each farm visit.

Milking was done with a machine designed for the separate collection of milk from the quarters of the udder; the brand of liners, the pulsators and the pulsation rate and ratio were those ordinarily used by the farmers. At each milking, the milk from each udder quarter of each cow was weighed, a sample taken, and a weighted composite sample of the evening and morning milk for each quarter and also of the herd bulk milk was prepared for chemical analysis. Immediately before the evening milking 10 ml of foremilk were taken aseptically from each quarter for bacteriological examination. The bacteriological and chemical methods and the definition of infection have been described previously (Walsh *et al.* 1968).

RESULTS

The results relate to the 2 herds studied previously (Walsh *et al.* 1968) and to the 6 additional herds. They are presented in the following way. First, the data from the heifers in each of the 6 additional herds are examined to determine whether they conform to the potassium–lactose relationship described by Walsh & Rook (1964). Then, using data from all herds, the predicted potential lactose contents and estimates of the depressions in lactose content caused by age and inter-quarter differences are given for individual cows and for herds. Estimates of the effect of udder infections are made also, since this effect is a major factor contributing to the calculated inter-quarter difference in milk lactose content. For the 2 herds studied previously the estimates based on mid-lactation values are also compared with the previous estimates based on values for the whole of the lactation. Finally, herd values for the various estimates as calculated from analyses of the milk of individual cows are compared with the corresponding estimates calculated from the analyses of the herd bulk milks.

The relationship between potassium and lactose in the milks of animals in their first lactation

For each heifer in herds C, D, F, G and H, the mean values for potassium (mg/100 g milk water) and lactose (g/100 g milk water) contents were calculated for the milk of infection-free quarters and the results are given in Fig. 1. Comparable results for herds A and B have already been reported (Walsh *et al.* 1968). In 4 of the 5 herds, the values for the individual animals were all within the 95 % confidence limits of the regression line established previously (Walsh *et al.* 1968). The values for heifers in

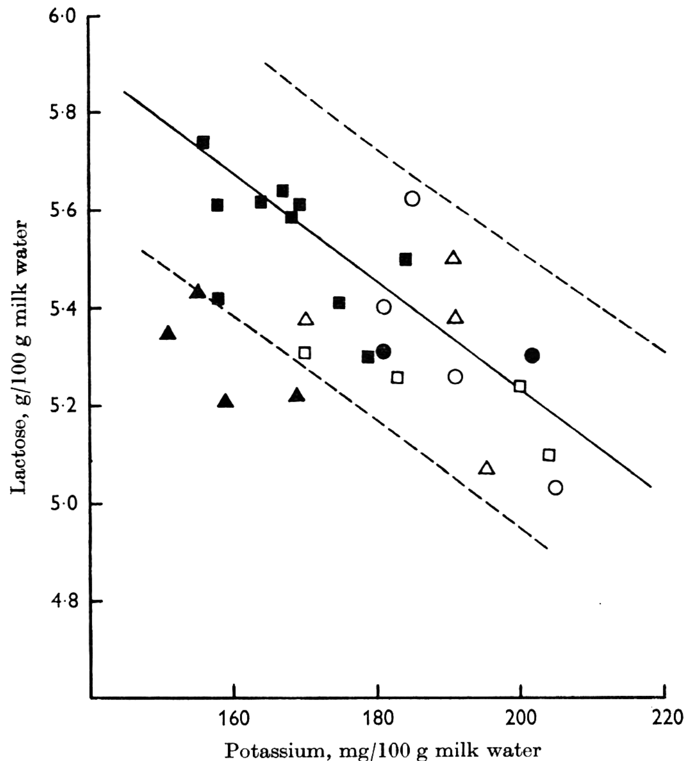

Fig. 1. The relationship between the potassium and lactose contents of the milks of the 24 heifers in herds C, D, F, G and H and of the 4 second-lactation cows in herd E. Values are for milk obtained from infection-free quarters of the udder when the animals were in mid-lactation. The line is $Y = 7{\cdot}4242 - 0{\cdot}01095X$ (see Walsh & Rook, 1964) and the broken lines indicate the 95 % confidence limits for lactose at given values of potassium. ○, Herd C; ●, herd D; □, herd E; ■. herd F; △, herd G; ▲, herd H.

herd H were not and the results for this herd have therefore not been reported here. The frequency of occurrence of such herds within the Friesian breed is now being investigated. None of the animals in herd E was in its first lactation, but the mean values for potassium and lactose concentrations in the milk of infection-free quarters for 4 animals in their second lactation were within the 95 % confidence limits for the regression line established for first-lactation heifers (Fig. 1).

Table 1. *Details for the cows in herd A*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of: Age	Inter-quarter difference	Infections of the udder
Beauty 2	24. ix. 62	1	—	36·0	4·02	9·11	4·97	5·61	+0·15	−0·04	0
Damsel 2	1. xii. 62	3	1	63·7	3·90	8·92	4·69	5·37	+0·05	−0·08	−0·03
Darkie	1. x. 62	1	—	35·3	3·93	8·86	4·87	5·50	+0·12	−0·04	0
Dimple 4	13. ix. 62	2	1	55·7	4·03	9·07	4·64	5·47	−0·07	−0·07	−0·04
Dimple 5	13. ix. 62	1	2	26·0	4·18	9·08	4·71	5·53	+0·01	−0·11	−0·10
Dimple 6	8. ix. 62	1	—	37·5	3·69	8·86	4·68	5·46	−0·10	−0·01	0
Doris 2	18. xi. 62	3	1	78·7	4·10	8·43	4·69	5·58	−0·01	−0·21	−0·16
Dreamie 2	6. xi. 62	5	3	74·9	4·22	8·77	4·47	5·51	−0·26	−0·11	−0·05
Dreamie 4	23. xii. 62	2	2	62·9	3·94	8·48	4·61	5·45	−0·08	−0·11	−0·07
Dreamie 6	24. ix. 62	1	—	36·9	3·85	8·98	4·88	5·60	+0·05	−0·05	0
Ida	20. xi. 62	1	—	41·8	3·96	8·88	5·13	5·66	+0·28	−0·06	0
T. Maude	1. i. 63	10	2	79·4	3·60	7·67	4·31	5·25	−0·26	−0·13	−0·01
C. Maude	17. xi. 62	4	1	82·4	4·09	7·89	4·24	5·26	−0·41	−0·04	−0·04
Maude 2	28. x. 62	2	—	68·8	3·82	8·17	4·39	5·19	−0·17	−0·03	0
Maude 3	16. xii. 62	1	—	45·9	4·47	8·93	4·62	5·38	+0·05	−0·09	0
Maude 4	13. ix. 62	1	1	36·8	3·09	8·23	4·42	5·29	−0·26	−0·05	−0·02
Sheila 2	13. ix. 62	1	—	38·0	3·71	8·79	4·75	5·56	−0·06	−0·07	0
Stella	17. ii. 63	8	2	90·6	3·62	8·36	4·42	5·32	−0·05	−0·26	0
Stella 2	29. viii. 62	2	2	67·2	3·70	8·76	4·59	5·40	−0·10	−0·06	−0·03
Sulky	23. viii. 62	4	3	73·5	3·63	8·08	4·20	5·18	−0·30	−0·12	−0·07
Sweetie	1. x. 62	2	3	69·1	3·40	8·46	4·56	5·30	0·07	−0·06	−0·06
Herd mean value		2·7	1·1	57·2	3·785	8·476	4·558	5·396	−0·100	−0·097	−0·037
Range				26·0 to 90·6 (64·6)	3·09 to 4·47 (1·38)	7·67 to 9·11 (1·44)	4·20 to 5·13 (0·93)	5·18 to 5·66 (0·48)	+0·28 to −0·41 (0·69)	−0·01 to −0·26 (0·25)	0·00 to −0·16 (0·16)

* Weighted mean values for the mid-lactation period.

Table 2. *Details for the cows in herd B*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of		
									Age	Inter-quarter difference	Infections of the udder
Blackbird 1	14. x. 62	8	4	49·0	2·83	7·97	4·23	5·29	−0·43	−0·12	−0·12
Blackbird 3	5. vii. 62	2	1	54·9	2·94	8·13	4·28	5·08	−0·21	−0·06	−0·03
Blackie	12. xii. 62	10	4	40·7	3·11	7·78	4·01	5·04	−0·33	−0·20	−0·20
Charm	11. viii. 62	2	1	48·1	2·62	7·52	4·16	5·09	−0·32	−0·14	−0·08
Clover	27. x. 62	6	3	51·6	3·92	8·09	4·26	5·23	−0·31	−0·08	+0·08
Clover 2	10. x. 62	4	2	58·7	3·89	8·43	4·70	5·49	−0·01	−0·12	−0·06
Collona	8. i. 63	1	3	32·0	3·53	8·56	4·84	5·50	+0·02	−0·02	−0·02
Colonist	6. xi. 62	4	3	51·8	3·50	7·90	4·26	5·48	−0·32	−0·36	−0·36
Contented	12. vii. 62	1	1	37·9	3·67	8·78	4·70	5·39	+0·03	−0·05	−0·04
Deirdre 1	14. vii. 62	3	—	55·1	3·37	8·22	4·30	5·06	−0·19	−0·01	0
Dot 2	31. x. 62	3	2	43·7	3·22	7·97	4·45	5·22	−0·19	−0·02	−0·02
Favourite 2	26. vii. 62	1	1	33·8	3·40	8·35	4·43	5·14	−0·06	−0·06	−0·02
Foundation 2	7. vii. 62	3	3	37·5	3·18	8·28	4·12	5·22	−0·44	−0·13	−0·13
Graceful	25. v. 62	7	2	60·5	3·18	8·38	4·53	5·35	−0·18	−0·06	−0·05
Jess	28. x. 62	6	3	47·4	3·41	8·20	4·41	5·27	−0·06	−0·21	−0·21
Jess 1	7. vii. 62	2	—	56·1	3·21	8·44	4·45	5·16	−0·09	−0·04	0
Marigold 1	26. xii. 62	3	1	36·4	3·59	8·29	4·53	5·20	−0·02	−0·03	−0·03
Marigold 2	18. vi. 62	1	3	35·2	3·16	8·89	4·75	5·38	+0·13	−0·11	−0·11
Martha	15. vii. 62	1	1	42·1	3·57	8·98	4·75	5·36	+0·11	−0·04	−0·01
Rhoda	8. vii. 62	3	2	48·8	3·27	8·32	4·34	5·23	−0·27	−0·05	−0·05
Rhoda 2	21. vii. 62	2	2	52·8	3·53	8·13	4·32	5·23	−0·13	−0·22	−0·13
Rhoda 3	26. vii. 62	2	—	41·3	3·59	8·73	4·49	5·20	−0·04	−0·04	0
Rita	18. xii. 62	6	3	38·1	3·87	8·63	4·49	5·37	−0·16	−0·08	−0·08
Rita 5	7. vii. 62	1	3	44·7	3·17	8·12	4·40	5·11	−0·06	−0·09	−0·09
Ruth 2	1. xi. 62	1	2	41·0	3·15	8·02	4·52	5·19	−0·07	−0·04	−0·04
Snowdrop 2	10. x. 62	3	3	47·4	3·14	7·83	4·08	5·32	−0·36	−0·38	−0·38
Suzette 1	28. v. 62	4	3	42·1	3·28	7·86	4·16	5·12	−0·37	−0·07	−0·07
Suzette 3	20. vii. 62	2	1	54·8	3·17	7·94	4·13	5·08	−0·33	−0·10	−0·04
Herd mean value		3·3	2·0	45·8	3·249	8·172	4·384	5·240	−0·175	−0·108	−0·080
Range				32·0 to 60·5 (28·5)	2·62 to 3·92 (1·30)	7·52 to 8·98 (1·46)	4·01 to 4·84 (0·83)	5·04 to 5·50 (0·46)	+0·13 to −0·44 (0·57)	−0·01 to −0·38 (0·37)	−0·00 t −0·38 (0·38)

Table 3. *Details for the cows in herd C*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of: Age	Inter-quarter difference	Infections of the udder
Actress 4	28. viii. 63	4	1	25·3	4·43	8·43	4·19	5·35	−0·37	−0·17	−0·06
Actress 6	22. iii. 64	4	1	66·8	3·14	8·07	4·24	5·15	−0·23	−0·16	−0·13
Angela	12. vii. 63	2	3	31·7	3·81	9·31	4·43	5·35	−0·17	−0·09	−0·09
Audrey	7. vi. 63	1	2	36·1	3·44	8·37	4·37	5·13	−0·08	−0·09	−0·07
Diana	19. iii. 64	7	3	47·4	3·01	8·66	4·59	5·40	+0·02	−0·22	−0·22
Fiona	23. ix. 63	8	4	69·4	3·48	8·38	4·44	5·35	−0·26	−0·05	−0·05
Fiona 2	9. vi. 63	3	3	39·6	3·53	8·65	4·60	5·52	−0·03	−0·26	−0·26
Flossie	25. iii. 63	1	—	30·0	4·55	9·38	4·87	5·46	+0·25	−0·04	0
Jessie	6. x. 63	3	1	62·5	4·21	8·44	4·68	5·44	0	−0·08	−0·04
Rachel	28. iii. 64	1	1	42·0	3·26	8·98	4·71	5·44	+0·04	−0·12	0
Rosaline	30. vi. 63	3	—	45·0	3·66	8·88	4·59	5·45	−0·17	−0·03	0
Ruby	23. viii. 63	5	—	49·1	4·26	8·71	4·76	5·46	+0·06	−0·05	0
Tansy	11. xi. 63	7	3	74·4	2·81	8·24	4·68	5·32	+0·03	−0·09	−0·02
Tansy 3	19. v. 63	4	3	44·1	3·60	8·67	4·55	5·46	−0·17	−0·11	−0·11
Tansy 5	12. i. 64	3	1	55·7	3·79	8·74	4·57	5·31	+0·08	−0·16	−0·06
Vanessa	5. v. 63	1	—	23·8	4·67	9·22	4·50	5·29	−0·02	−0·04	0
Vanity	19. vii. 63	6	1	48·0	3·31	8·44	4·28	5·18	−0·22	−0·11	−0·05
Violet	16. viii. 63	3	2	22·5	3·32	9·06	4·62	5·32	+0·04	−0·09	−0·06
Herd mean value		3·7	1·6	45·2	3·595	8·616	4·538	5·351	−0·073	−0·109	−0·069
Range				22·5 to 74·4 (51·9)	2·81 to 4·67 (1·86)	8·07 to 9·38 (1·31)	4·19 to 4·87 (0·68)	5·13 to 5·52 (0·39)	+0·25 to −0·37 (0·62)	−0·03 to −0·26 (0·23)	−0·00 t[o] −0·26 (0·26)

* Weighted mean values for the mid-lactation period.

Table 4. *Details for the cows in herd D*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of: Age	Inter-quarter difference	Infections of the udder
F. Cherry	29. ii. 64	3	—	36·8	4·52	8·41	4·39	5·32	−0·24	−0·03	0
Bonny	15. x. 63	5	3	34·5	3·07	8·39	4·34	5·46	−0·39	−0·18	−0·17
Bonnybell	20. viii. 63	7	3	29·9	2·89	8·00	3·78	5·52	−0·89	−0·39	−0·39
Bonnybell 2	16. x. 63	4	2	43·7	3·57	8·39	4·41	5·34	−0·07	−0·27	−0·23
Bonnybell 3	11. x. 63	3	—	32·8	4·26	8·91	4·66	5·54	−0·09	−0·09	0
Bonnyboll 4	24. iii. 64	2	1	44·5	3·99	8·27	4·43	5·33	−0·13	−0·14	−0·04
Buttercup 4	10. vi. 63	6	3	28·0	3·08	7·87	4·13	5·53	−0·56	−0·33	−0·33
Buttercup 5	23. ii. 64	6	1	51·3	4·26	8·52	4·64	5·48	0	−0·16	−0·13
Buttercup 6	26. ix. 63	2	—	31·9	3·80	9·16	4·64	5·44	−0·03	−0·08	0
Dinah 5	24. ix. 63	8	4	35·5	3·58	7·87	3·35	5·41	−0·72	−0·90	−0·90
Dinah 6	20. x. 63	3	2	27·0	3·74	8·98	4·54	5·37	+0·03	−0·21	−0·20
Dinah 8	6. xii. 63	1	—	27·8	3·48	8·98	4·65	5·41	−0·07	−0·03	0
Gracie	10. x. 63	7	4	40·2	2·82	7·83	3·95	5·41	−0·56	−0·43	−0·43
Gracie 2	11. x. 63	2	3	8·8	4·44	8·06	4·16	5·53	−0·66	−0·10	−0·10
Hiltress 3	1. iv. 64	2	2	19·5	3·02	8·55	4·61	5·39	−0·09	−0·09	−0·04
Judy	7. ix. 63	4	1	33·4	2·89	8·97	4·40	5·58	−0·26	−0·33	−0·15
Pansy 2	13. xi. 63	8	3	32·8	2·77	7·75	3·91	5·56	−0·68	−0·51	−0·51
Torse	18. viii. 63	4	—	22·4	2·66	8·50	4·36	5·59	−0·62	−0·06	0
Torse 4	12. i. 64	7	2	28·0	3·90	8·06	4·19	5·51	−0·30	−0·44	−0·44
Topsy	9. x. 63	5	2	38·0	3·44	8·25	4·32	5·45	−0·24	−0·32	−0·29
Trix	3. xii. 63	10	4	29·1	3·13	8·32	3·79	5·50	−0·66	−0·55	−0·55
Trix 2	14. xi. 63	6	—	37·1	3·16	8·56	4·64	5·39	−0·03	−0·10	0
Wildfire	26. x. 63	11	2	40·8	3·47	8·25	4·39	5·26	−0·16	−0·12	−0·11
Wildfire 2	20. x. 63	6	2	43·6	3·50	8·23	4·22	5·36	−0·18	−0·40	−0·38
Wildfire 3	4. x. 63	5	2	32·5	3·95	8·94	4·55	5·38	+0·06	−0·22	−0·07
Wildfire 4	5. ix. 63	3	—	24·7	2·12	8·61	4·54	5·49	−0·34	−0·07	0
W. Lady 3	25. xi. 63	7	4	40·8	3·80	7·91	4·12	5·49	−0·62	−0·20	−0·20
W. Lady 5	26. xi. 63	5	4	29·1	3·15	8·18	4·23	5·57	−0·26	−0·54	−0·54
W. Lady 6	29. ii. 64	4	—	25·6	3·56	8·80	4·61	5·44	−0·09	−0·09	0
W. Lady 7	27. ix. 63	3	—	32·8	3·67	9·20	4·60	5·34	+0·02	−0·08	0
W. Lady 8	18. ix. 63	1	—	35·3	3·43	8·65	4·63	5·24	+0·10	−0·07	0
Herd mean value		4·8	1·7	32·1	3·481	8·419	4·330	5·431	−0·266	−0·251	−0·207
Range				8·8 to 51·3	2·12 to 4·52	7·75 to 9·20	3·35 to 4·66	5·24 to 5·59	+0·10 to −0·89	−0·03 to −0·90	0·00 to −0·90

Table 5. *Details for the cows in herd E*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of: Age	Inter-quarter difference	Infections of the udder
Doris	22. xii. 63	3	2	37·2	2·96	8·32	4·33	5·35	−0·11	−0·36	−0·24
Fifty-eight	8. xii. 63	2	1	46·4	3·39	8·34	4·56	5·24	+0·05	−0·13	−0·09
Kitty	21. vi. 63	3	2	19·1	5·74	9·06	4·33	5·55	−0·11	−0·36	−0·01
Lucy	13. vi. 63	4	3	18·0	4·62	8·64	4·24	5·53	−0·28	−0·36	−0·36
Lulu	16. i. 64	2	2	31·4	2·25	8·43	4·57	5·39	−0·12	−0·16	−0·15
Monica	27. vii. 63	7	4	23·3	3·19	7·86	3·97	5·29	−0·43	−0·39	−0·39
Newlands Blackie	16. vi. 63	4	3	38·0	3·33	8·49	4·60	5·56	−0·19	−0·15	−0·15
Molly	21. x. 63	2	1	44·3	3·46	8·20	4·43	5·17	−0·03	−0·13	−0·08
Tina	19. i. 64	2	2	28·6	3·70	8·61	4·62	5·53	−0·20	−0·06	−0·04
Herd mean value		3·2	2·2	31·8	3·459	8·400	4·439	5·372	−0·128	−0·208	−0·155
Range				18·0 to 46·4 (28·4)	2·25 to 5·74 (3·49)	7·86 to 9·06 (1·20)	3·97 to 4·62 (0·65)	5·17 to 5·56 (0·39)	+0·05 to −0·43 (0·48)	−0·06 to −0·39 (0·33)	−0·01 t[o] −0·39 (0·38)

* Weighted mean values for the mid-lactation period.

Table 6. *Details for the cows in herd F*

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of: Age	Inter-quarter difference	Infections of the udder
Cherry 7	21. i. 64	1	—	40·2	3·82	8·48	4·92	5·67	0	−0·06	0
Garlette 17	26. xii. 63	3	—	53·6	3·62	8·37	4·73	5·42	−0·02	−0·02	0
Garlette 18	5. xii. 63	2	1	34·5	3·58	8·51	4·44	5·24	−0·11	−0·08	−0·03
Garlette 21	16. ix. 63	1	—	46·2	3·64	8·78	4·94	5·61	+0·06	−0·03	0
Gipsy 5	7. viii. 63	11	2	36·6	3·42	8·35	4·55	5·52	−0·23	−0·13	−0·10
Gipsy 6	14. vii. 63	6	2	47·2	3·21	8·40	4·58	5·54	−0·24	−0·13	−0·08
Gipsy 7	3. xii. 63	5	—	52·0	3·25	8·05	4·62	5·64	−0·36	−0·07	0
Gipsy 10	20. xii. 63	2	—	31·9	4·18	9·07	4·75	5·55	−0·06	−0·02	0
Gipsy 11	10. ix. 63	2	—	38·4	3·94	8·96	4·86	5·58	+0·10	−0·10	0
Gipsy 12	22. ix. 63	1	2	31·6	3·65	8·94	4·86	5·58	+0·03	−0·04	−0·03
H. Esme	28. ix. 63	1	—	42·6	3·75	8·68	4·92	5·58	+0·06	−0·04	0
Henderaiko 7	21. x. 63	1	—	25·9	3·75	8·67	4·65	5·42	−0·07	−0·04	0
Hyphen 11	21. ix. 63	1	2	23·8	4·21	9·12	4·93	5·71	+0·15	−0·18	−011
Joanna 3	11. xii. 63	7	1	33·7	4·14	8·28	4·51	5·53	−0·17	−0·21	−0·16
Joanna 7	17. ix. 63	1	—	26·5	3·48	8·82	4·75	5·62	−0·13	−0·07	0
Minnie 2	13. xii. 63	3	1	42·0	3·81	8·42	4·63	5·52	−0·13	−0·11	−0·11
Minnie 4	28. ix. 63	1	1	33·0	3·97	9·09	4·84	5·62	+0·04	−0·10	−0·06
R. Esme	25. xii. 63	5	1	58·2	3·65	8·29	4·69	5·37	+0·04	−0·09	−0·05
R. Esme 2	14. xii. 63	4	3	37·0	3·65	8·34	4·50	5·32	−0·06	−0·16	−0·16
S. Content 52	11. iv. 64	2	—	60·3	3·82	8·97	4·97	5·54	+0·20	−0·05	0
S. Content 53	29. ii. 64	2	—	55·8	3·79	8·58	4·70	5·47	−0·04	−0·06	0
S. Content 54	27. iii. 64	2	2	55·6	3·44	8·65	4·68	5·33	+0·04	−0·04	−0·04
S. Content 55	26. iii. 64	2	2	51·3	4·16	8·67	4·59	5·38	−0·02	−0·09	−0·07
S. Content 57	11. ix. 63	2	—	46·6	4·16	8·81	4·81	5·40	+0·14	−0·01	0
S. Content 58	14. xii. 63	2	—	35·6	3·59	8·30	4·54	5·29	−0·10	−0·03	0
S. Content 61	30. ix. 63	1	1	41·6	3·44	8·56	4·65	5·46	−0·05	−0·12	−0·13
S. Content 62	24. ix. 63	1	—	39·3	3·71	8·82	4·81	5·43	+0·11	−0·04	0
Herd mean value		2·7	0·8	41·5	3·719	8·606	4·721	5·486	−0·028	−0·074	−0·038
Range				23·8 to 60·3 (36·5)	3·21 to 4·21 (1·00)	8·05 to 9·12 (1·07)	4·44 to 4·97 (0·53)	5·24 to 5·71 (0·47)	+0·15 to −0·36 (0·51)	−0·01 to −0·21 (0·20)	0·00 to −0·16 (0·16)

* Weighted mean values for the

Cow	Calving date	Lactation no.	No. of quarters infected	Mean daily yield, lb	Fat, %*	SNF, %*	Lactose, %*	lactose content, g/100 g milk water	(g/100 g milk water) of		
									Age	Inter-quarter difference	Infections of the udder
Amy	23. x. 63	3	2	40·8	2·90	7·96	4·37	5·23	−0·07	−0·26	−0·22
Anita	14. ii. 64	2	1	33·7	3·71	9·02	4·74	5·47	+0·03	−0·06	0
Aster	9. x. 63	1	—	33·5	3·27	8·01	4·50	5·21	−0·12	−0·02	0
Dolly	29. viii. 63	3	2	47·7	3·73	8·36	4·60	5·42	−0·11	−0·08	−0·08
Dora	28. i. 64	6	3	56·6	3·20	8·16	4·35	5·48	−0·24	−0·34	−0·34
Eira	9. vii. 63	4	1	35·1	4·10	8·63	4·26	5·45	−0·35	−0·22	−0·20
Lucy	23. ix. 63	1	—	30·8	3·86	8·61	4·70	5·34	+0·08	−0·05	0
Mandy	5. vi. 63	1	—	29·2	4·02	8·55	4·80	5·38	+0·16	−0·06	0
Marie	13. xi. 63	2	3	18·5	3·94	8·29	4·37	5·53	−0·16	−0·39	−0·35
H. Rhoda	21. vii. 63	2	2	31·6	3·62	8·73	4·51	5·42	−0·07	−0·20	−0·20
H. Rita	27. xii. 63	5	3	64·2	3·18	8·11	4·52	5·40	−0·10	−0·20	−0·20
H. Ruby 2	30. viii. 63	3	2	41·7	3·85	8·70	4·58	5·35	−0·01	−0·10	−0·05
H. Ruth	19. vii. 63	6	3	32·9	3·67	8·08	3·78	5·16	−0·12	−0·74	−0·74
Rose	5. ix. 63	1	3	23·9	4·33	8·90	4·73	5·52	−0·03	−0·04	+0·05
Sooty	23. i. 64	4	3	39·3	3·01	7·83	4·06	5·12	−0·32	−0·24	−0·24
Herd mean value		2·9	1·9	37·3	3·552	8·355	4·451	5·361	−0·106	−0·203	−0·178
Range				18·5 to 64·2 (45·7)	2·90 to 4·33 (1·43)	7 83 to 9·02 (1·19)	3·78 to 4·80 (1·02)	5·12 to 5·53 (0·41)	+0·16 to −0·35 (0·51)	−0·02 to −0·74 (0·72)	+0·05 t −0·74 (0·79)

* Weighted mean values for the mid-lactation period.

Table 8. *Herd mean values for milk fat, SNF and lactose contents, predicted potential lactose content and the effects on milk lactose content of age, inter-quarter difference and infections of the udder*

Herd	Fat, %	SNF, %	Lactose,		Predicted potential lactose content, g/100 g milk water	Effect on lactose content (g/100 g milk water) of		
			%	g/100 g milk water		Age	Inter-quarter difference	Infections of the udder
F	3·719	8·606	4·721	5·384	5·486	−0·028	−0·074	−0·038
A	3·785	8·476	4·558	5·199	5·396	−0·100	−0·097	−0·037
C	3·595	8·616	4·538	5·169	5·351	−0·073	−0·109	−0·069
G	3·552	8·355	4·451	5·052	5·361	−0·106	−0·203	−0·178
E	3·459	8·400	4·439	5·036	5·372	−0·128	−0·208	−0·155
B	3·249	8·172	4·384	4·957	5·240	−0·175	−0·108	0·080

The predicted potential lactose content and the effects of age, inter-quarter difference and udder infections on milk lactose content in animals in each of the 7 herds

The predicted potential lactose content and the effects of age, inter-quarter difference and udder infections on milk lactose content for animals in herds C–G were calculated from the results for the 3 sampling occasions and for animals in herds A and B from the results for the 7 sampling occasions between the 11th and the 112th days of lactation. The details of the calculations have been described previously (Walsh *et al.* 1968). Results for the individual cows tested in each of the 7 herds are given in Tables 1–7, together with information on calving date, lactation number, the number of quarters infected and the mean values for fat, SNF and lactose contents of the milk of the whole udder. Herd average values are summarized in Table 8.

Table 9. *The relationship between lactation number and the effect of age on milk lactose content*

Lactation no.	No. of cows	Effect of age on milk lactose content, g/100 g milk water: Mean	Range
1	36	+0·024	−0·26 to +0·28
2	32	−0·095	−0·66 to +0·20
3	25	−0·108	−0·36 to +0·08
4	17	−0·260	−0·62 to −0·01
5	9	−0·166	−0·39 to +0·06
6	11	−0·193	−0·56 to ±0·00
7	9	−0·344	−0·89 to +0·03
8	5	−0·427	−0·72 to −0·05
10	3	−0·420	−0·66 to −0·26
11	2	−0·200	−0·23 to −0·16

Table 10. *The relationship between the number of quarters infected and the effect of inter-quarter difference and infections of the udder on the lactose content of milk*

No. of infected quarters	No. of cows	Effect on lactose content, g/100 g milk water of: Inter-quarter difference	Infections of the udder
1	31	−0·116	−0·068
2	35	−0·161	−0·111
3	32	−0·210	−0·195
4	9	−0·376	−0·376*

* Comparison made with the best udder quarter.

The average SNF contents for the milks of the different herds varied from 8·17 % in herd B to 8·62 % in herd C and within each herd there was a range of more than 1·0 percentage units in the SNF contents of the milks of individual animals. Much of the variation in SNF content was related to variations in lactose content; herd averages for lactose content varied from 4·33 % in herd D to 4·72 % in herd F, about 88 % of the corresponding range in SNF content, and within each herd the range for individual animals was at least 0·5 percentage units and in some herds more than 1·0.

The overall range in all herds for the predicted potential lactose content of indi-

vidual cows was 5·04–5·71 (0·67) g/100 g milk water. The range for individual animals in a single herd was less, varying from 5·24 to 5·59 (0·35) in herd D to 5·18–5·66 (0·48) in herd A. Herd average values for predicted potential lactose content were, however, generally similar. At the two extremes were herd F with a mean value of 5·49 and herd B with a mean value of 5·24, and these were the 2 herds that had made recent use of farm bulls in their breeding programme.

Table 11. *Details of the recorded udder infections in 7 herds*

	Herd A	Herd B	Herd C	Herd D	Herd E	Herd F	Herd G
No. of cows in the herd	21	28	18	31	9	27	15
Cows with infections, %	62	89	78	68	100	48	80
Udder quarters infected, %	29	51	40	45	56	19	47
No. of cows infected in all quarters	0	2	1	5	1	0	0
Pathogen	No. of infections						
Staphylococcus aureus	8	32	5	16	8	10	3
Micrococcus sp.	1	11	5	4	3	1	4
Streptococcus agalactiae	2	0	0	9	1	0	8
Str. dysgalactiae	3	3	1	3	0	1	3
Str. uberis	0	4	2	4	4	4	2
Mixed infections	3	3	5	7	2	0	5
No pathogen found	7	4	11	11	2	5	3
Total	24	57	29	54	20	21	28

Table 12. *A comparison of the effect of inter-quarter difference and infections of the udder on the lactose content (g/100 g milk water) of the milk produced in the mid-lactation period when infections were caused by (i) staphylococcal, (ii) micrococcal or (iii) streptococcal pathogens*

			Mean effect/cow		Mean effect/infected quarter	
Organisms	No. of cows	No. of infected quarters/cow	Inter-quarter difference	Infections of the udder	Inter-quarter difference	Infections of the udder
(i) *Staphylococcus*	36	2·0	−0·155	−0·119	−0·310	−0·238
(ii) *Micrococcus* sp.	6	2·2	−0·205	−0·152	−0·373	−0·276
(iii) *Streptococcus*	23	2·3	−0·253*	−0·230*	−0·440	−0·400

* Significantly ($P > 0·05$) greater than the effect of staphylococcal infections.

Both the range of values for individual cows and the herd average values for the effects of age, inter-quarter difference and udder infections showed important variations from herd to herd. As noted previously (Walsh *et al.* 1968), the effect of age tended to increase with an increase in lactation number (Table 9), but there was a considerable variation within each lactation number group. Also, inter-quarter difference and the effects of udder infection were highly correlated, the correlation coefficient being 0·93, and these effects on average increased with the number of quarters infected (Table 10). There was, however, a variety of pathogens (Table 11) and the most marked effects on milk lactose content were associated with streptococcal infection (Table 12). It is of interest that micrococcal infection depressed the lactose content

of milk to the same extent as staphylococcal infection but only 6 micrococcal infections were recorded in the absence of other infection and, of these, 4 were in one herd. The overall ranges for the effects of age, inter-quarter difference and udder infections were similar and the average values for each of the effects also were of the same order in a given herd. Each of the effects was at a minimum in herd F, a herd with a low average lactation number of 2·7 and a low incidence of udder infection, and at a maximum in herd D, the herd with the highest average lactation number of 4·8 and a high incidence of streptococcal infections.

A comparison of measurements made in herds A and B over the mid-lactation period with those made over the whole lactation

Results for herds A and B calculated for the mid-lactation period only are compared in Table 13 with those calculated previously (Walsh *et al.* 1968) for the whole lactation. The estimates of potential lactose content by the 2 methods were closely similar in each of the 2 herds and the estimates of the effects of age and of inter-quarter difference, though in less good agreement, invariably differed by not more than 0·03 percentage units. Small differences in the estimates of the effects of age and inter-quarter difference by the 2 procedures are to be expected because of possible differences in the quarter selected as the best quarter and because estimates made during the mid-lactation period only would exclude the effects of infections of the udder occurring after this period.

Table 13. *Herd values for predicted potential lactose content (g/100 g milk water) and the effects of age, inter-quarter difference and infections of the udder on milk lactose content (g/100 g milk water) calculated for (i) the mid-lactation and period (ii) the whole of the lactation*

	Herd A		Herd B	
	(i) Mid-lactation period	(ii) Full lactation period	(i) Mid-lactation period	(ii) Full lactation period
Predicted potential lactose content	5·396	5·394	5·240	5·244
Effect of				
Age	−0·100	−0·127	−0·175	−0·197
Inter-quarter difference	−0·097	−0·073	−0·108	−0·078
Udder infections	−0·037	−0·037	−0·080	−0·060

The estimation of herd predicted potential lactose content and of the combined effects of age, inter-quarter difference and changes with stage of lactation from analyses of herd bulk milk

The herd potential lactose content and the combined effects on the herd bulk milk of age, inter-quarter difference and changes with stage of lactation as estimated directly from the analyses of the herd bulk milk are compared in Table 14 with the corresponding values calculated as the mean of the values for individual cows within the herd. There was close agreement in all herds between the 2 estimates of potential lactose content. The combined effects of age, inter-quarter difference and changes with stage of lactation (which would be small since over the period of sampling most

of the cows were in mid-lactation) estimated from analyses for herd bulk milk were invariably greater, by 0·012–0·127, than the combined figures for age and inter-quarter difference calculated from the results for individual cows. However, the herd ranking was still maintained and the figures obtained from analyses on the herd bulk milk would be a useful guide to the likely order of the effects of age and inter-quarter difference.

Table 14. *Herd values for milk lactose content, predicted potential lactose content and the combined effects on lactose content of age and inter-quarter difference calculated from (i) the results for individual cows and (ii) analyses of herd bulk milk*

	No. of samples			Lactose, g/100 g milk water	
Herd	(i)	(ii)		(i)	(ii)
C*	216	10	Predicted potential	5·351	5·383
			Effect of age and inter-quarter difference	−0·182	−0·309
			Actual content	5·169	5·074
D*	384	10	Predicted potential	5·431	5·490
			Effect of age and inter-quarter difference	−0·517	−0·555
			Actual content	4·914	4·935
E†	108	8	Predicted potential	5·372	5·314
			Effect of age and inter-quarter difference	−0·336	−0·348
			Actual content	5·036	4·966
F*	324	10	Predicted potential	5·486	5·499
			Effect of age and inter-quarter difference	−0·102	−0·197
			Actual content	5·384	5·302
G†	180	7	Predicted potential	5·361	5·360
			Effect of age and inter-quarter difference	−0·309	−0·340
			Actual content	5·052	5·020

* Bulk milk samples were taken from the mixed milk of all animals in the herd.
† Bulk milk samples were taken from the mixed milk of cows being sampled individually on the occasion of farm visits.

DISCUSSION

Lactose is the major component of the SNF fraction of cow's milk, accounting on average for about 60 %. Comparatively little importance has, however, been attached to variations in milk lactose content as a source of variation between cows and between herds in the SNF content of their milk. This is the result partly of the emphasis given in recent years to the effect of the energy nutrition of the cow on the protein content of her milk as a source of variation in SNF content, and also of the evidence from some experimental investigations (Rowland, Neave, Dodd & Oliver, 1959; O'Donovan, Dodd & Neave, 1960) that the effects of infections of the udder on milk SNF content, which are the result mainly of changes in lactose content, may not be quantitatively important in commercial herds.

There is surprisingly little published information on the lactose content of the milk of individual animals or herds under commercial conditions. The few investigations in which the lactose content of milk was determined directly are consistent, however,

Table 15. *Variation in the SNF content of milk between individual cows, between herds, and between individual cows within herds and the reduction in this variation when adjustment is made for variation in lactose content*

	D.F.	Sum of squares SNF content	Sum of squares due to the regression of SNF content on lactose content	Reduction in SNF content sum of squares due to adjustment for variation in lactose content, %	Regression coefficient (*b*) of SNF content on lactose content with S.E.
(*a*) Between cows within herd A	20	3·4515	2·1784	63·1	1·39 ± 0·243***
Between cows within herd B	27	3·3800	2·2062	65·3	1·30 ± 0·185***
Between cows within herd C	17	2·3799	0·5434	22·8	0·97 ± 0·447*
Between cows within herd D	30	5·1622	3·0990	60·0	1·01 ± 0·153***
Between cows within herd E	8	0·8743	0·1155	13·2	0·56 ± 0·546
Between cows within herd F	26	2·1539	1·0285	47·8	1·30 ± 0·272***
Between cows within herd G	14	1·8810	0·7653	40·7	0·85 ± 0·285**
Individual cows within herds	142	19·2828	9·5641	49·6	1·09 ± 0·092***

	D.F.	SNF content mean square	Lactose content mean square	D.F.	Adjusted SNF content mean square
(*b*) Between herds	6	0·6038***	0·4836***	6	0·3047***
Individual cows within herds	142	0·1358	0·567	141	0·0689

	Herd A	Herd B	Herd C	Herd D	Herd E	Herd F	Herd G
(*c*) Unadjusted mean SNF content, g/100 g milk	8·476	8·172	8·616	8·419	8·400	8·606	8·355
Mean lactose content, g/100 g milk	4·558	4·384	4·538	4·330	4·439	4·721	4·451
Adjusted mean SNF content, g/100 g milk	8·487	8·514	8·474	8·561	8·494	8·475	8·498

*** Significant, $P < 0·001$. ** Significant, $P < 0·01$. * Significant, $P < 0·05$.

in indicating that variations in milk lactose content and milk protein content make a similar contribution to variations in SNF content. In a study of the bulk milk of a representative selection of herds of the Ayrshire, Friesian, Guernsey and Shorthorn breeds located in the southern counties of England (Rowland & Rook, 1949), the ranges, within breeds, for the annual averages in individual herds of the percentage of lactose and protein varied from 0·16 to 0·31 and from 0·17 to 0·34, respectively. For individual cows in commercial herds of Ayrshire cattle in Scotland, Robertson, Waite & White (1956) found that the standard deviations for fat, SNF, protein and lactose percentages were 0·37, 0·26, 0·20 and 0·15, respectively. In a similar study with cows of the Friesian breed in Friesland, Politiek (1957) obtained corresponding values of 0·26, 0·17, 0·16 and 0·12. These conclusions are fully supported by other investigations (Purchase & Reverberi, 1946; Cullity, Hood & Needham, 1949; Edwards, 1958) in which, with one exception (Edwards, 1958), lactose content was estimated not directly but as the difference between SNF content and the contents of total protein plus ash.

Our results for 7 commercial herds confirm that variations in lactose content are a major source of variation in SNF content both between individual animals within a herd and between herds. The variation in SNF percentage in the milks of individual cows within herds and the extent to which this is reduced by removing variation due to lactose were calculated from the data in Tables 1–7. The results are given in Table 15. If the effect of herd difference is removed half of the total variation in the SNF content in the milks of individual cows is due to variation in the percentage lactose. Within particular herds, however, the importance of lactose variation in creating variation in SNF ranged from 65 % in herd B to 13 % in herd E. The slope of the regression relating SNF and lactose contents was calculated for each herd. Comparison of the regression coefficients for the different herds showed no significant differences, and it was possible, therefore, to use a common regression coefficient (b = 1·09) for adjustment of herd mean values of milk SNF percentage to allow for covariation with lactose content. This adjustment reduced the SNF variation between herd means by about a half.

The experimental approach adopted in this study has also given information on the possible source of this considerable variation in lactose content. The variance in milk lactose content (g/100 g milk water) between animals within herds and between herd means and the proportions of these variances associated with variations in predicted potential lactose content, age effects and effects of inter-quarter difference have been calculated from the results in Tables 1–7. The values are given in Table 16. The total variances of the 3 factors do not equal the variance of actual milk lactose content without the introduction of covariance terms, but expressing the variance of the separate factors as a percentage of the variance of the actual milk lactose content indicates the contribution of each factor to the observed variation of lactose content. There was a considerable variation from herd to herd in the importance of the 3 factors, but on average the effect of age was greatest, accounting for as much variation in actual milk lactose content as the other 2 factors combined. Between herds, age was a less important factor, accounting for 25 % of the variation in milk lactose content, with differences in predicted potential and inter-quarter difference accounting for 24 and 15 %, respectively.

Table 16. *The variance between cows within herds and between herd mean values of actual milk lactose content, predicted potential lactose content and the effects of age and inter-quarter difference on milk lactose content*

	D.F.	Variance of: (*a*) Actual milk lactose content, g/100 g milk water	(*b*) Predicted potential lactose content, g/100 g milk water	(*c*) Effect of age, g/100 g milk water	(*d*) Effect of inter-quarter difference, g/100 g milk water	$b+c+d$, g/100 g milk water	$\frac{b \times 100}{a}$	$\frac{c \times 100}{a}$	$\frac{d \times 100}{a}$
Within herds									
Herd A	20	0·0874	0·0207	0·0275	0·0026	0·0508	23·7	31·5	3·0
Herd B	27	0·0754	0·0178	0·0262	0·0088	0·0528	23·6	34·7	11·7
Herd C	17	0·0531	0·0121	0·0234	0·0041	0·0396	22·8	44·1	7·7
Herd D	30	0·1467	0·0085	0·0772	0·0405	0·1262	5·8	52·6	27·6
Herd E	8	0·0590	0·0210	0·0197	0·0146	0·0553	35·6	33·4	24·7
Herd F	26	0·0360	0·0148	0·0167	0·0036	0·0351	41·1	46·4	10·0
Herd G	14	0·1129	0·0170	0·0190	0·0355	0·0715	15·1	16·8	31·4
Cows within herds	142	0·085	0·0154	0·0340	0·0162	0·0656	18·1	40·0	19·1
Between-herd means (variance component)	6	0·0316	0·0075	0·0078	0·0048	0·0201	23·7	24·7	15·2

In the light of these observations it appears that the scheme that we have put forward could offer a useful basis for assessing the quantitative contributions of inherent and environmental factors to differences between herds in the composition of their bulk milk. Two schemes have been described, one a simple system based on the analysis of herd bulk milk, which could be used to determine whether the low lactose content of a herd's bulk milk is due to genetic or environmental factors. This method does not partition the effects of the specific factors involved, but would provide a useful preliminary observation. The complete method described, using analysis at regular intervals of the milk from individual quarters of each cow in the herd, would require considerable laboratory support. An intermediate scheme involving analysis of the milks of individual quarters over a short period of time would provide a more practical method for investigating herd problems.

Some theoretical considerations concerning the validity of the techniques have been discussed previously (Walsh *et al.* 1968). They are mainly concerned with the factors which influence the accuracy of prediction of the potential lactose content of milk and a fuller examination of these factors is now being undertaken.

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Rate of infection of milked and unmilked udders

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Summary. The teats of milked and unmilked quarters of 6 cows were deliberately contaminated twice daily by immersion in a suspension of streptococci and staphylococci. Only the unmilked quarters became infected, 7 with *Staphylococcus aureus* and 3 with *Streptococcus uberis.* Neither previous infection with the same strain or a different strain of pathogen nor existing infection with *Corynebacterium bovis* appeared to influence the results.

It was shown by Neave, Dodd & Henriques (1950) that, in the absence of hygiene, intramammary infection rate in a herd of 50 cows was highest in the early dry period. A preliminary trial was made in 1956 to try to confirm that there is a difference in infection rate between milked and non-milked quarters when teats are equally exposed externally to the common mastitis pathogens. Publication was delayed as we had hoped in a further study to explain the difference in the rate of infection by examining the effect of milking, the milk yield of individual quarters, udder pressure, teat patency, teat erosion, and the composition of the secretion and its inhibitory or stimulating effect on pathogens. This study has not yet been carried out, and therefore the results of the preliminary trial are published because of their theoretical and practical interest.

METHODS

Because animals vary greatly in their susceptibility to infection the best practical method of comparing the ease with which milked and unmilked quarters become infected might seem to be to make the comparison between quarters within udders. However, this would cause milk ejection in the unmilked quarters every time the other quarters are milked. The use of monozygotic twins, milking one member and drying-off the other would avoid this difficulty and largely retain the equal susceptibility required. Both experimental methods were adopted.

Animals

Six animals were used, 2 sets of monozygotic twins (T31, T32 and T47, T48) and 2 unrelated animals (T42 and Y46) in their first lactation. The only twins available were those in which the 2 members were of different lactation ages, i.e. first and second lactation animal in each set. Thus, in one set the older animal, in terms of

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Table 1. *A within udder comparison of new infections in milked and unmilked quarters*

Quarter	Previous infection: Type	Previous infection: Terminated	Examination of fresh foremilk. Days before start of experiment: 6 (Bacteria/0·05 ml. milk)	1 (Bacteria/0·05 ml. milk)	0 (Bacteria/0·05 ml. milk)	1 MA	1 Wh.	0 OS	Experimental treatments from 22. xi. 56: Dried off	Dipped in culture	Examination of fresh secretion. Days after start of experiment: 4 (Bacteria/0·05 ml secretion and appearance of secretion (MA))	7	13	13 MA	20	21*	Secretion before calving: Bacteria	MA	Secretion after calving: Bacteria	MA	Remark
Cow T 42. 1st lactation. Milked from left side. Daily yield at start of experiment 13 lb.																					
RF	*Str. uberis*	iii. 56	150Cb	50Cb	10Cb	F	3	0	**Yes**	**No**	0	400Cb	∞ Cb	QC4	0	0	·	·	100Cb	—	·
	Staph. aureus t	x. 56																			
RH	*Str. uberis*	iii. 56	60Cb	20Cb	10Cb	F	3	0	**Yes**	**Yes**	0	∞ Cb	200Cb **300HS**	QC3	∞ **HS**	∞ **HS**	·	·	∞ **HS**	DC4	T. not cured
	Staph. aureus t	x. 56																			
LF	*Str. uberis*	iii. 56	50Cb	20Cb	20Cb	—	3	0	**No**	**No**	100Cb	100Cb	200Cb	—	50Cb	0	·	·	∞ III	—	T. cured
	Str. agalactiae β	ix. 56																			
LH	*Str. uberis*	iii. 56	60Cb	40Cb	50Cb	F	2	0	**No**	**Yes**	150Cb	100Cb	200Cb	F	100Cb	0	·	·	50Cb	—	·
	Str. agalactiae β	viii. 56																			
	Staph. aureus t	x. 56																			
Cow Y 46. 1st lactation. Milked from right side. Daily yield at start of experiment 9 lb.																					
RF	*C. bovis*	—	∞ Cb	40Cb	100Cb	—	3	0	**No**	**No**	250Cb	0	300Cb	—	100Cb	60Cb	0	V3	0	C2	·
RH	*Str. uberis*	vii. 56	400Cb	50Cb	100Cb	—	2	0	**No**	**Yes**	200Cb	0	200Cb	—	200Cb	60Cb	0	V3	0	C1	·
	Str. agalactiae β	viii. 56																			
LF	*Staph. aureus* ? t	vii. 56	200Cb	60Cb	50Cb	F	3	0	**Yes**	**No**	400Cb	0	50Cb	D	∞ Cb	∞ Cb	0	V2	300Cb	C1	·
	Str. agalactiae β	ix. 56																			
LH	*C. bovis*	—	150Cb	40Cb	100Cb	—	1	0	**Yes**	**Yes**	**16HS** ∞ **III**	**70HS** **200III**	**500HS**	DF	∞ **HS**	∞ **HS**	∞ **HS**	—	∞ **HS**	C1	T. not cured

* Experiment ended after 21 days and all quarters dried off.

RF, RH, LF, LH, refer to right fore, right hind, left fore and left hind quarters.

Bacteria: Cb, *C. bovis*; HS, *Staph. aureus*; III, *Str. uberis*; mic, non-haemolytic coagulase-negative staphylococci. Numbers in bold indicate the quarters infected during the experiment. ∞, innumerable colonies.

Wh., Whiteside test; OS, teat apex swab, i.e. swab of external opening of the teat canal including 2–3 mm of the canal; T, antibiotic therapy; MA, macroscopic appearance of milk; F, small wh flakes; D, discoloured; C1–C4, increasing number of milk clots, C1, a few and C4, mainly clo P1–P3, increasing amount of blood or blood clots in the milk; V1–V3, increasing viscosity of seru like secretion; —, normal for the period, i.e. milk, watery milk or colostrum.

Quarter	Previous infection: Type	Previous infection: Terminated	Examination of fresh foremilk, Days before start of experiment: 6 (Bacteria/0·05 ml milk)	1 (Bacteria/0·05 ml milk)	0 (Bacteria/0·05 ml milk)	1 MA	1 Wh.	0 OS	Experiment started 22. xi 56: Dried off	Dipped in culture	Examination of fresh secretion, Days after start of experiment: 4 (Bacteria/0·05 ml secretion and appearance of secretion (MA))	7	13	13 MA	20	21*	Secretion, Before calving: Bacteria	Before calving: MA	After calving: Bacteria	After calving: MA	Remarks
Cow T 31. 2nd lactation. Milked from left side. Daily yield 8 lb.																					
RF	*C. bovis* (Cb)		250Cb	60Cb	0	D	3	0	**No**	**No**	80Cb	50Cb	40Cb	F	100Cb	0	0	V2	0	—	.
LF	*Staph. aureus* m	vii. 55	200Cb	20Cb	0	—	3	0	**No**	**No**	50Cb	30Cb	50Cb	F	50Cb	200Cb	0	V2	20Cb	—	.
	Staph. aureus t	vii. 56																			
RH	*Str. agalactiae* γ	xii. 55	0	0	0	—	3	0	**No**	**Yes**	1HS	0	0	—	0	0	0	V2	30Cb	—	.
	Str. agalactiae β	viii. 56																			
LH	*Str. agalactiae*	iii. 55	0	0	0	—	3	0	**No**	**Yes**	0	0	0	—	0	0	0	V2	10Cb	—	.
	Staph. aureus	v. 55																			
Cow T 32. 1st lactation. Milked from left side. Daily yield 13 lb.																					
RF	*Staph. aureus* d	ix. 56	60Cb	0	0	—	2	0	**Yes**	**No**	**100Cb**	**300Cb** 1HS	**∞ Cb**	D	**∞ Cb** **50 HS**	**400Cb** **200HS**	.	.	.	.	Cow sold while dry
LF	*Staph. aureus* f & d	vii. 56	0	0	0	—	3	0	**Yes**	**No**	0	0	0	D	0	0	.	.	.	.	.
	Staph. aureus t	x. 56																			
RH	*Staph. aureus* b	ix. 56	200Cb	0	0	—	1	0	**Yes**	**Yes**	0	**300Cb** **2HS**	**∞ Cb** **45HS**	D	**∞ Cb** **50HS**	**150III** **100HS**	.	.	.	.	.
LH	*Str. agalactiae* γ	viii. 56	0	0	0	—	1	0	**Yes**	**Yes**	0	0	0	D	1HS	0	.	.	.	.	.
Cow T47. 2nd lactation. Milked from right side. Daily yield 9 lb.																					
RF	*C. bovis*	vi. 56	0	0	0	DC_3	3	0	**Yes**	**No**	0	0	0	DC1	0	0	0	P1C1	Mic	DC1	T. cured
	Staph. aureus d	viii. 56																			
LF	Pseudomonad	iii. 56	0	0	0	—	1	0	**Yes**	**No**	0	0	0	DC2	**100HS**	**250HS**	**∞ HS**	P3T	0	—	Cured
	Str. agalactiae β	viii. 56																			
	C. bovis	vi. 56																			
RH	O		50Cb	0	0	—	1	0	**Yes**	**Yes**	0	0	1HS	DC3	**∞ HS**	**300HS**	**300HS**	C4T	**50HS**	—	Cured
LH	Pseudomonad	iii. 56	0	0	10Cb	C1	2	0	**Yes**	**Yes**	0	**200HS** **400III**	**500HS** **500III**	DC3	**50HS** **100III**	**70HS** **300III**	**200HS** **∞ III**	C4T	**∞ HS** **50III**	C1	T. cured
	Staph. aureus d & t	ix. 56																			
	Str. agalactiae β	ix. 56																			
Cow T 48. 1st lactation. Milked from right side. Daily yield 11 lb.																					
RF	*Str. agalactiae* β	viii. 56	∞ Cb	0	10Cb	F	3	0	No	No	200Cb	100Cb	50Cb	—	0	20Cb	∞ Cb	V2	∞ Cb	—	.
	C. bovis																				
LF	O		0	0	0	F	2	0	No	No	0	0	0	F	0	0	0	V3	0	—	.
RH	*Staph. aureus* m	iv. 56	30Cb	0	0	—	2	0	No	Yes	0	0	50Cb	F	0	60Cb	0	V2	300Cb	—	.
LH	*C. bovis*		30Cb	0	10Cb	F	1	0	No	Yes	30Cb	20Cb	50Cb	F	0	50Cb	0	V3	0	—	.

* Experiment ended after 21 days and all quarters dried off.

Legend as Table 1.

lactation age, and in the other set the younger animal, was dried off. The animals were near the end of lactation and yielded 8–13 lb of milk a day.

All animals had acquired, previous to this experiment, intramammary infections with *Staph. aureus*, *Streptococcus agalactiae* and *C. bovis* in one or more quarters. Also 2 cows had been infected with *Str. uberis* and one cow with a pseudomonad (Tables 1, 2). Infected quarters other than those infected with *C. bovis* had received one or more courses of antibiotic therapy and the infections were eliminated at least 5 weeks before the experiment started, as determined by 8 or more bacteriological tests on foremilk. Teat orifice swabs taken at the start of the experiment yielded no *Staph. aureus* and no streptococci though *Staph. aureus* was recovered from the teats of one cow (T31) but only after incubation of the swab. This cow did not develop an intramammary infection.

All animals were free from teat sores and had little or no teat canal erosion. The right fore (RF) quarter of cow T47 was nearly 'blind' as a result of a severe infection with *Staph. aureus* strain *d* 5 months earlier. The quarter was milked regularly though it yielded only a few ounces of milky fluid.

Exposure of udders to pathogens

Milking infected cows (donors) before experimental or test cows does not give equal or constant contamination of the teats of the test cows. This is more nearly achieved by simply dipping each teat in a standardized suspension of pathogens.

The teats of both milked and unmilked quarters were dipped twice daily in a suspension of bacteria prepared in litmus milk. The suspension contained 3 types of bacteria, isolated from cases of mastitis in 1956 by Mr C. D. Wilson. *Staph. aureus* strain *t* (Sharpe, Neave & Reiter, 1962) was grown in Lemco broth for 24 h at 37 °C, *Str. dysgalactiae* and *Str. uberis* were seeded in warmed litmus milk using a 2 % inoculum and grown for about 8 h at 37 °C. The grown cultures were refrigerated, a plate count determined in nutrient aesculin ox blood agar and a suspension prepared in litmus milk to contain 8×10^6/ml of each strain, i.e. a total of 24×10^6/ml. The suspension was distributed into 16 bottles of 100 ml and these were refrigerated at 2–4 °C. Immediately before each milking the contents of one bottle were poured into a plastic beaker and used to contaminate the hind teats by dipping the teats in the beaker which was pushed against the udder to form a seal and then shaken. In this way the whole of the teat was wetted with the suspension of bacteria.

A fresh suspension was prepared weekly, there being only a slight fall in the numbers of viable cells of each strain in this period.

Procedure

The main experiment lasted for 21 days and during this time some quarters were milked and some not milked but all hind teats were dipped twice daily in the bacterial suspension. After 21 days all quarters in milk were dried off and the udders of all 6 cows washed thoroughly and dipped in Hibitane (Imperial Chemical Industries, Ltd, London, England) disinfectant containing 2 % chlorhexidine.

During the experiment all quarters of cows T31 and T48, members of different sets of twins, were milked twice daily; the foremilk cup was used and the whole udder washed with a cloth and sodium hypochlorite solution containing 600 ppm. available

chlorine before milking. Immediately after milking the hind teats were dipped in the bacterial suspension. The twin mates of these 2 cows, T 32 and T 47, were not milked after 22 November; they were brought into the cowshed twice daily for teat contamination only and were turned out of the cowshed unmilked before milking of the rest of the herd commenced.

Cows T 42 and Y 46 each had 2 quarters milked and 2 quarters dried off (Table 1). The 2 milked quarters were on the side from which the cows were milked, i.e. the left-hand side of cow T 42 and the right-hand side of cow Y 46. The foremilk cup was used on the milked quarters only but all 4 teats were washed before milking with the same cloth and sodium hypochlorite solution.

Before the experiment started all teats were dipped after every milking in Hibitane disinfectant.

All animals were put on experiment on the same day.

Milking machines

All animals were milked with ordinary bucket units. From the start of the experiment cows T 42 and Y 46 were milked using a half-udder teatcup cluster. The machines were operated at a vacuum of 15 inHg, 60 pulsations/min and an approximate pulsation ratio of 1 : 1.

Before and during the experiment the teatcup clusters were pasteurized with circulating hot water at 85 °C for 5–7 s before milking each animal.

Foremilk samples and skin swabs

Foremilk samples were taken at intervals as shown in Tables 1 and 2, after scrubbing the ends of the teat with 70 % (w/v) ethanol. About 25 ml were taken from milked quarters and about 8 ml from unmilked quarters. The fresh milk was plated (0·05 ml) on aesculin ox blood agar and 5 ml of each sample seeded with pathogens and a pH indicator. These samples and the plates were incubated at 39 °C for 40 h. The growth or lack of growth of the pathogens in the milks and dry secretions will be reported elsewhere.

The identification of the organisms found on the plates was by inspection only, except that isolates of *Staph. aureus* from suspected new infections were phage-typed by our colleague Dr M. E. Sharpe.

Milks were examined macroscopically for abnormalities and by the Whiteside test. Teat swabs and teat apex swabs were taken at intervals of several days before the experiment started using the methods described by Neave & Oliver (1962).

RESULTS

The experimental treatments and the main results for each udder quarter are shown in Tables 1 and 2. A summary of the main bacteriological results is given in Table 3.

None of the 12 quarters milked twice daily became infected but 7 of the 12 quarters that were dried off became infected and these 7 quarters had a total of 10 infections. Seven of the 10 infections were caused by *Staph. aureus* and 3 by *Str. uberis*. The *Str. uberis* infections were mixed infections with *Staph. aureus*. In 2 of these cases the streptococci and staphylococci were isolated at the same time and in the 3rd case

Table 3. *New infections found in the milked and unmilked udder quarters during the experimental period of 21 days*

No. of animals on experiment	Comparison	Quarters milked twice daily		Quarters not milked						
							Type of infection†			
		No.	Became infected	No.	Became infected*	No. of infections*	HS	II	III	Other infections
2 twin sets	Within twin sets	8	0	8	5	7	5	0	2	2Cb
2 unrelated	Within udders	4	0	4	2	3	2	0	1	0
	Total	12	0	12	7	10	7	0	3	2

* Infected with the types of pathogen used to contaminate the exterior of the RH and LH teats.
† HS is *Staph. aureus*. II is *Str. dysgalactiae*. III is *Str. uberis*. Cb is *C. bovis*.

Table 4. *New infections found in the unmilked udder quarters that had been exposed externally, (a) directly and (b) indirectly,* to pathogens for 21 days*

Comparison	Quarters (teats) exposed directly								Quarters (teats) not directly exposed*							
					Type of infection‡								Type of infection			
	No.	Became infected	No. of infections	Infection found, days†	HS	II	III	Cow	No.	Became infected	No. of infections	Infection found, days†	HS	II	III	Cow
Within pairs of twins	4	1	2	5·5	1	—	—	T 32	4	1	1	16·5	1	—	—	T 32
				20·5	—	—	1	T 32		1	1	16·5	1	—	—	T 47
		1	1	10	1	—	—	T 47								
		1	2	5·5	1	—	—	T 47								
		—	—	5·5	—	—	1	T 47								
Within udders	2	1	1	10	1	—	—	T 42	2	0	—	—	—	—	—	—
		1	2	< 4	1	—	—	Y 46								
		—	—	< 4	—	—	1	Y 46								
Total	6	5	8	—	5	0	3	—	6	2	2	—	2	0	0	—
Mean	—	—	—	< 8	—	—	—	—	—	—	—	16·5	—	—	—	—

* Teats of these quarters not dipped in bacterial culture but other teats of the same udder were contaminated.
† Estimated number of days from start of dipping teats in suspension of pathogens to intramammary infection.
‡ See Table 3.

Str. uberis was not found until 2 weeks after recovering *Staph. aureus*. *Str. uberis* apparently disappeared spontaneously from one quarter in which *Staph. aureus* persisted (cow Y 46). In addition, it is possible that 2 quarters of cow T 32 became infected with *C. bovis* when not being milked. Indeed, about half the total of 24 quarters were possibly already infected with *C. bovis* at the start of the experiment (Tables 1 and 2). *Str. dysgalactiae* was not recovered from any of the secretions.

Eight of the 10 infections were in quarters exposed deliberately to external contamination with pathogens (Table 4). The other 2 infections, both caused by *Staph. aureus*, occurred in quarters not exposed deliberately (cows T 22 RF and T 47 LF), but they were not detected until the 20th day, whereas the other *Staph. aureus* infections were detected within 4–13 days (average < 8 days). Only one of 6 quarters (T 32 LH) exposed deliberately did not become infected.

The foremilk of all 24 quarters was Whiteside-positive at the start of the experiment, but the quarters not becoming infected had, with one exception (T 32 LH), the highest Whiteside reading.

At the start of the experiment, the foremilk of only one quarter had more than a few visible clots or flakes, and this quarter (the RF of cow T 47), which would normally be regarded as a 'dry' quarter, did not become infected. About 3 weeks after the start of the experiment the secretion of several of both the infected and uninfected quarters showed visible clots. One cow was sold before calving. The secretion of the remaining cows was examined pre-calving or at calving; that of 3 of the 5 quarters infected during the experiment showed extensive clots, that of another showed many blood clots. The remaining quarter gave, a week before calving, a milky fluid instead of the usual viscous serum-like secretion found at this time. Of the remaining 15 quarters not infected during the experiment, one apparently became infected with *Str. uberis* before calving and another with a coagulase-negative non-haemolytic staphylococcus. Some of the other quarters may have been infected with *C. bovis*, but only the secretion from one quarter (RF quarter of cow Y 46) contained more than a few clots.

DISCUSSION

It is evident from this experiment that natural infection in cows exposed to pathogens at the end of lactation occurred quite rapidly and much more readily in unmilked than in milked quarters of the udder. The findings were similar when the experimental comparison was made between quarters within cows or between cows. Only one unmilked quarter of 6 that were deliberately exposed externally twice daily for 3 weeks to large numbers of pathogens resisted infection.

The udder undergoes very considerable changes after regular milking ceases. Up to this time the products of secretion are removed at the milkings which normally occur twice daily. At each of these milkings the teats are manipulated by hand or machine, milk ejection occurs and most of the milk within the udder is removed, thereby flushing the streak canal of the teats. After the animal has been dried off the teats normally remain untouched, milk ejection probably ceases, internal udder pressure increases for a short period followed by much resorption of the milk—the greatest changes in volume and composition occur in the first week (Wheelock, Smith, Dodd & Lyster, 1967; Smith, Wheelock & Dodd, 1967).

There is no direct evidence of a relationship between the changes in the dry udder and the high susceptibility to infection. It would appear that either the pathogens gain entry to the udder more easily in the early dry period or that the conditions within the udder at this time are more favourable for their multiplication.

The evidence of Oliver, Dodd & Neave (1956*a*, *b*) that new dry-period infection occurs more frequently in cows yielding over 20 lb/day of milk at drying-off, and of Newbould & Neave (1965) that extending the milking interval increases the chance of pathogens penetrating the streak canal, may indicate that the high internal udder pressure in the first few days after drying-off is an important factor. It is probable that at this time milk leaks through the streak canal more frequently, allowing easier access for bacteria through growth or capillary movement, and that under these conditions the pathogens would be less affected by any inhibitory properties of the canal epithelium.

That the streak canal is important gains further support from the evidence of Dodd & Neave (1951) that fast-milking cows (i.e. those with the most patent streak canals) are much more liable to contract infection in the dry period.

Reiter & Oram (1967) have measured the rapid increase in the natural defence mechanisms after cessation of milking but it is not clear why these are not more effective in preventing new infection in the dry cow.

It is our experience that most new dry-period infections are caused by *Staph. aureus* and *Str. uberis* and few by *Str. dysgalactiae* (Oliver *et al.* 1956*a*, *b*) and also that nearly all infections found at the end of lactation persist through the dry period (Smith, Westgarth, Jones, Neave, Dodd & Brander, 1967).

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With 1 *plate*

Studies on the change in the properties of the fat globule membrane during the concentrating of milk

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SUMMARY. Previous work had shown that when milk is boiled in a climbing film evaporator a reaction occurs between the casein micelles and the fat globule membrane, probably at the liquid–gas interface. It now appears that the micelle–globule complex is formed by combination of the κ-casein components of the micelles and the membrane of the fat globule. The reaction is not significantly affected by increases in concentration of individual constituents, or by changes in temperature or pH.

The history of the milk before its concentration is an important factor in its subsequent behaviour. Thus, cooling and stirring of the milk during storage, as normally occurs in a bulk collection tank, promotes the reaction in the evaporator and, for some milks, is essential for starting the reaction.

A high retention of whey by curds made from the concentrated milks, diluted with water to their original volume before renneting, indicates that curd properties other than fat retention are also altered during concentration.

INTRODUCTION

In a previous paper (Mabbitt & Cheeseman, 1967) it was shown that during the process of concentrating milk in a climbing film evaporator the properties of the fat globule were changed so that it became more firmly retained within the curd during cheese-making. Furthermore, it was shown that this change was not brought about by the concentrating process *per se* but by some other factors associated with it.

The appearance of this 'fat retention' property was attended by changes in the protein composition of the water-soluble fraction of the globule membrane. New protein components of the soluble fraction were identified as caseins and it was suggested that during the concentration process the casein micelles were bound to the fat globule membrane, and that after rennet coagulation of the milk the complex of fat globule and casein formed strong links with other casein micelles of the gel matrix.

It was also suggested in the previous paper that the history of the milk prior to processing was of some importance in connexion with the phenomenon.

The work reported in this paper was undertaken to discover the change or changes occurring during the process of concentration which may be responsible for the casein-micelle–fat-globule interaction and also to show how the treatment of the milk prior to concentrating affects the reaction.

MATERIALS AND METHODS

Milk

Bulked milk (mixed evening and morning milk) from the Institute's herd of Friesian cows was used. The content of the bulk storage tank was held at 4 °C with intermittent stirring. In experiments using milk from individual cows, the milk was collected at the morning milking and held at 35–37 °C in a Dewar flask until used.

Methods

The method of concentration of the milk using a laboratory climbing film evaporator and the technique for determining the fat retention by the curd were as previously described (Mabbitt & Cheeseman, 1967). In the present experiments, however, the rate of stirring during rennet coagulation of the milk was increased to 150 rev/min. This modification gave better replicates and resulted in fat contents of 2·5–3·0 % in the whey from untreated whole milk (fat content, 3·4 ± 0·2 %) and of about 0·8 % in whey from milk concentrated threefold and diluted to its original volume. All the determinations were done in duplicate.

In a number of experiments the milk was only concentrated to two-thirds of its original volume rather than to the more usual one-third. This modification enabled changes in the degree of the fat-retention property to be more easily observed.

The term 'diluted concentrated milk' used in this paper refers to milk that was concentrated in the evaporator and after concentration diluted with water to its original volume.

Preparation of glycomacropeptide (GMP)

The glycomacropeptide, the component of κ-casein released by the action of rennin, was recovered from 1 l of renneted skim-milk in the fraction soluble in 12 % trichloroacetic acid (TCA) as described by Alais (1956). The TCA was removed by dialysis against water at 4 °C and the GMP solution concentrated to 300 ml in a rotary evaporator.

Preparation of casein fractions

κ-Casein was prepared by the method of Zittle & Custer (1963). α_s-casein and β-casein were prepared by refractionating the α- and β-casein fractions obtained by the urea fractionation method of Hipp, Groves, Custer & McMeekin (1952). All the casein solutions were tested by polyacrylamide gel electrophoresis using buffers containing urea and were found to contain predominantly the expected casein fraction.

In experiments to determine the contribution of casein to the fat-retention property, casein solutions were added to milk to give about a 10 % increase in the respective casein constituent. These were, per 400 ml of milk, 600 mg α_s-casein, 300 mg β-casein and 200 mg κ-casein. Calcium chloride solution (1 %, w/v) was added to the casein solutions to avoid altering the calcium balance of the milk at the rate of 4·63 ml with the α_s-casein, 1·42 ml with the β-casein and 1·07 ml with the κ-casein.

Emulsions of butterfat in casein solutions were prepared as described by Mabbitt & Cheeseman, 1967.

Preparation of slides for photography of fat globules in milk

To reduce the clumping of the globules to a minimum and to get a clear matrix for photography the milk was incorporated into a polyacrylamide gel as follows: 2 ml of a 10 % (w/v) solution of Cyanogum in citrate buffer (2 % (w/v) tri-sodium citrate dissolved in dilute alkali, 0·1 % (w/v) NaOH and the pH adjusted to 6·5 with N-HCl) was mixed with 1 ml of milk, and to this mixture was added 0·1 ml of 30 % tetramethyl ethylene diamine and, just before making the slide, 0·1 ml of 10 % ammonium persulphate. Slides were prepared by pipetting 5 μl of the final mixture onto equal areas circumscribed with a light film of silicone grease. A cover-slip was then placed over the mixture, which at room temperature gelled about 1 min after adding the ammonium persulphate.

Syneresis studies

The rate and extent of syneresis of diluted concentrated milk and raw milk were determined by the method of Cheeseman (1962).

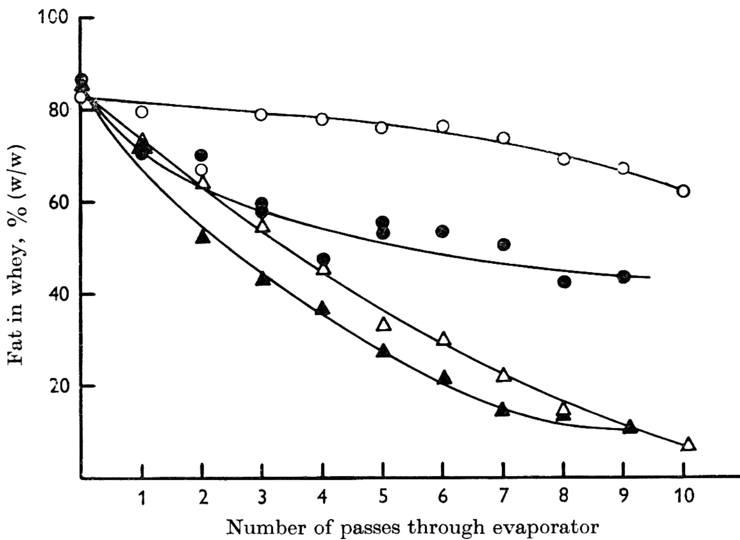

Fig. 1. The relationship of the fat-retention property of the curd and the treatment of the milk in the evaporator. Circles: no vacuum applied in the evaporator, nitrogen gas was used to form the climbing film of milk; ○, heating temperature 50 °C; ●, 80 °C. Triangles: normal method of concentration with reduced pressure, heating temperature 80 °C; △, repeated concentration until milk was ¼ of its original volume; ▲, diluted with water to its original volume after each pass.

RESULTS

Effect of changes in the concentrating process on fat retention by curd

Treatment of the milk in the evaporator. In normal operation the milk continuously circulates through the evaporator, but in this experiment the milk was retained in the holding column after each pass in order to ensure that all the milk was receiving the same amount of treatment and that samples could be obtained with known degrees of treatment.

An examination of the fat losses in whey made from milk subjected to different numbers of passes is shown in Fig. 1. Similar results were obtained whether the milk was diluted with water to its original volume after each pass or further concentrated at each cycle so that after 10 passes it was reduced to 25 % of its original volume.

Table 1. *The effects of various ions and agitation treatments on the fat retention property of raw milk*

Compound	Concentration, mM	Treatment*	Fat in whey, % (w/v)
Calcium chloride	20	None	1·85
	20	Nitrogen	1·00
	10	None	2·45
	10	Nitrogen	2·30
	2·5	None	2·07
	2·5	Nitrogen	2·10
	2·5	Stirred	2·28
	2·5	Stirred, nitrogen	2·57
Barium chloride	4	None	2·70
	4	Nitrogen	1·58
	2·5	None	2·45
	2·5	Nitrogen	2·25
	2·5	Stirred	2·80
	2·5	Stirred, nitrogen	2·20
Calcium citrate	2·5	None (20 °C)	3·17
	2·5	Nitrogen (20 °C)	2·60
	2·5	Stirred (20 °C)	3·15
	2·5	Stirred, nitrogen (20 °C)	2·65
	2·5	None	3·10
	2·5	Nitrogen	2·65
	2·5	Stirred	3·17
	2·5	Stirred, nitrogen	2·95
Citric acid	2·5	None	2·43
	2·5	Nitrogen	1·45
	2·5	Stirred	2·70
	2·5	Stirred, nitrogen	2·05
Ferrous sulphate	1·0	None	3·15
	1·0	Nitrogen	2·77
	0·1	None	3·10
	0·1	Nitrogen	3·07
Cupric nitrate	1·0	None	2·60
	1·0	Nitrogen	2·42
	0·1	None	2·80
	0·1	Nitrogen	2·70

* Unless stated otherwise, nitrogen was bubbled for 15 min through a column of milk held at 40 °C. The column of milk was formed from 100 ml of milk in a 250-ml measuring cylinder and the nitrogen pressure was so adjusted as to give a head of foam equal to about half the original volume of milk. Stirring the milk before treatment was carried out at 20 °C for 30 min.

The results of experiments in which nitrogen gas was bubbled into the milk to create a climbing film in the evaporator at 50 and 80 °C instead of boiling under low pressures are also given in Fig. 1, and show that there was an increase in fat retention as the number of passes increased. However, the increase in retention was much slower than in the low-pressure climbing film system. In addition, it can be seen that, using the nitrogen bubbling technique, the rate at which fat retention increased in the curd was higher at the higher temperature.

Changes in concentration of milk constituents. It is possible that various changes occurred in the concentrations of the free and bound constituents of the milk during evaporation and that one or more of the changes may initiate the observed change in the protein composition of the soluble membrane fraction. This possibility was examined.

Table 2. *Effect of addition of casein components to raw milk and to milk before concentration on the fat content of whey from the renneted milks*

	Fat in whey, % (w/w) of total fat	
Addition*	Milk untreated	Milk concentrated to $\frac{2}{3}$ original volume and then diluted to original volume with water
None	82	57
α-casein	82	51
κ-casein	75	47

* 600 mg α_s-casein or 300 mg β-casein or 200 mg κ-casein/400 ml milk.

Table 3. *Fat content of whey made from mixtures of skim-milk and emulsions of butter oil and caseins*

Emulsifying agent	Fat in whey, % (w/w) of total fat
Separated milk	8
α_s-casein	29
β-casein	7
κ-casein	6
Control: bulk raw milk	68

These are average results for 2 experiments each with a freshly prepared emulsion.

Inorganic constituents. Compounds containing one or more of the ions found in milk were added to the milk prior to treatment. These were calcium chloride, barium chloride, calcium citrate, citric acid, ferrous sulphate, and cupric nitrate. They were added to milk at the concentrations given in Table 1 and the conditions obtained in the evaporator were simulated by passing nitrogen gas through a column of the mixture. For comparison, curd was also made from milk mixtures not subjected to bubbling with nitrogen. However, as can be seen from Table 1, only relatively small improvements in the retention of fat were obtained. Some improvement was found when the milk was bubbled with nitrogen as compared with no treatment, which is in accord with the results shown in Fig. 1. The higher level of calcium chloride (20 mM) had a good effect; barium chloride (4 mM) and citric acid (2·5 mM) had a slight effect. Other concentrations of these ions and all concentrations of the other ions had little effect and it does not seem likely that changes in the inorganic ions were responsible for the relatively rapid changes which occur in the evaporator.

Organic constituents. Solutions of α_s-, β- or κ-casein were added to milk to give an increase in concentration of 10% of the normal value for each of these casein components. No significant change in the fat-retention property of these supplemented milks occurred (Table 2). In another experiment, creams were prepared by emulsifying butter oil with solutions of the 3 casein fractions and with separated milk, and

Table 4. *Effect of temperature of the milk, before and during concentration, on the fat-retention property*

Treatment	Pre-heating temperature, °C	Temperature of heating jacket, °C*	Milk temperature at top of column, °C*	Fat in whey, % (w/w) of total fat
Concentrated to ⅓ volume†	45	79	39·5	12
Concentrated to ⅓ volume	61	85	48·5	15
Concentrated to ⅓ volume‡	79	90	53	12
None	45	—	—	83
Concentrated to ⅔ volume	45	80	40	42
None	60	—	—	65
Concentrated to ⅔ volume	60	80	40	31
None	70	—	—	66
Concentrated to ⅔ volume	70	80	40	36
None	80	—	—	65
Concentrated to ⅔ volume§	80	80	40	10
None§	90	—	—	65
Concentrated to ⅔ volume§	90	80	40	11
Cream removed after initial heat treatment and added to unheated skim-milk	80	—	—	52
Concentrated to ⅔ volume, cream removed and added to unheated skim-milk	80	80	40	39
Cream removed after initial heat treatment and added to unheated skim-milk	90	—	—	49
Concentrated to ⅔ volume, cream removed and added to unheated skim-milk	90	80	40	31

* Mean temperature—range ± 2 °C.
† All milks diluted back to original volume before testing.
‡ Clotting time increased 3-fold.
§ The curd formed after these treatments was very fine and could not be filtered using a cheese-cloth as in the standard technique. The mixture of curds and whey was spun in 250-ml containers for 10 min at 250g and the curds and whey separated.

these emulsions were then added to skim-milk to give a similar fat content to that of whole milk. The fat-retention properties of the reconstituted milks are summarized in Table 3 and show that stabilizing the fat globule with casein components gave a high degree of fat retention. The retention was greater with κ-casein, β-casein and separated milk than with α_s-casein. Mabbitt & Cheeseman (1967) found that the casein micelles were more effective than was sodium caseinate in giving an emulsion with good fat-retention properties. The greater effect of the individual casein components obtained in the present work may have been in part due to the added calcium (p. 136). However, the higher fat loss obtained with α_s-casein is similar to that previously obtained for sodium caseinate; this suggests that the effective component may be κ-casein. The marked effect of the β-casein is, therefore, difficult to explain.

It has been suggested that the outside of the casein micelle contains proportionately more κ-casein than does the rest of the micelle (Sullivan, Fitzpatrick & Stanton, 1959). This could, therefore, account for the fat-retention property of emulsions made with suspensions of micelles and butter oil.

Further evidence in support of this reaction was obtained by adding a solution of GMP (the hydrophilic component of κ-casein split off by rennin action) to milk before concentration to see if the GMP interfered with the binding of the micelles to the fat globule membrane. The GMP recovered from 1 l of skim-milk was added in 300 ml aqueous solution at pH 6·5, to 300 ml of bulk raw milk and the mixture was then concentrated to 300 ml. A control which consisted of 300 ml milk and 300 ml water was also concentrated to the same volume. When these milks were coagulated with rennet the whey from milk with GMP contained 42 % of the total fat, that from the ordinary milk only 21 %.

Effect of temperature of milk during concentration and heating treatment before concentration. The effect of temperature both in pretreatment of the milk and during concentration was studied. The results are summarized in Table 4 and show that there was no effect on the fat-retention property when milk was preheated for 10 min in the temperature range 45 °C to just below 80 °C. Heating at 80° C and above caused denaturation of the whey proteins and gave poor curds which had to be separated by centrifugation. However, the fat loss from these curds was very low. This fat retention was a function of this type of curd and not of the casein-micelle–fat-globule interaction, as was shown by removing cream from concentrated and non-concentrated milk preheated at 80 °C and 90 °C and adding it to skim-milk before testing. Under these conditions similar values were obtained to those for milks preheated at the lower temperatures. Changing the boiling temperature of the milk within the evaporator from 39 to 54 °C by altering the temperature of the heating jacket had no effect on the fat-retention properties of the concentrated milk.

Effect of the pH value during concentration on the fat-retention property. The pH values of several samples of raw milk were adjusted with N-HCl or N-NaOH to give values of 5·5, 6·0, 6·5, 7·0 and 7·5 before concentration. After concentration to two-thirds volume the milk was diluted to the original volume and the cream separated. Each sample of cream was then added to an appropriate volume of untreated skim-milk and the fat-retention property of each reconstituted milk was examined. In all cases the fat loss in the whey was similar; thus alteration of pH in the range 5·5–7·5 did not influence the rate of appearance of the fat-retention property.

Effects of other factors on fat retention by curd

Effect of washing the cream upon the rate of appearance of the fat-retention property. It was previously suggested (Mabbitt & Cheeseman, 1967) that some of the membrane material of the fat globule must be displaced before the membrane can become appreciably associated with casein micelles. To see if this was a limiting step in the conferring of the fat-retention property during concentration, cream was separated and then washed with water before reconstituting with skim-milk and concentrating. It has been well established that washing cream with water removes phospholipoproteins which are associated with the fat globule membrane (Hayashi & Smith, 1965).

The experimental results are summarized in Table 5 and show that this treatment before concentration or homogenization had no effect on the fat content of the wheys made from these milks.

Effect of milk agglutinins on the fat-retention property. As agglutinins have been shown to be important in the clustering of cream globules (Dunkley & Sommer, 1944) an experiment was undertaken to see if an increase in the amount of agglutinins in the milk would affect the fat-retention property during and after concentration. An

Table 5. *Effect of washing cream before processing on the fat-retention property of diluted concentrated milk and homogenized milk*

Experiment	Concentrated to $\frac{2}{3}$ volume and rediluted before making curds and whey. Fat in whey, % (w/w) of total fat	Homogenized at 300 lb/in^2 at 75 °C. Fat in whey, % (w/w) of total fat
Control, separated milk and cream*	46	26
Separated milk and cream washed once	46	27
Separated milk and cream washed twice	43	28
Raw milk + 50 ml water†	41	33

* Cream from 8 l bulk raw milk was separated and $\frac{1}{3}$ added back to skim-milk as control. Rest dispersed in 3 l water at 50 °C and again separated; half the recovered cream was added back to 2 l skim-milk. Remainder of cream dispersed in 2 l water at 50 °C, separated and added back to skim-milk.

† 50 ml water added to compensate for the effect of water in the washed cream.

Table 6. *Effect of agglutinins* on the fat-retention property of concentrated milk*

Experiment†	Fat in whey, % (w/w) of total fat
Control, raw milk concentrated to $\frac{1}{3}$ volume	19
Solution of agglutinins added before redilution	22
Control, raw milk concentrated to $\frac{2}{3}$ volume	46
Concentrated with agglutinins to $\frac{2}{3}$ volume	46
Bulk raw milk	74

* Cold agglutinins obtained by desorption from cream. For preparation see Methods.

† All milks diluted to original volume before renneting.

agglutinin-rich solution was prepared according to the technique of Dunkley & Sommer, by first separating cream from milk at 10 °C, and then warming the cream to 50 °C and centrifuging to isolate an agglutinin-rich solution from the fat globules. This solution, in which the presence of agglutinins was confirmed, was added to raw milk before and after concentration. The results of fat losses in whey from curds made with these milks are given in Table 6 and show that the agglutinins had no effect upon the fat-retention property.

Effect of storage of diluted concentrated milk and homogenized milk on the fat-retention property. The effect of storage at room temperature and 4 °C is shown in Table 7. No significant change was found up to 25 h.

Effect of using milk from individual cows and history of the milk before concentrating. Milk from 6 cows in the Institute's Friesian herd was compared with bulk raw milk.

The milk sample from the individual cows was taken at morning milking and its temperature was maintained at 35–37 °C until the milk was examined and concentrated about 3 h later. All the cows were in the middle part of their lactation and 3 samples of milk were taken at intervals of 4 weeks. The feeding régime was changed during this period and the last 2 sets of samples were taken when the cows were on grass.

Table 7. *Effect of storage on the fat-retention property of diluted concentrated milk and homogenized milk*

*Diluted concentrated milk		Milk homogenized at 400 lb/in², 75 °C		Milk homogenized at 1000 lb/in², 75 °C	
Storage time,† h	Fat in whey, % (w/w) total fat	Storage time, h	Fat in whey, % (w/w) total fat	Storage time, h	Fat in whey, % (w/w) total fat
0·5	21	1	28	1·25	12
1·5	22	2	27	2·25	13
3·5	22	4	28	4·25	12
4·5	23	5	28	5·25	13
5·5	21	7	26	6·25	12
23·5	20	25·5	25	26·75	11

* Concentrated to ⅓ volume and then diluted with water to original volume.

† After 6 h at 20 °C the milks were stored overnight at 4 °C. The rennet-clotting times of the milk samples changed with time, from 2 min 0 s to 2 min 47 s for the homogenized milk and from 2 min 10 s to 45 s for the concentrated milk.

Table 8. *The fat in wheys obtained from milks of individual cows subjected to various preconcentration treatments*

(Results expressed as % w/w of total fat in milk)

	Sampling periods 1				2				3			
	A	B	C	D	A	B	C	D	A	B	C	D
Bulk herd milk	—	—	82	20	—	—	74	24	—	—	68	25
Cow 1	65	38	67	14	38	32	57	27	28	34	28	21
2	66	28	72	20	60	38	69	25	35	39	40	29
3	53	37	65	22	42	49	52	32	45	55	57	34
4	55	27	49	16	45	37	44	24	31	29	35	26
5	74	65	82	40	81	53	87	50	79	76	77	53
6	58	27	62	27	42	49	64	30	47	36	58	36

A, untreated raw milk, temperature maintained 35–37 °C; B, milk concentrated to ⅓ of its volume and diluted with water to original volume before renneting, temperature maintained 35–37 °C until milk was concentrated; C, milk cooled at 10 °C for 4 h with intermittent stirring; D, milk treated as in C and then concentrated to ⅓ of its volume and diluted with water to original volume before renneting.

Each individual milk was compared at the time of sampling with the bulk milk from the herd. The bulk milk consisted of the previous evening's milk plus the morning's milk and was held in a refrigerated collecting tank at 4 °C. The fat-retention property of the curd obtained from individual milks was compared with that obtained from curd of the same milk after concentration and dilution. A further portion of the untreated milk was cooled and held at 10 °C for 4 h with intermittent stirring.

The fat retention property of this milk and that of the concentrated milk made from it were also compared.

The results of these experiments are given in Table 8. There was a trend to lower fat losses with the untreated bulk tank milk over the period of sampling which may have been due to changes in the feeding régime. However, this trend was not consistent in the results for the milk from individual cows.

The untreated milks from individual cows gave significantly lower fat losses in the whey made from them than did the corresponding bulked milks. Keeping the individual milks at 10 °C for 4 h gave some increase in fat losses for most of the samples examined.

When the uncooled individual milks were concentrated only those samples taken during the first period gave a significantly lower fat loss in the whey. With milk taken at the other 2 sampling periods no significant fat-retention property was conferred by concentration. However, if these milks were first cooled then good fat-retention properties obtained during the concentrating process.

Table 9. *Effect of concentration and homogenization on the rate of creaming*

(Fat left in milk after centrifuging, % w/w total fat in milk)

Centrifuging steps	Milk untreated	Milk concentrated to ⅓ volume and diluted to original volume	Milk homogenized at 1000 lb/in², 50 °C	1000 lb/in², 70 °C	600 lb/in², 70 °C	300 lb/in², 70 °C	Separated milk plus cream from concentrated milk	Separated milk from concentrated milk plus untreated cream
1st	15	44	71	94	98	60	35	30
2nd	9	33	60	85	71	51	25	10
3rd	7	23	50	73	53	35	13	6

In each experiment 200 ml milk at 40 °C were taken for the initial centrifuging. Each centrifuging step was carried out for 10 min at 250*g*, the cream removed and a sample of the milk tested for fat content.

Comparison of creaming rates of diluted concentrated milk and homogenized milk

The creaming rate of the treated milks was compared with that of raw milk by a 3-stage centrifuging process, the fat content of the skim-milk being determined after each centrifuging. The results are summarized in Table 9, and show that the creaming rate of diluted concentrated milk is considerably less than that of the raw milk but not so low as that of the mildest homogenization treatment (300 lb/in², 70 °C). The fat content in whey from some of these milks, expressed as percentage of total fat in the milk, was 68 % for the raw milk, 27 % for the diluted concentrated milk, 58 % for the milk homogenized at 1000 lb/in², 50 °C, and 15 % for the milk homogenized at 1000 lb/in², 70 °C. Separation of the cream from concentrated milk when added to skim-milk gave a milk with creaming rates somewhat greater than the original concentrated milk but still much less than the raw milk. Diluted concentrated skim-milk plus untreated cream gave creaming rates similar to the raw milk. The similarity in

size of the globules of untreated milk and diluted concentrated milk and their difference from the homogenized milks are shown in Plate 1.

Syneresis of diluted concentrated milk

The results of 2 experiments are given in Fig. 2 and show that at pH 6·0 the whey retention by the diluted concentrated milk was very much higher than that by the untreated raw milk. Approximately twice as much whey was held by the curd from the treated milk compared with the curd from the untreated milk. At lower pH values, the results for which are not shown in the figure, the difference was less and at pH 5·5 and below no significant difference in whey retention was detected.

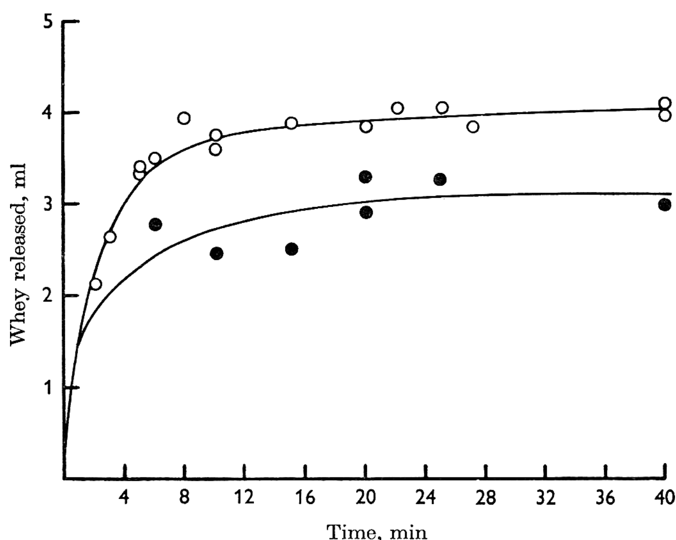

Fig. 2. The release of whey from curd made with raw milk and diluted concentrated milk. ○, Raw milk; ●, diluted concentrated milk. Each point represents the volume of whey obtained from the curd made with 5 ml of renneted milk after the curd was heated to 60 °C for the corresponding time.

DISCUSSION

Mabbitt & Cheeseman (1967) investigated the fat-retention property of curds formed from milk which had been concentrated in a climbing film evaporator and then diluted to its original volume before renneting. They suggested that the property was conferred as a result of the removal of a soluble lipoprotein fraction from the globule membrane followed by the association of the modified membrane with casein micelles. The reaction by which the casein micelles combined with the membrane was not understood but it was thought that the properties of the surface films of milk formed in the evaporator were in some way responsible.

The present findings strengthen this hypothesis. In the experiments using the milks of individual animals it was clearly demonstrated that a cooling step is beneficial and in some instances essential. This requirement can be explained by the change in the physical state of the fat because at the lower temperatures it will be mainly solid, and the consequent rearrangement of physico-chemical forces at the surface may well weaken the binding between components of the membrane and the

fat, thus allowing removal of components and permitting the modified membrane to react with casein micelles. Extensive washing of the fat globule after its initial cooling treatment did not, however, appear to enhance the casein-micelle–membrane interaction during concentration. Either sufficient change had occurred in the cooling stage or rapid changes in the evaporator cancelled out any advantages that the washing treatment may have given.

Change in concentration of inorganic ions and some casein fractions of the milk before concentration, and variation of heating temperatures both before and during concentration, had little effect upon the fat-retention property compared with that of bubbling and the formation of milk films. It would appear that the reaction between the casein micelle and the membrane is independent of changes in the milk composition but that it takes place during the formation of the milk–gas interphase, when it would be expected that the physico-chemical forces at the globule surface would be considerably modified. Higher concentrations of calcium in the milk did give some increase in fat retention but the reason for this is obscure. It is unlikely that a decrease of micelle stability, which would be one of the effects of increased calcium, would be responsible, as no similar increase was observed when milk was treated at lower pH values, at which micelle stability would also be reduced.

The results reported in this paper and by Mabbitt & Cheeseman (1967) show that the reaction between the casein micelles and the fat globule membrane is not greatly enhanced by increasing the concentration of many of the milk constituents. Nevertheless, there is some evidence that the κ-casein moiety of the micelle may be involved in the binding between the micelle and the membrane. This is suggested from the considerable decrease in fat retention when GMP is present during concentration and where it is likely to be in competition with κ-casein for binding sites on the membrane, and also from the very good fat-retention properties of 'creams' made with κ-casein and butter oil.

The properties of curds from renneted diluted concentrated milk are very different from those made from untreated milk. The high whey retention may be due to the casein-micelle–fat-globule interaction; the presence of more micelle–globule complexes would be expected to interfere with the casein–casein interactions which occur during syneresis.

It is interesting to note that the properties of the milk from one cow of the 6 examined were outstandingly different from those of the others (Table 9). This suggests that differences in the composition of the fat globule membrane do occur.

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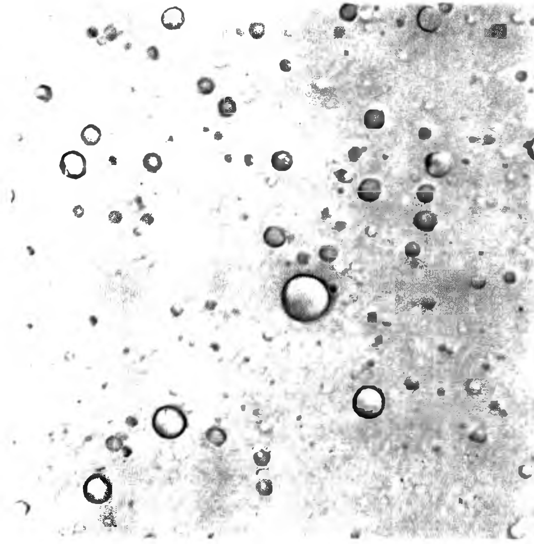

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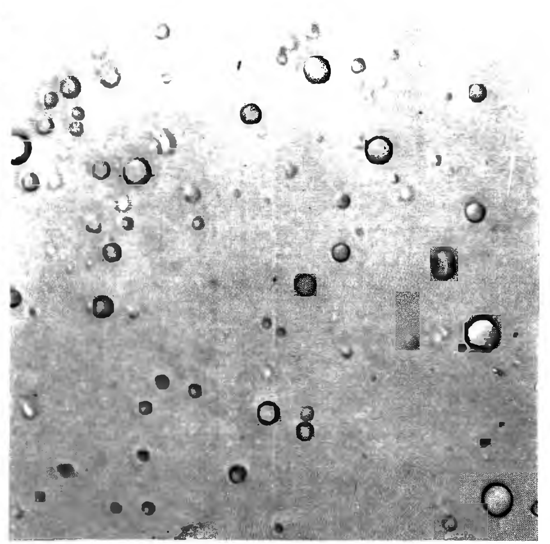

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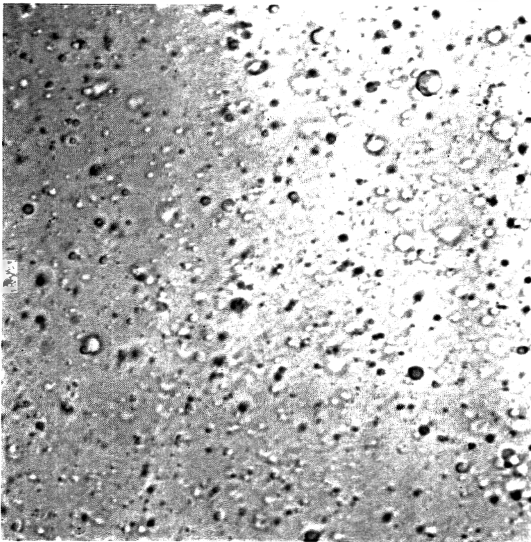

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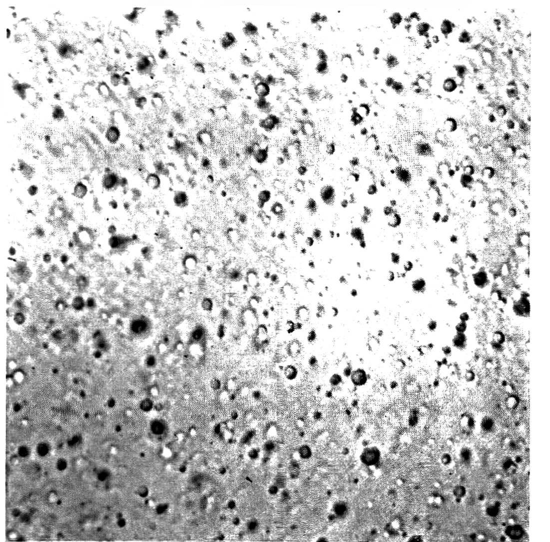

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EXPLANATION OF PLATE

The appearance of fat globules obtained from milks subjected to various treatments. (*a*) Raw untreated milk. (*b*) Concentrated to $\frac{1}{3}$ volume and diluted with water to original volume. (*c*) Homogenized, 300 lb/in^2 and 70 °C. (*d*) Homogenized, 1000 lb/in^2 and 70 °C. Magnification $\times 800$.

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Reviews of the progress of dairy science

Section D. Nutritive value of milk and milk products. Fat soluble vitamins in milk and milk products

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INTRODUCTION

The fat soluble vitamins in milk were last discussed in this series of reviews by McGillivray & Gregory (1962). The present review covers publications that have appeared during the period from January 1962 to January 1967; in most cases the original publication was seen and is cited, but when only an abstract could be consulted the appropriate reference in Dairy Science Abstracts is also listed.

In 1965, the American Dairy Science Association published a comprehensive review by Hartman & Dryden of the literature to 1963 covering the whole field of vitamins in milk and milk products. The book *Nutrition and the Composition of Milk* by Kirchgessner, Friesecke & Koch (1967) also contains much relevant information about fat-soluble vitamins, and has a full bibliography up to the end of 1963. Methods for the estimation of fat-soluble vitamins are discussed and described in the book by Strohecker & Henning (1965).

Vitamin A and carotenoids

General, including methods of measurement

Owen (1965) reviewed the effects of diet, season, climate, hormones, mastitis and stage of lactation on the levels of vitamin A and carotene in human and in cow's milk. Most of the references quoted are to papers which appeared before 1962 and which have been discussed in earlier reviews in this series. Von Aust (1963) reviewed the literature on the content and stability of carotenoids in fats and dairy products. Zechmeister's (1962) book on *cis–trans* isomerism in carotenoids and vitamin A will be of interest to those concerned with the study of the effects of heat treatment on milk and milk products which could cause isomerization.

Only 3 papers have been published that deal with methods of estimation. Gori, Cantagalli & Grande (1962) described a simple method for the determination of vitamin A which involves saponification, purification of the non-saponifiable residue dissolved in diethyl ether by the addition of calcium phosphate, followed by filtration and spectrophotometric measurement of the optical densities of the filtrate at 311, 325 and 332·5 nm. Using this method they obtained values within the expected ranges for the vitamin A content of raw and homogenized cow's milk and of butter and cheese. Makrushin & Dement'ev (1961) described the estimation of vitamin A and carotene in colostrum and milk using a new Russian-built photoelectric colorimeter (KFE′-I). Samel & Schimelmitz (1966) applied the method of Budowski & Bondi (1957), in which vitamin A is converted to the anhydro form and then measured spectrophotometrically at 399 nm, to the estimation of vitamin A in a number of dairy products. They found the method to be as accurate as are methods involving chromatography, and less time-consuming.

In many countries there is a need for analytical methods that allow milks of different species to be distinguished. One such procedure for distinguishing between the milk of the cow and that of the buffalo is based on the measurement of carotenoids, of which only traces are found in buffalo milk. Singh, Yadav & Pathak (1962; 1963) reported that the carotenoid content of buffalo's milk ranged from 0·25 to 0·48 μg/100 ml, compared with 4·5–19·5 μg/100 ml in cow's milk.

In Canada, where there is a marked consumer preference for richly coloured butter, Giroux (1961) found that a reflectometric method was more suitable than visual, colorimetric or spectrophotometric methods for measuring the capacity of added annatto and β-carotene to colour butter. The advantage of the reflectometric method was that it could be applied to intact butter and thus gave results according more closely than the other methods with the effect on the human eye.

In their studies on the effect of milking 3 or 4 times daily on the vitamin A and carotene content of cow's milk, Gazo & Landau (1958) found little effect of frequency of milking on vitamin A content, but the carotene content of the milk of cows milked 3 or 4 times daily showed a peak at the second milking. The values obtained were 16·0, 19·4, 16·3 and 16·6 μg carotene/100 g milk, for milking at 5 a.m., 8 a.m., midday and 6 p.m., respectively. These changes do not follow those in fat content, which was the lowest in the morning and highest in the midday and evening milk. In a later paper, Gazo & Landau (1964) reported that the vitamin A and carotene content and the fat content of milk increased throughout milking, and that the concentration of

vitamin A in the fat increased, whereas that of carotenoids did not. This is surprising, since McGillivray (1957) has shown that milk serum contains small quantities of carotenoids bound to protein; thus it might have been expected that foremilk, which has the lowest concentration of fat, would have yielded fat having the highest concentration of carotenoids, since the protein-bound carotenoids will be extracted and measured with the carotenoids in milk fat.

Fractionating the components of the non-saponifiable matter of butter by chromatography on Al_2O_3, Lopez Lorenzo, Sanz Perez & Burgos (1962) obtained, per g fat, 3·8–4·5 μg vitamin A, 13·0–18·0 μg α-tocopherol, 1·9–2·4 mg cholesterol and 0·82–1·2 μg ubiquinone.

A new metabolic role for vitamin A may have been established by Haubold, Heuer, Loew & Rohuschinsky (1965), who found that the vitamin was necessary for the uptake of fat emulsions into the cell cytoplasm of rat tissue cultures. Since the fat globules of raw cow's milk were taken up under the same conditions they concluded that for absorption into the cell the fat globules of milk must be emulsified and contain vitamin A.

Cow's milk

Effect of dietary source of carotene

Pasture. It is now well established that there is a direct relationship between the vitamin A potency of milk and the carotene intake of the cow. Thompson, Henry & Kon (1964) showed conclusively from nation-wide surveys carried out in 1943 and in 1958–60 that market milk in Great Britain obtained from 14 large dairies contained levels of vitamin A and carotene in the milk fat that were closely correlated with the abundance of pasture. The highest levels found were in milk from a large farm in Hampshire where the cows were given diets almost exclusively of grass and grass silage. The mean values obtained during a period of 28 months for vitamin A and carotene, 9·7 μg and 6·8 μg/g fat, respectively, for milk from the Hampshire farm were somewhat higher than those of 8·5 and 5·8 for milk from dairies in southern England, and considerably higher than those of 7·2 and 3·5 for milk from dairies in the northern part of the country. The differences in levels between milks from the north and from the south is undoubtedly due to the longer growth season in the south. In the north, the differences between summer and winter milks were more marked than in the south. In winter milk, the mean vitamin A potencies were 5·8 and 7·7 μg/g fat for northern and southern areas, respectively; corresponding values for β-carotene were 2·0 and 4·5 μg/g fat. In summer milk, the mean values for northern and southern areas were, respectively, 8·8 and 9·7 μg/g fat for vitamin A, and 5·1 and 6·5 μg/g fat for carotene. The summer values show increases over winter values of about 52 and 26 % for vitamin A, and about 155 and 44 % for β-carotene.

Umezu *et al.* (1957–60) found in the Tohoku district of Japan that cows grazing native grasses, mainly *Zoysia japonica* and *Miscanthus sinensis*, needed a larger area of pasture to produce milk as rich in carotene and vitamin A as did cows grazing a pasture consisting of 54 % red top fescue and 34 % Ladino clover and supplying 3–4 g of carotene per cow per day. The carotene content of the various native grasses was measured and found to be correlated with the carotene and vitamin A content of the milk. In a further study, in the same part of Japan, Nakanishi & Nakae (1966)

reported widely differing carotene intakes in different localities, and in summer and winter. In the Takasago area the carotene intake varied from 700 mg per cow per day in summer to 156 mg in winter. They suggested that this low winter intake was responsible for the extremely low vitamin A potency of 3·5 i.u./g fat that they found in winter milk. However, more normal levels of vitamin A in milk fat in the winter were found for the Kawatabi district, where the cows received in the summer 3–4 g carotene daily. Such intakes were similar to those found by Thompson *et al.* (1964) in England. It seems likely, therefore, that the low level of vitamin A in winter milk from the Takasago area was not due solely to a low carotene intake during the winter, but was the cumulative effect of a low carotene intake during the preceding summer, followed by a low intake during the winter.

Further interesting work is reported from New Zealand by McDowall & McGillivray (1963*a*, *b*) and by Hawke (1963) who have continued their studies of factors influencing the carotene and vitamin A content of butter, and have now published their findings in full (see McGillivray & Gregory, 1962, for preliminary discussion). Their earlier work had suggested that the stage of maturity of the pasture influenced the utilization of carotene from rye-grass and these later papers now show that the milk of cows given immature rye-grass contained more unsaturated fatty acids and had a higher carotene and vitamin A content than did that of cows given mature rye-grass. They interpreted their findings by suggesting that some lipid—probably leaf-cuticle wax—present in larger amounts in the more mature pasture decreased the uptake of carotene and its conversion to vitamin A. Studies bearing on the absorption of carotene are of topical interest in England as the present trend towards the stall feeding of cows, with the consequent lower carotene intake, has led to consumer complaints about poor milk colour.

Thompson & Ascarelli (1962) studied the changes in levels of vitamin A ester, alcohol and carotene in the blood and milk of 4 Shorthorn and 42 Friesian cows at the time of change-over from a winter ration providing 20–60 mg carotene per cow per day to pasture supplying 2–6 g per cow per day. The total vitamin A content of the milk and the vitamin A ester content of the blood in general reflected the carotene intake; they increased rapidly and reached a maximum value in milk after the animals had been 2–5 days on pasture. The carotene content of blood and milk showed a close relationship, but increased more slowly than did vitamin A, taking about 3 weeks to reach maximum values. On reverting to a low carotene intake the levels of vitamin A in blood and milk decreased rapidly, but those of carotene remained constant for a day or two and then slowly declined. The level of vitamin A alcohol in blood was unaffected by changes in the carotene intake.

Unexpectedly high contents of carotene and vitamin A found by Viana, Viana & Moreira (1962) in milk produced in the Belo Horizonte province of southern Brazil in June and July, in the middle of the dry season, were followed by unusually low contents, particularly of carotene, towards the end of the dry season in October.

From East European countries Belyaeva (1962), Dzhurkov (1962) and Emelina (1965) all reported that the vitamin A potency of milk fat was correlated with the abundance of pasture.

Grass silage and artificially dried grass. Kiermeier & Renner (1962) in southern Germany found that butter made from the milk of cows given grass or clover silage

had a higher carotene content and iodine value, and a lower flavour score, after 1 week's storage than had butter from cows given no silage, or maize silage, or sugar-beet-top silage; however, on further storage the flavour of butter from cows given sugarbeet-top silage deteriorated more rapidly than that of butter from cows given the other diets.

Dijkstra (1958*a*, *b*) gave 3 groups of cows hay, grass silage made after wilting, or grass silage made with molasses, and found little difference in milk yield between the groups, but the highest carotene and vitamin A contents and iodine values were found in the milk of cows given the molasses silage and the lowest in the milk of those given hay. The lower values found with the wilted grass silage were due to loss of carotene from the grass during wilting. Thompson (1959) reported that when, after the end of the pasture season, grass silage was fed to cows to provide 700 mg carotene per cow per day, the carotene and vitamin A contents of milk fat were not restored to the high levels existing at the end of the grazing period. When the grass silage intake was increased to provide 2 g carotene/day there was a large increase in vitamin A but only a small increase in carotene. However, when these cows changed from silage to pasture feeding providing 4–6 g of carotene/day, there was a decrease from the high vitamin A level reached on silage feeding and a marked increase in the carotene content. Possible reasons for this different utilization of carotene from grass and from grass silage were discussed.

Further evidence of the effect of silage on the vitamin A potency of milk was given by Luksho (1962), who for 60 days gave Brown Latvian cows clover silage prepared with and without the addition of H_2SO_4 and HCl, and found a higher content of vitamin A but not of carotene in the milk of cows fed the acid-treated silage.

The expected increase in vitamin A and carotene levels in milk was found by Brzozowski (1963), who replaced up to 75% of the oats in the rations of 16 Polish Pied cows by artificially dried grass containing 90 mg carotene/kg.

The increased use of silage in Sweden was found by Hellström & Anderson (1965) to have increased the carotene but not the vitamin A content of 180 butter samples from 15 dairies.

Kale, hay and other winter fodders. Thompson *et al.* (1964) and Thompson & Ascarelli (1962) drew attention to the low carotene intake of cows in winter in Britain after the end of the kale and grass silage feeding period, when the only source was hay supplying some 20–60 mg per cow per day. On such low intakes milk fat in Britain during January, February and March had mean contents per g of 5·9 μg vitamin A and 2·5 μg carotene. Thompson (1959) further showed that the normal intakes of kale did not provide enough carotene to prevent a drop in carotene and vitamin A in the milk after the end of the pasture season. Steger, Piatkowski, Busch & Püschel (1964) found that the exclusive feeding of kale reduced the milk yield and vitamin A content of the milk but increased the carotene content.

Golyarkin (1965) found that supplementing the cow's diet with pine and fir twigs increased the vitamin A content of the milk. Surprisingly, Kadiiski & Shishkov (1962), in Bulgaria, found only traces of carotene in the milk of 2 cows fed kale.

Other sources of carotene. Estimates from various workers of the carotene intake needed to maintain summer levels of vitamin A and carotene in milk were discussed in a review by Thompson (1963), who concluded that between 0·3 and 2 g of carotene

per cow per day is needed, depending on the source and composition of the diet. Thompson (1963) showed that cows utilized 1 g carotene poorly when it was given in oil, but efficiently when it was given as a microcrystalline gelatin beadlet preparation (Roche Products, Ltd); in fact, 1 g of carotene from the latter preparation was as well utilized as 4 g of carotene from pasture grass.

Rezevskaya (1962) gave known amounts of carotene of unspecified origin to cows and concluded that a 500-kg cow needed in winter 350 mg carotene for maintenance and a further 10 mg carotene/l of milk produced. On this basis, a cow giving 15 l/day of milk requires a daily intake of 500 mg.

The value of vitamin A supplements to diets low in carotene was studied by Rusoff, Drude & Lovell (1965), who fed 2 groups of cows low- or high-roughage diets (supplying 56 or 217 mg carotene/day) with concentrates; half the cows in each group were given daily 82500 i.u. vitamin A/cow. The vitamin A contents of the milk ranged from around 45 i.u./100 ml, for the unsupplemented low-roughage group, to around 77 i.u./100 ml for the supplemented high-roughage group; the increased potency was largely attributable to the added vitamin A.

It is well known that diets containing high levels of nitrates may give rise to serious toxic effects, but there have been conflicting reports about the possible relationship of feeding such diets to vitamin A deficiency and the conversion of carotene to vitamin A. However, careful investigation by Jones, Weswig, Bone, Peters & Alpan (1966) showed that the vitamin A content of the milk was unaffected when cows were given an adequate diet that contained maize of high nitrate content.

Effect of season and geographical location

The effect of season is inextricably mixed with that of diet, and seasonal variation reflects the response to different carotene-containing feeds at different times of the year. The general trends are well known: high intakes of carotenoids from pasture, usually in the spring and summer months, result in the fat of summer milk being rich in carotene and vitamin A, whereas low intakes in the winter months cause falls in the milk levels. Surveys covering periods of a year or more and showing these trends have been reported in England (Thompson *et al.* 1964); Bulgaria (Dardzhonov, 1962; Tsvetkova, Ikonomov & Todorov, 1962; Dzhurkov, 1962); Italy (Sebesta & Dalma, 1965); Yugoslavia (Naumovic, 1964); Poland (Luczak, Barska & Kwinta, 1961); and France (Hugot & Causeret, 1966). In Brazil, Viana *et al.* (1962) found mean annual levels in pasteurized milk of 3% fat content for carotene and vitamin A of 1·3 μg and 234·6 i.u. (70·4 μg) per 100 ml, respectively. The value for vitamin A seems improbably high and further work is needed to confirm the finding.

Thompson *et al.* (1964) showed that both breed and feed affect the proportion of biologically active carotenes in butterfat, but that feed is by far the more important. They showed that in Britain the carotenoids of milk fat contained about 80% of carotene during the grass-growing season, but that in winter the percentage of biologically active carotenes fell and was lower in the north (62%) than in the south (74%). The lowest percentages were found in the north of Scotland, where values of 25 and 33% were recorded in March 1944 and 1958, respectively. In winter, cows receive not only much less carotene, but also a greater variety of inactive carotenes,

some of which, such as lycopene in yellow turnips and breakdown products of xanthophylls in silage and hay, are absorbed by the cow and pass into the milk.

It is of interest that Hellström & Anderson (1965) found that winter butter from northern Sweden had a higher carotene content than that from southern Sweden. They postulated that either the colder northern climate allowed better conservation of carotene in hay or that the difference was due to the breed of the cow. In summer, the levels of vitamin A in butter were higher in the south than in the north due to the better grazing conditions.

Effect of breed

It is very difficult to find reliable comparisons for the vitamin A and carotene contents of the milk of different breeds, for such comparisons are only valid when the breeds being compared are on the same farm under identical conditions of management and feeding. Even if such conditions can be found, the numbers of animals of each breed are generally small. Thompson *et al.* (1964) compared the carotene and vitamin A content of milk from Guernsey, Shorthorn and Friesian cows kept at the National Institute for Research in Dairying and from Ayrshire, Friesian and Jersey cows kept on a nearby farm. They found the concentration of vitamin A in the milk fat was essentially the same for Ayrshires, Friesians, Jerseys and Shorthorns but somewhat lower for Guernseys; the concentration of carotene was higher for the Channel Island breeds, particularly Guernseys, than for the other breeds studied. Guernsey milk contained about twice as much carotene as Shorthorn milk, which was in turn a little richer than Friesian milk.

Further information has been reported about the carotene and vitamin A levels in the milk fat of breeds in Eastern Europe. Belyaeva (1962) studied the vitamin A activity of the milk of 2 breeds of Russian cows and of their cross-breeds, and also measured the carotene content of the fodders used. The highest vitamin A activity was found in the milk of the Siberian breed Yakatsh, which was 17·1 % higher than that of the Kholmogor breed, but only 3·5 % higher than that of the breeds when they were crossed. In another paper from Russia, Dobrynina (1961) reported that the vitamin A activity was higher in the milk of Jersey than of Black Pied cows or of crosses of the 2 breeds. Herzen (1966) found, in a study involving over 100 animals in the Ukraine, that Lebedinsky × Jersey and Red Steppe × Jersey cows produced milk richer in vitamin A than their pure-bred Lebedinsky and Red Steppe mothers, 29·8–37·4 and 30·1–33·9 μg, as against 26·9–28·0 and 22·1 μg vitamin A/100 ml, respectively. In Yugoslavia, Naumovic (1964) compared the vitamin A and carotene contents of 3 breeds—Friesians, Simmental and native cattle—but his detailed results await publication.

Effect of stage of lactation

Until recently, it was extremely difficult to maintain cows throughout a complete lactation on a constant carotene intake. It was necessary either to keep the cows constantly on pasture and to limit their carotene intake to a predetermined level, by measurement of the carotene content of the pasture at frequent intervals and regulation of the amount of pasture grazed, or alternatively to keep the cows indoors on a carotene-low diet and then give carotene-rich feed such as grass silage. Both

courses involved very careful control of the carotene content of the feed and of the many dietary factors which could alter the utilization of the carotene. However, during the last 10 years the introduction of synthetic carotene has markedly lowered the cost of carotene, and Thompson (1962) was able to maintain a group of 10 cows from 1 month before parturition until the end of lactation on a carotene-low diet supplemented with 1 g of synthetic carotene in gelatin beadlet form (Roche Products, Ltd). Results showed that colostral levels of 52 μg vitamin A and 74 μg carotene/g fat declined rapidly to minimum vitamin A and carotene levels of 7·7 and 7·7 μg/g fat, respectively. during the 2nd and 3rd months of lactation, after which there was little change in vitamin A but a slight increase in carotene level towards the end of lactation, when the values were 8·3 μg vitamin A and 9·4 μg carotene/g fat. Vlasova (1961) also reported that the vitamin A and carotene contents of the colostrum of cows in Moldavia declined rapidly *post partum* and that the colostral vitamin A was increased markedly by feeding carotene during the last month of pregnancy. Other studies of the effect of stage of lactation include that of Naumovic (1964), who found in Friesian and Simmental cows a clear relationship between the carotene content of milk and the stage of lactation, but not between vitamin A or tocopherol and stage of lactation.

Effect of exposure to electro-magnetic radiation, to heat treatment and to storage

Ford, Gregory & Thompson (1962) reported that raw liquid milk irradiated with 1·0 megarad of γ-radiation lost 64 and 68 % of its content of vitamin A and carotene, respectively. Lowering the oxygen tension of the milk by flushing with nitrogen before irradiation was found to reduce these losses. Glew (1962) found that flavour defects appeared in milk irradiated with 0·02 megarad and that at sterilization levels (1–2 megarad) these flavour defects were quite objectionable. In fact, it seems unlikely that sterilization of liquid milk by γ-irradiation will become a commercial process.

Side effects of ultra-violet light irradiation as used for the conversion of provitamin D to vitamin D were studied by Wodsak (1965), who found that such irradiation also caused some isomerization of vitamin A to neo-vitamin A.

Sinha (1963) studied the effect on the vitamin A and β-carotene contents of milk of irradiation for 6 and 12 h with fluorescent light. He found, rather surprisingly, an increase in the level of vitamin A from 88 to 119 i.u./100 ml and a corresponding decrease in carotene from 25 to 20 μg/100 ml after 6 h irradiation; after 12 h irradiation the level of vitamin A had decreased to its initial value. Preliminary supplementation of the milk with carotene promoted an even greater increase in vitamin A. It seems probable, as Sinha himself suggested, that breakdown products of carotene were contributing to the measured vitamin A. Attempts by the present author to confirm this finding were unsuccessful as exposure of milk to bright fluorescent light of about half the intensity of daylight resulted in a progressive loss of vitamin A after 3, 6 and 24 h exposure.

Agrawal & Singh (1960) reported unexpectedly high losses of 4 and 7 % of carotene and vitamin A during heating cow's milk for 16 s at 161 °F and greater losses (26 and 17 %) on boiling for 15 min. Heating buffalo milk, which contains little or no carotene, in the same way caused similar losses of vitamin A. In another study, Maqsood Ali, Haque & Khan (1963) showed that the more severe heating as used in the preparation

of ghee and vitaminized vanaspati caused losses of vitamin A of 19, 44 and 72 % after heating for 1 h in an oven at 100 °C, 1 h over boiling water, or 15 min at 200 °C, respectively. It seems surprising that the loss was greater during heating over boiling water than in an oven at 100 °C, and it is possible that exposure to light may have been the factor responsible for the losses of vitamin A in both these studies.

Davidov, Gul'ko & Bekhova (1962) found that pasteurization alone caused no change in the vitamin A and carotene content of milk, but that when this process was followed by evaporation as in the manufacture of condensed milk, there was a loss of 20 % of the vitamin A, although carotene was not affected. After 2 years storage at 8–12 °C the loss of vitamin A was 56 % and of carotene 47 %. The same authors (1963) studied the losses on drying and storage of milk. They found only 6 % loss of vitamin on drying, but a total loss of 65 % on drying and storing for 2 years. Luczak *et al.* (1961) found that pasteurization caused a 4·7 % loss of vitamin A and 2·9 % loss of carotene in winter milk, with no further losses by the time the milk was retailed. In summer milk, the losses on pasteurization were 4·0 % for vitamin A and 5·2 % for carotene, with additional losses of 0·70 and 2·0 %, respectively, by the time the milk was retailed. Lobmaier (1960) found no loss of carotene in the spray-drying of raw milk, but up to 30 % loss when pasteurized or pasteurized homogenized milk was spray-dried. He found a further 30 % loss during 5 months' storage of the spray-dried milk at 4–15 °C; exposure to sunlight or to higher temperatures during storage increased the loss still further. It is interesting to note that Causeret, Hugot, Goulas-Scholler & Mocquot (1961) found that vitamin A was stable for up to 6 months in sterilized milk stored at 4 or 20 °C in the dark, but that at 38 °C the loss was 50 % in 6 weeks, with no further loss on subsequent storage; in diffused or direct light 80 % of the vitamin A was lost in 6 weeks; carotenoids were stable under all the conditions of storage tested. Hugot, Lhuissier & Causeret (1962) stored sterilized milk in clear- and amber-glass bottles for 6 months at room temperature in the dark, in diffused daylight and in direct sunlight. The vitamin A content of the original milk was 28 μg/100 g but this decreased by 20 % during 6 months storage in the dark in both amber- and clear-glass bottles; in diffused light the loss was 25 % in amber- and 40 % in clear-glass bottles. In sunlight, 10 % was lost after 6 weeks in amber- and 70 % in clear-glass bottles.

Most butter is manufactured during the grazing season when there is a surplus of milk for the liquid market. Thus, it is important to ensure that butter retains its vitamin content and flavour during storage for some months. Tsvetkova & Ikonomov (1960) found that butter made from cow's, buffalo's or ewe's milk lost no vitamin A activity when stored for 15 days at 18–20 °C or for 3 months at −5° C, and lost only 5 % after storing at −18 °C for 9 months. It is generally considered that clarified fat keeps better than butter, and it is interesting to note that the Finnish workers Antila & Antila (1963) found that the addition of *Propionibacterium jensenii* to clarified milk fat made from sweet churned summer butter prevented, during 1 year's storage at room temperature and in diffused daylight, the increase in peroxides and free fatty acids and the decrease of 70 % in vitamin A and 96 % in carotene that were found in fat stored without addition of the bacterial culture. Shebanin (1965) found that the addition of β-carotene to winter milk fat did not alter the normal peroxide development.

Carotene and vitamin A content of butter and cheese

Keilling *et al.* (1966) found that care was necessary in the treatment of Emmenthal cheese lest glycerides, in which the carotenoids are dissolved, are expressed and allow bleaching and destruction of carotenoids.

Devyatin, Mel'nikova & Chepenko (1959) reported mean vitamin A contents of 0·13–0·40 mg/100 g dry weight in Russian hard and soft cheeses. Gori *et al.* (1962) in Italy found vitamin A contents of butter and cheese to be respectively 16·9–26·5 and 2·3–10·5 i.u./g.

Vitamin A and carotene in nutrition

There is evidence that one or more of the constituents of milk aid the uptake of carotene and vitamin A in animals, and the utilization of these substances from milk and milk products is usually superior to that when they are given with other carriers. Thus, Berger, Gronowska-Senger & Chabrowska (1966) found that the vitamin A from a vitamin-A–casein complex, formed when vitamin A acetate in acetone solution was added to an aqueous suspension of casein, was better utilized by young rats than was vitamin A from an oily solution. Dvorak (1963) found that the absorption by calves of vitamin A from an oily solution of the vitamin, as judged by levels of vitamin A in blood serum, was low during their first 14 days of life but improved as the animals became older; emulsification of the oil with Tween 80 improved absorption of vitamin A in the younger calves: best absorption was achieved when the vitamin was given with milk.

Continuing their studies on the nutrition of the Ayrshire calf, Roy *et al.* (1964) and Roy, Shillam, Thompson & Dawson (1961) found that animals reared for veal production and receiving a whole-milk diet had a vitamin A requirement of 44 mg/kg body weight, and that when fat was emulsified with lecithin or was homogenized into reconstituted dried skim-milk, the plasma levels of vitamin A were similar and higher than when the calves were given unhomogenized fat or fat without lecithin.

Swanson, Carpenter & Thomas (1962) found no significant differences in liveweight gain between groups of calves receiving from 7 days of age a skim-milk starter ration, with or without supplements of vitamins A and D, when the diet from birth consisted of colostrum followed by whole milk.

Addition of vitamin A to milk products

The United States of America does not allow fortification of dried skim-milk with vitamins. Nevertheless, because of the growing market for this product—the demand in the U.S.A. has risen from 2 million lb in 1948 to 200 million lb in 1963—and the possibility of helping underdeveloped countries with gifts of this valuable source of proteins, Bauernfeind & Allen (1963) and Bauernfeind & Parman (1964) felt it was worth while to study the stability of added vitamin A in case such fortification became desirable and permissible. Their experiments were carried out both on a pilot plant and on a commercial scale, and included tests of stability and palatability in Central and South America, Indonesia and Africa. They aimed at fortification with 2000 i.u. (600 μg) vitamin A/qt of reconstituted milk and achieved it either by homogenizing a blend of coconut oil and vitamin A into skim-milk and then spray-drying

or by mixing the vitamin in a dry beadlet form into the dried skim-milk. Results indicated that it was practicable to produce enriched dried skim-milk by either method, that the loss of vitamin A fell within acceptable limits, and that the flavour of the enriched milk could not be distinguished from that of the unfortified control. On the other hand, Thomas, Coulter & Kudale (1965) found that in instantized dried skim-milk, to which vitamin A was added at the same rate, there was a loss of about 15 % of the vitamin and a stale flavour developed after only 3 months' storage at 70 °F.

Marmori (1963) added to milk vitamin A acetate, made water-soluble by a non-ionic surface active agent. The enriched milk was then stored in a deep-freeze, or sterilized and stored at room temperature. After 30 days storage the frozen sample showed no loss of vitamin A, whereas the sterilized sample showed a 26 % loss; however, it is not stated whether the latter sample was stored in the dark or in light. Gorbunova, Zolstovskaya & Milytina (1966) homogenized into milk an oily concentrate of vitamins A and D and also, separately, a solution of ascorbic acid, to give concentrations of 430 i.u. (129 μg) vitamin A, 100 i.u. vitamin D and 10 mg ascorbic acid/100 g milk. Subsequent pasteurization and the making of kefir caused little loss of these vitamins.

Stalberg & Radaeva (1966) found that the browning or Maillard reaction during the storage of sweetened condensed milk was either prevented or decreased by the addition of 10 mg vitamin A/100 g.

Addition of colouring matter to milk products

A review by Bunnell & Bauernfeind (1962) deals comprehensively with the chemistry and properties of carotenoids—some of them biologically active—which could be used for colouring foods, including, where legislation permits, dairy produce. The Bulgarian workers, Bonev, Yankov, Penev & Prodanski (1962) described the enrichment of sterilized milk with carotene from strained carrot purée, and with protein from dried skim-milk or whey protein. The process involved homogenization at 150–200 atmospheres at 85 °C, deaeration, bottling and sterilization.

Richardson & Rege (1961) outlined a method for controlling the colour of butter in order to produce butter of a predetermined colour intensity: this was done by separating the fat from cream by the use of the U.S. Bureau of Dairy Industry (BDI) detergent method as described by Sager, Sanders, Norman & Middleton (1955), and measuring the colour. It was suggested that 12 μg carotene/g fat is a desirable standard concentration to aim at in butter.

Giroux (1961) described methods for measuring the colouring properties of annatto and β-carotene added to butter (see p. 150).

Berglöf & Kjell (1963) concluded that β-carotene imparts a better colour to cheese than does annatto, and pointed out that β-carotene is the only colouring additive permitted in cheeses for export from Sweden.

Human milk

Saito *et al.* (1965) analysed 551 samples of human milk obtained at 3 health centres in Tokyo and 2 in Osaka and gave results for total solids, fat, protein, lactose, ash, calcium, phosphorus, iron, sodium, potassium, chlorine, amino acid composition of

the milk proteins, fatty acid composition of milk lipids and levels of vitamin A, carotenoids, thiamine, riboflavin and ascorbic acid. They found on average 152 i.u. (45·6 μg) vitamin A and 20·9 μg carotenoids/100 g milk; only 20–30 % of the carotenoids was contributed by β-carotene. These values agree well with those reported for human milk in Britain by Kon & Mawson (1950).

A paper by Ashdir & Puri (1962) reported on the lactational trends of the milk of 10 women in India. The mean vitamin A content of colostrum was 375 ± 98 i.u. (113 ± 29 μg)/100 ml. In the transitional period from colostrum to mature milk it was 230 ± 79 i.u. (69 ± 24 μg)/100 ml and in the mature milk 159 ± 30 i.u. (48 ± 9 μg)/100 ml. There was a significant correlation between dietary vitamin A and milk vitamin A. Venkatachalam, Belavady & Gopalan (1962) found that women in India in a low socio-economic group gave milk having a vitamin A content of 55 i.u. (16·5 μg)/100 ml, which was lower than the level of 70 i.u. (21 μg)/100 ml in the milk of better-nourished women in another district. Similar findings were reported by Contreras, Arroyave & Guzman (1962), who studied the composition of milk from 43 mothers of a high socio-economic level in the city of Guatemala and 69 poorer mothers living in the rural village of Santa Maria Conque. During the first 3 months of lactation the levels of carotenoids and vitamin A were 22·3 μg and 169 i.u. (51 μg)/100 ml in the milk from the city mothers and 43·5 and 129 i.u. (39 μg)/100 ml in the milk of mothers from the rural areas; these differences were not statistically significant. During the second 3 months, the levels of carotenoids and vitamin A were 16·2 μg and 164 i.u. (49 μg)/100 ml in the milk of city mothers and 29·1 μg and 46·9 i.u. (14·1 μg)/100 ml in the milk of rural mothers; the differences in vitamin A levels were significant.

Ajans, Sarrif & Husbands (1965) in the Lebanon found that one dose of 180 000 μg of vitamin A given orally to women at about the time of parturition caused a significant rise in the level of vitamin A in the colostrum, whereas the same dose given intramuscularly was without effect. They suggested that a single large oral dose of vitamin A given to the mother at the time of delivery could form a practical prophylactic measure against subsequent vitamin A deficiency in the infant.

Studies with human milk were made in Hungary by Tarjan *et al.* (1965), who investigated the effect of stage of lactation on vitamin levels in milk samples obtained during the first 140 days. The vitamin A content in colostrum was 248 i.u. (74 μg)/100 ml. It remained at this level for 2 weeks and then decreased to 110 i.u. (33 μg)/100 ml in mature milk. It is noteworthy that although the content of vitamin A was low, Tarjan, Kramer, Szöke & Lindner (1963) reported that ingestion by the mothers of sufficient grated raw carrot on one day to supply 12–24 mg carotene was without effect on the vitamin A level in the milk. However, ingestion of 150 g liver, which supplied 16·5–39 mg vitamin A, produced a transient increase of 2 to 4·5-fold; a peak was reached in 12 h which declined to the original level after 48 h. The direct addition to acidified milk of varying fat content of carrot powder was found by Ferola & Tiberio (1964) to cause in infants a doubling of the vitamin A and trebling of the carotene content of the blood serum.

Table 1. *The content of fat-soluble vitamins in the colostrum and milk of different species*

	Fat, g/100 g	Vitamin A, μg /g fat	Vitamin A, μg /100 g or ml milk	Carotene, μg /g fat	Carotene, μg /100 g or ml milk	References
Buffalo: colostrum	9·55	—	184	—	—	Mazziotti di Celso (1963)
Buffalo: milk	7·6	—	28	—	—	Mazziotti di Celso (1963)
Buffalo: milk	—	—	—	0·24–0·48*	—	Singh *et al.* (1962, 1963)
Buffalo: milk	—	3·1	—	None	None	Tsvetkova & Ikonomov (1960)
Buffalo: milk	—	—	32–52	None	None	Sirry & El-Said Saleh (1962)
Buffalo: milk	7·5	—	35	—	3·8	Grigorov, Shalichev & Goranov (1962*b*)
Camel (Bactrian): milk	—	—	—	—	46–16†	Bestuzheva (1964)
Ewe: milk	—	4·2	—	0·16	—	Tsvetkova & Ikonomov (1960)
Ewe: milk	—	—	44	—	—	Gori *et al.* (1962)
Ewe: milk	—	—	30	—	8·1	Grigorov, Shalichev & Goranov (1962*a*)
Ewe: milk	—	8·0	—	0·8	—	Oravcova & Görner (1962)
Giraffe: milk	12·5	6·1	—	None	None	Aschaffenburg, Gregory, Rowland, Thompson & Kon (1962)
Goat: colostrum	—	—	270–980‡	—	—	Owen *et al.* (1965)
Goat: colostrum	—	—	124–206§	—	—	Owen *et al.* (1965)
Goat; milk	—	—	20–23	—	—	Owen *et al.* (1965)
Goat: milk	—	—	20	—	7·7	Grigorov *et al.* (1962*a*)
Kangaroo: milk	1·35–12·77	5·4–21	—	None	None	Aschaffenburg, Ford, Kon, Rowland & Thompso (1965)
Okapi: milk	4·4	6·0	—	None	None	Gregory, Kon, Rowland & Thompson (1965)
Rabbit: colostrum	17·7	—	670	None	None	Coates, Gregory & Thompson (1964)
Rabbit: milk	12·3	—	84	None	None	Coates, *et al.* (1964)
Reindeer: milk	9·2–16·9	7·5–11	—	None	None	Aschaffenburg, Gregory, Kon, Rowland & Thomp son (1962)
Rhinoceros: milk	0·0–0·45	None	None	None	None	Gregory, Rowland, Thompson & Kon (1965)
Sow: colostrum	—	—	187	None	None	Heaney, Hoeffer, Ullrey & Miller (1963)
Sow: milk	—	—	22	None	None	Heaney *et al.* (1963)
Sow: colostrum	5·9	—	210	None	None	Nielsen, Højgaard-Olsen, Hjarde & Leerbeck (1965)
Sow: milk	6·4	—	51	None	None	Nielsen *et al.* (1965)

* Total carotenoids. † 6th–13th week of lactation. ‡ Grazing animal. § Stall-fed animal.

Milk of other milch animals and of animals not normally used for milk production

Recently reported values for the contents of vitamin A and carotene in the milk of various species are summarized in Table 1.

The values for vitamin A content of the milk of the ewe, goat and buffalo are within the expected ranges and it is of interest that several workers found small amounts of carotene in the milk of these species.

Oravcova & Görner (1962) studied the distribution during cheese-making of carotenoids and vitamin A from ewe's milk, and found that the whey fat contained slightly more carotene and considerably more vitamin A than did the fat of the curd.

The vitamin A content of the sow's ration determines the level of vitamin A in the colostrum and milk and a wide range of values has been reported (cf. Evans, 1959). Nielsen, Højgaard-Olsen, Hjarde & Leerbeck (1965) found the colostrum and milk of cows given a daily supplement of 40000 i.u. (12000 μg) of vitamin A contained 350 and 54 μg/100 g when the vitamin was supplied in a dry powder form and 210 and 51 μg/100 g when it was given as cod-liver oil. Heaney, Hoeffer, Ullrey & Miller (1963) maintained 4-month gilts for 6 months on a ration low in vitamin A; then, after receiving daily 2·5, 5 or 16 μg of vitamin A/kg liveweight, the gilts gave colostrum containing 187, 75 and 94 and milk containing 22, 14 and 9 μg/100 g, respectively.

VITAMIN D

Methods of measurement

There is still no satisfactory chemical method for the determination of vitamin D in unfortified milk products, and for these materials it is necessary to use the expensive and time-consuming biological assays. However, some success has been achieved in the estimation of vitamin D in fortified milk products. Thus, to determine the vitamin D content of irradiated milk Tancredi (1962) used paper chromatography and spectrophotometric estimation at 500 nm of the colour formed between an antimony trichloride reagent and vitamin D. By these means samples of irradiated milk were found to contain 34·6 i.u./100 ml, and recoveries of added vitamin D of 95–98 % were claimed. The U.S.P. method for vitamin D was modified by Osadca & Ritter (1964) to make it more specific by including an additional chromatographic separation on alumina, between the separation on Celite and on Florex; the vitamin was determined after reaction with antimony trichloride. By this modified procedure a dried skim-milk preparation fortified with 10 U.S.P. units vitamin D/g was found to contain 11·6 units/g, as against a content of 21·6 units/g found by the unmodified method. Jones & Libby (1966) found that a similar modified procedure was necessary in the estimation of vitamin D in certain brands of evaporated milk. In their procedure the non-saponifiable residue of the fat was fractionated, first on a column of Celite, polyethylene glycol-600 and *iso*-octane, and then on Florex XXS. The fraction containing vitamin D in solution in 1,2-dichloroethane was treated with antimony trichloride, and the resulting colour read 15 and 90 s later at 500 and 550 nm, respectively. This procedure gave results with 2 samples of evaporated milk that agreed well with those of rat bioassay.

An attempt by Kurashvili (1965) to determine vitamin D in 5 ml samples of human milk by reaction with antimony trichloride after paper and column chromatography must be judged unsuccessful. The results obtained ranged from 7750 to 25000 i.u./100 ml and are several thousandfold higher than the accepted values (cf. Kon & Mawson, 1950) of around 1 i.u./100 ml found in biological tests.

Cow's milk

Natural factors affecting the concentration in milk

Günther & Tekin (1964) studied the vitamin D content of the liver and milk of lactating cows during different seasons and found that the amount of vitamin D stored in the liver and later mobilized was insufficient to compensate for the vitamin D_3 secreted in winter milk during times of low intake. In a subsequent paper, Günther, Tekin & Lenkeit (1965) found that foetal liver contained no vitamin D, and that the small reserves found in the liver of calves came from their mother's milk and that 90% was present in the form of vitamin D_3.

Thompson *et al.* (1964) found little difference between breeds in the vitamin D content of milk fat from 60 Ayrshire, 60 Friesian and 50 Jersey cows, maintained under identical conditions of feeding and management. The milk fat produced by these herds contained mean winter and summer contents of 0·08 and 0·53 i.u./g, respectively. A mean content of 0·23 i.u./g fat was found in samples of milk from 13 large dairies in Great Britain; during November to April the vitamin D content of the milk fat ranged from 0·08 to 0·23 i.u./g, whereas during the period May–October, when the cows were exposed to sun and skyshine, the values were higher, ranging from 0·14 to 0·51 i.u./g fat. The higher mean annual value of 0·25 i.u./g found for samples from the north of England and Scotland than that of 0·21 i.u./g for samples from southern England may have been due to more hay being fed in the north, since hay is a better source of vitamin D than grass, or it may have been the result of a lower intensity of ultra-violet light in the south due to industrial pollution of the atmosphere.

Thompson *et al.* (1964) also measured the vitamin D content in samples of butterfat from New Zealand and found that the contents in winter were similar to those found in Great Britain, but that the summer levels of 0·64 i.u./g fat were higher.

Vitamin D in nutrition

Argument continues about the value of the enrichment of milk with vitamin D. It is now well recognized that in the developed countries infants are more likely to be given too much of the vitamin, which may lead to hypercalcaemia, than too little, which may allow rickets to develop. Causeret (1963) has reviewed the evidence from the U.S.A. and Germany and concludes that there is little justification for advocating routine fortification in France, where the daily intake of vitamin D by adults is around 100 i.u., though he considered that fortification may be desirable for milk used for infant feeding.

The widespread use of supplementary sources of vitamin D is deplored by Guthrie (1963), who found that 40 infants aged from 9 months to 2 years received an adequate intake of vitamin D from fortified milk, and by the Committee on Nutrition of the

American Academy of Pediatrics (1965), which has recommended that only fluid milk and infant milk preparations should be fortified with vitamin D and that the daily intake of the vitamin from all sources should be restricted to 400 i.u.

The vitamin D content of irradiated market milk from 4 Italian cities was found by Sebesta & Dalma (1964) to range from 270 to 320 i.u./l. For dried milk Bauernfeind & Allen (1963) and Bauernfeind & Parman (1964) (see also p. 158) found that satisfactory enrichment with vitamin D could be achieved either by homogenizing a blend of the vitamin with coconut oil into skim-milk and then spray-drying or by mixing the vitamin in a dry beadlet form into dried skim-milk. However, Thomas *et al.* (1965) found that instantized, dried skim-milk that had been fortified with vitamins A and D developed a stale flavour after storage for 3 months at 70 °F.

Sow's milk

Lenkeit & Günther (1964) found that sow's milk, sampled in October–November, contained 0·41 i.u./g fat and that most of this was in the form of vitamin D_3.

Vitamin E

General, and methods of measurement

A comprehensive bibliography compiled by Kujawski (1965), covering the period 1960–4, includes a number of references to milk tocopherol. The chemistry and bioassay of vitamin E were reviewed by Dicks & Matterson (1961).

Procedures for the chemical analysis of vitamin E are discussed critically in a report prepared by the vitamin-E panel of the Analytical Methods Committee of the Society of Public Analysts (1959). The panel examined in detail through collaborative tests the various steps involved in the determination of the tocopherols and evolved a working procedure that was successfully applied to the assay of tocopherols in oils, foods and feeding stuffs. The 7 stages were: solvent extraction for quantitative extraction of all tocopherols and lipid material present; saponification; separation and rejection of steroid material by freezing from solution in methanol; column chromatography on floridin earth to remove carotenoids and residual steroids; two-dimensional paper chromatography to separate tocopherols into 5 zones, and individual elution; treatment of eluates with the Emmerie–Engel reagent (ferric chloride and 2,2-dipyridyl) and finally, measurement of extinction at 520 nm.

Applying this procedure to milk fat, Thompson *et al.* (1964) found that the steroid content of milk was so low that the methanol-freezing step could be omitted. In further modifications they replaced the purification on floridin earth by the simpler procedure with Decalso F described by Crane, Lester, Widmer & Hatefi (1959) and followed this with 2-dimensional separation on paper, using as first solvent 25 % (v/v) benzene in cyclohexane and as second solvent 95 % (v/v) ethanol.

Simplifying the procedure even further, and omitting chromatographic purification of the saponified milk fat, Wako & Kuratani (1962) found cow's milk to contain 123–207 μg tocopherol/100 ml and Markuze (1962) obtained values for 50 samples of butter ranging from 12 to 52 μg/g fat. It is probable that these results are too high, since no correction was made for interference from carotenoids. However, such

simplified procedures are of value for the detection of the presence in butterfat of vegetable oils, which have much higher contents of tocopherols.

Erickson & Dunkley (1964) used a method involving chromatography on silicic acid, followed by reaction with ferric chloride and 4,7-diphenyl-1,10-phenanthroline, to determine the tocopherol content of milk. They corrected for interference from carotenoids and found values for individual cows of from 11·6 to 25·0 μg/g fat. Subsequently, Erickson, Dunkley & Smith (1964) studied the distribution of vitamin E in the fat globule and found that the concentration in the lipid of the membrane was 3 times that in the lipid inside the fat globule, and that the tocopherol associated with the fat globule membrane was particularly susceptible to oxidation. This latter observation may be of particular relevance to the role of vitamin E in preventing the development of flavours associated with the oxidation of milk fat.

Sheppard, Ford, Boehne & Libby (1965), as part of a study of gas–liquid chromatographic methods for the determination of fat-soluble vitamins, found that vacuum distillation could be used to isolate tocopherol from butter.

Krukovsky (1964) has confirmed the earlier finding of Nobile & Moor (1953) that the addition of ascorbic acid before saponification prevented loss of tocopherol, vitamin A and carotenoids.

Cow's milk

Factors influencing the level of vitamin E in milk and milk products

Changes in tocopherol levels in early lactation were studied by Thompson (1962), who found that the content of 88 μg/g fat in colostrum fell by the 21st day of lactation to 15 μg, and thereafter remained at between 12 and 14 μg/g fat.

In a later study, Thompson *et al.* (1964) determined tocopherol levels in the milk fat of 60 Ayrshire, 60 Friesian and 50 Jersey cows at monthly intervals for 28 months and found mean values for each breed of 30, 27 and 29 μg/g fat, respectively, and no evidence of seasonal variation. Throughout the experimental period the 3 groups of cows were maintained in southern England under identical conditions of management and given diets of grass, or grass silage. However, there was some evidence of seasonal variation in the levels of tocopherol in other samples, for the content in milk from dairies in the north and south of Great Britain was 23 and 29 in summer, and 15 and 23 μg/g fat in winter, respectively. The appreciably higher summer than winter levels in the north accorded with the shorter grazing season in this area. It was noteworthy that in samples of butter from New Zealand, where the cows are on grass all the year round, there was no evidence of a seasonal trend. In most cases the fluctuations in tocopherol levels in milk were found to follow those of carotene more closely than those of vitamin A. Although it is unusual to find tocopherols other than α-tocopherol in butterfat, samples from Aberdeen and Glasgow contained, during the months of January, February and March, 12–15 μg α-tocopherol and 3–5 μg ζ-tocopherol/g fat; the main source of the latter in the cow's feed was distiller's or brewer's grains.

In Yugoslavia, Naumovic (1964) compared the vitamin E content in the milk of a native breed of cow with that in the milk of imported Friesian and Simmental cows. He found no differences due to breed or stage of lactation, but summer levels were higher than those in winter. Similar seasonal variations were shown in the values

reported for the α-tocopherol content of raw and homogenized milk in the U.S.A. (Herting & Drury, 1965), for butter in Finland (Antila, Nordlund & Antila, 1965) and for butter and cream in Spain (Lopez Lorenzo *et al.* 1962).

Bunnell, Keating, Quaresimo & Parman (1965) in the U.S.A. reported abnormally low values for the vitamin E content of milk and butter of 36 μg/100 g and 10 μg/g, respectively.

Effect of processing

Ford *et al.* (1962) found that irradiation of raw liquid milk with 1 megarad of γ-radiation caused a 57 % loss of α-tocopherol; lowering the oxygen tension of the milk by flushing with nitrogen before irradiation was found to reduce this loss. Wodsak (1965) showed that irradiation of milk with ultra-violet light, under the commercial conditions used to increase the content of vitamin D, did not affect vitamin E.

The effects of processing on the vitamin E content of milk were studied by Davidov *et al.* (1962, 1963) who found no loss after pasteurization or evaporation, but a 9 % loss after drying and reconstituting. Dried and condensed milks showed losses of vitamin E after storage for 2 years at 8–12 °C of 24 and 17 %, respectively.

Abbot & Waite (1965) found that added dodecyl gallate was more effective than α-tocopherol alone, or a mixture of α-, γ- and δ-tocopherols, in the prevention of oxidative changes during the drying of milk.

Human milk

Several workers have reported values for the vitamin E content of human milk throughout lactation and it is evident that the level is generally about twice that in cow's milk. Thus Tarjan *et al.* (1965) found values in human colostrum and milk of 1330 and 280 μg/100 ml respectively; Herre (1965) reported values in early lactation milk in winter and summer of 857 and 1470, and in late lactation milk in winter and summer of 568 and 1130 μg/100 ml, respectively; Wako & Kuratani (1962) found human milk to contain 297–1098 μg/100 ml, and Herting & Drury (1965) reported that human milk fat contained about 40 μg/g fat.

Herre (1965) claimed that heating milk at 65 °C for 30 min or at 100 °C for 5 min caused 25 or 46 % loss of vitamin E.

Milk of giraffe, reindeer, okapi, kangaroo

Aschaffenburg, Gregory, Rowland, Thompson & Kon (1962) found that a milk sample from a 16-year-old giraffe at Bristol Zoo in the 5th month of lactation contained 12·5 % fat, and 2·7 μg α-tocopherol/g fat; Aschaffenburg, Gregory, Kon, Rowland & Thompson (1962) found that a sample of the mixed milk of 4 reindeer, from a herd in Scotland during the 13–16th weeks of lactation contained 16·9 % fat and 21 μg α-tocopherol/g fat; Gregory, Kon, Rowland & Thompson (1965) found 4·4 % fat and 19·6 μg α-tocopherol/g fat in the milk of an okapi, and a sample of kangaroo milk contained 12 μg/g fat (Aschaffenburg, Ford, Kon, Rowland & Thompson, 1965).

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