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The temperature-dependent dissociation of β-casein from bovine casein micelles and complexes

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SUMMARY. The temperature-dependent dissociation of β-casein from the casein micelles of milk and from the soluble casein complexes of colloidal phosphate-free (CPF) milk was investigated by high-speed centrifugation and gel-filtration. The percentage of the total casein in supernatants prepared by high-speed centrifugation of mid-lactation milks increased from approximately 6 to 15 % on cooling the milks from 30 to 5 °C; β-casein accounted for about 46 % of this increase, while α_s- and κ-casein constituted 30 and 23 %, respectively. On gel-filtration both of skim-milk and CPF milk on Sepharose 2B at 0, 2, 5, 10 and 25 °C, maximum amounts of free β-casein (c. 60 % of total) were obtained at 5 °C. The remainder of the β-casein appeared to be more strongly bound to the α_s- and κ-casein and may be involved in the internal cohesion of casein micelles. The free β-casein of both milk preparations appeared to be in equilibrium with the bound β-casein. On Sephadex G-200 columns at 5 °C, approximately 5 and 60 % of the β-casein of skim-milk and CPF milk, respectively, was eluted in the free form in the expected position for a globular protein of molecular weight about 200000. At low temperatures, particularly at 5 °C, colloidal phosphate appeared to play an integrating role in the association of over half the total β-casein with the other casein components of native micelles. However, when the equilibrium between micellar and free β-casein was disturbed by gel-filtration on Sepharose 2B, the presence of colloidal phosphate did not prevent the release of most of the β-casein from casein micelles. Some problems encountered in the use of densitometry for the estimation of individual caseins on electropherograms are described.

It has been suggested (Sullivan *et al.* 1955) that β-casein is removed from casein micelles when skim-milk is chilled. More recently Rose (1968) demonstrated that β-casein constituted about 55 % of the total increase in serum casein obtained by storing milk overnight at 4 °C.

In the present investigation the relative amounts of free and bound β-casein present in milk and in CPF milk (Pyne & McGann, 1960) at various temperatures between 0 and 30 °C were determined by high-speed centrifugation and gel-filtration. The hypothesis (cf. Rose, 1969) that β-casein constitutes the internal lattice or framework of casein micelles was further investigated by studying the effect of

β-casein release on the gel-filtration behaviour of the casein micelles of milk and of the soluble casein complexes (Downey & Murphy, 1970) of CPF milk.

The evidence presented for the various models proposed for casein micelles (cf. Rose, 1969) was derived largely from studies involving the interactions of purified α_s-, β- and κ-caseins, under ionic conditions which in most instances were markedly different from those prevailing in milk and not compatible with the native structure of casein micelles. By equilibrating the columns with buffer (referred to as synthetic milk serum) similar in composition to milk ultrafiltrate, we have attempted to maintain the casein micelles and complexes in an environment similar to that in milk. Some of these results have been briefly reported (Downey, Murphy & Aherne, 1969).

MATERIALS AND METHODS

Purified β-casein was kindly provided by Dr G. C. Cheeseman.

Skim-milks. Milks were collected from 15 individual Friesian cows. The cream was immediately separated by centrifugation at $1000g$ for 20 min, care being taken not to sediment casein micelles.

High-speed supernatants. Skim-milks were held for 4–6 h at 5, 20 and 30 °C and centrifuged ($38000g$ for 2 h). Most of the high-speed supernatant was removed by pipette so as to exclude the slowly sedimented casein micelles and residual fat.

CPF milk. Colloidal calcium phosphate was removed from skim-milk, by controlled acidification to pH 4·85 at 0 °C, followed by dialysis against skim-milk (Pyne & McGann, 1960; Downey & Murphy, 1970).

The total casein content of the milk preparations was determined as described by Aschaffenburg & Drewry (1959).

Synthetic milk serum. The composition of synthetic milk serum was similar to that described by McGann (1960). It was prepared by the addition of 8·8 m-moles of citric acid, 11·7 m-moles of potassium dihydrogen orthophosphate, 25 m-moles of sodium chloride, 10 m-moles of calcium chloride and 2·5 m-moles of magnesium chloride to 800 ml of distilled water. The pH was adjusted to 6·7 by addition of 60 ml of 0·5 M-NaOH and 0·12 moles of lactose were added. The volume was then made up to 1 l and the pH checked and if necessary readjusted to 6·7. The buffer was stored at 5 °C and filtered immediately before use.

Gel-filtration. Sepharose 2 B (lot no. 4203) and Sephadex G-200 (lot no. To-5212) columns (of length 70 cm and diameter 2·4 cm) were prepared at various temperatures (0, 2, 5, 10 and 25 °C) as previously described (Downey & Murphy, 1970) and equilibrated overnight at the appropriate temperature with synthetic milk serum. Freshly prepared skim-milks and CPF milks were held for 4–6 h at the required temperature before gel-filtration and at least 5 experiments were carried out at each temperature.

On repeated use of the same column, particularly at temperatures > 10 °C, some degradation of casein occurred during gel-filtration, as evidenced by an increase in the number of artifact protein bands on electropherograms. These bands were located mainly in front of the α_s-casein and on the cathode side of the origin. Casein degradation was not observed when columns were repacked after 2 days (1–2 expts) at temperatures > 10 °C or after 1 week (3–4 expts) at lower temperatures. To minimize bacterial contamination, the column materials were suspended overnight in a solu-

tion of 1 M-NaCl containing 0·003 M sodium azide before repacking. Furthermore, only freshly prepared skim-milks and CPF milks were used for gel-filtration.

Proteins in the effluent fractions were estimated by spectrophotometric measurement at 280 nm. Fractions which were cloudy, due to the presence of micellar casein, were also read at 360 nm and their protein content expressed as the difference between the absorbance at 280 and 360 nm. When necessary, the protein content of the fractions was also determined by micro-Kjedahl N analysis. Based on the protein elution pattern, consecutive effluent fractions were combined to give 4–6 pooled fractions from each column run.

Electrophoresis. As soon as possible after preparation, the caseins in 1 ml of skim-milk or CPF milk, in 10 ml of supernatants from high-speed centrifugation, and in pooled effluent fractions were precipitated at 2 °C by careful adjustment of the pH to 4·6 with 2 N-HCl. To ensure complete precipitation of casein, the acidified preparations were held at 20 °C for 1 h and then centrifuged for 15 min at 38000g. The sedimented casein was dissolved in 0·076 M tris–citrate buffer of pH 8·6 (Poulik, 1957). In general, 1 ml of the buffer was used. However, with pooled effluent fractions which were low in casein, the precipitates were dissolved in 0·5 ml of buffer. One hour before electrophoresis 0·65 g urea and 0·08 ml mercaptoethanol/ml were added to the samples. The individual caseins were identified by urea starch gel electrophoresis (Aschaffenburg & Thymann, 1965) and quantified by densitometry.

Electrophoresis and densitometry were carried out as previously described (Murphy & Downey, 1969; Downey & Murphy, 1970) but in addition the following precautions were taken. Selected strips of Whatman 0·3 mm chromatography paper measuring 0·1 × 0·7 cm and weighing between 1·4 and 1·6 mg were immersed in the test solution for 5 s and the excess removed by draining on the side of the sample tube. After electrophoresis, the gels were immersed for 6 min in 500 ml of Amido Black 10B solution (Aschaffenburg, 1964) and exhaustively de-stained by washing for successive periods of 30 min, 3 and 18 h in 1-l volumes of a mixture of methanol, acetic acid and water (50:10:50). To eliminate inaccuracies due to the small inherent differences between electropherograms, related preparations were analysed on the same gel.

For densitometric analysis, electropherograms were sliced longitudinally and the protein pattern obtained with each sample scanned by means of a Chromoscan densitometer (Joyce, Loebl & Co. Ltd, London), using the following instrument settings; aperture 1 mm diam.; specimen/drive gear ratio, 1:3; cam no. 5-077/C; filter, 620. The densities of the α_s-, β- and κ-casein bands were calculated by measuring the areas under the appropriate peaks.

The distribution of the α_s-, β- and of the κ-casein in supernatants from high-speed centrifugation relative to that in the original skim-milk was expressed as $A/B \times 100$. A = density of the individual casein in the high-speed supernatant and B = density of the same casein in the original skim-milk.

To estimate the relative distribution of the α_s-, β- and of the κ-casein between the pooled fractions obtained from a column effluent, similar calculations were used. In this instance A = density of the individual casein in the pooled fraction and B = the sum of the densities for the same casein in the 4–6 constituent pooled fractions obtained from the total column effluent.

RESULTS

Sedimentation experiments. The percentage of the total casein soluble at the various temperatures varied between milks from individual cows (Table 1). In most of the milks examined (cows 1–11), on average 6 % of the total casein was soluble at 30 °C and a further 10 % (approximately) was solubilized by cooling to 5 °C for 4 h. On extending storage time at 5 °C to 24 h, a further 1 % of the total casein remained in the supernatant after high-speed centrifugation. High-speed supernatants (column *B*, Table 1) prepared from skim-milks which had been stored overnight (14 h) at 5 °C

Table 1. *Percentage of total casein not sedimented on centrifugation*
$(38000g$ *for 2 h) at 30, 20 and 5* $°C$

| Cow no. | Total casein,* g/100 g of skim-milk | Non-sedimented casein,* % of total casein | | | | |
		A 30 °C	*B* 30 °C	*C* 20 °C	*D* 5 °C	*D–A* (Increase on cooling from 30 to 5 °C)
1	2·1	2·4	4·7	4·3	9·9	7·5
2	2·4	4·9	6·2	4·5	12·7	7·8
3	2·0	4·4	5·4	5·9	14·8	0·4
4	2·5	4·8	6·4	3·2	9·6	4·8
5	2·5 (2·3)†	4·4 (4·2)†	4·8 (5·4)†	4·0 (5·2)†	10·5 (11·5)†	6·1 (7·3)†
6	2·5	4·7	—	6·7	17·3	12·6
7	2·6	4·1	—	6·4	12·6	8·5
8	1·9	9·8	—	14·0	20·7	10·9
9	2·5	9·7	—	15·3	20·2	10·5
10	2·3	9·6	—	14·7	16·8	7·2
11	2·4	8·1	—	10·2	20·6	12·5
Mean	2·34	6·1	—	8·1	15·1	9·0
12‡	2·8	13·6	—	22·6	28·7	15·1
13‡	2·8	20·0	—	23·6	31·3	11·3
14‡	3·0	24·2	—	27·5	33·2	9·0
15§	2·6	37·4	—	41·4	54·6	17·2

* Casein (nitrogen × 6·38) determined as described by Aschaffenburg & Drewry (1959).

† Mean of milks 1–5 only.

‡ Late lactation milks; all other milks were from cows in mid-lactation.

§ Clinical mastitis.

The supernatants in columns *A*, *C* and *D* were prepared from fresh skim-milks which had been incubated at 30, 20 and 5 °C for 4 h prior to centrifugation. Those in column *B* were held at 5 °C for 14 h followed by 4 h at 30 °C prior to centrifugation.

and then incubated at 30 °C for 4 h before centrifugation contained slightly more casein than the corresponding supernatants (column *A*, Table 1) obtained from skim-milks which had not been cooled below 30 °C.

While the factors responsible for the between-cow variations in soluble casein were not investigated, late lactation milks (cows 12–14) which were high in total casein contained elevated levels of soluble casein (*c.* 30 % at 5 °C). The extremely high level of non-micellar casein (55 % at 5 °C) detected in milk from cow 15 is attributed to compositional changes resulting from clinical mastitis. In the milk examined by Rose (1968) a similarly high level of soluble casein (43 % at 5 °C) was obtained.

Because of this variation in the soluble casein content of individual milks (Table 1)

and to avoid possible difficulties due to differences in the association behaviour of individual casein variants (Thompson & Pepper, 1964; Schmidt & Payens, 1964) the subsequent investigation was confined to milks in which less than 10 % of the casein was soluble at 30 °C and which contained α_s- casein B and β-casein A.

The percentage of α_s-, β- and κ-casein soluble at the various temperatures (cows nos 1–5, Table 1), as determined by densitometry, is shown in Table 2. Approximately 1–2 % of each of the 3 caseins was soluble both at 20 and at 30 °C. At 5 °C about 10 % both of the β- and κ-caseins and 4 % of the α_s-casein was not sedimented on high-speed centrifugation.

Table 2. *Percentage of α_s-, β- and κ-caseins not sedimented by centrifugation (38 000 g for 2 h) of fresh skim-milks* at 30, 20 and 5 °C as determined by densitometry*

	30 °C				20 °C				5 °C		
Cow no.	α_s	β	κ		α_s	β	κ		α_s	β	κ
1	1	1	3		2	3	4		5	10	8
2	1	1	0		1	1	1		4	10	7
3	1	1	0		1	2	0		4	10	14
4	1	2	2		1	2	2		6	11	12
5	1	1	0		1	1	0		2	8	7
Mean, % of total of each in milk	1	1	1		1	2	1		4	10	10
			Total $\alpha_s = \beta$- and κ-casein				Total $\alpha_s = \beta$- and κ-casein				Total $\alpha_s = \beta$- and κ-casein
Mean, mg/100 ml supernatant†	13	8	4	25 (1 %)	13	16	4	35 (1·5 %)	62	80	40	182 (8 %)

* Fresh skim-milks were held for 4–6 h at the 3 temperatures before centrifugation.

† These values were calculated from the data of Schmidt (1969) for the relative $\alpha_s = \beta$- and κ-casein contents of milk (2·5 g total casein/100 ml of milk; α_s-casein, 50 %; β-casein, 30 %; κ-casein, 14 %).

As shown by densitometry, α_s-, β- and κ-caseins accounted for approximately 95 % of the protein present in the acid casein precipitates obtained from skim-milk (Fig. 1a) and column effluents. However, as a result of the 10-fold concentration required for electrophoretic analysis of the small amounts of α_s-, β- and κ-caseins present in high-speed supernatants, minor proteins present in the acid casein precipitates were detected. The bands located on electropherograms (A–C; Fig. 1b, c) in the expected positions (Murphy & Downey, 1969) for α-lactalbumin, β-lactoglobulin and serum albumin, respectively, indicate coprecipitation of some whey proteins with the acid casein. The protein between κ-casein and the origin may have been due to γ-casein (El-Negoumy, 1967). Proteins other than α_s-, β- and κ-casein constituted about 20, 40 and 45 % of the acid precipitates obtained from high-speed supernatants prepared at 5, 20 and 30 °C, respectively (Fig. 1b, c). The high proportion of these proteins in the acid precipitates may account for the difference between the total casein content of high-speed supernatants as determined by Kjeldahl N analysis (cows 1–5, Table 1) and that calculated for the sum of the α_s-, β- and κ-caseins only, as estimated by densitometry (Table 2).
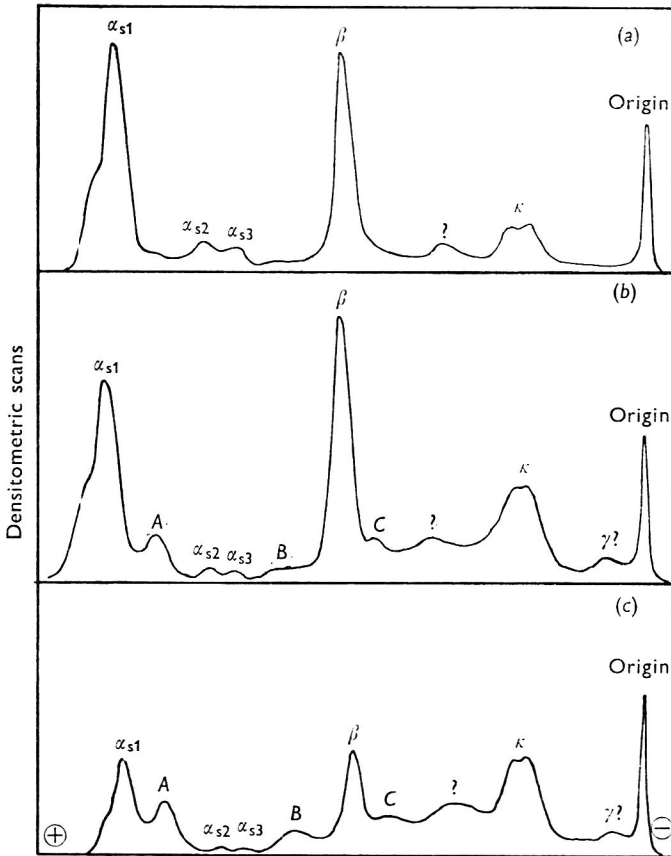
Fig. 1. Electrophoretic analysis of the caseins present in acid precipitates obtained from (a) 1 ml of skim-milk; (b) and (c) from 10 ml of high-speed supernatants prepared at 5 and 20 °C respectively. Similar electrophoretic patterns were obtained with high-speed supernatants prepared at 20 and 30 °C. Acid casein precipitates were dissolved in 1 ml of 0·076 M tris–citrate buffer. A, α-lactalbumin; B, β-lactoglobulin; C, serum albumin.

Gel-filtration

Sepharose 2B. The proteins in 4 ml of skim-milk were fractionated on Sepharose 2B columns into 2 well-defined peaks (Fig. 2a).

The fractions eluted at the void volume (V_e 80 ml) were quite opaque and contained α_{s1}-, α_{s2}-, α_{s3}-, β- and κ-casein (Plate 1a, b), apparently in micellar form. The β-lactoglobulin, serum albumin and, depending upon the temperature of fractionation, β-casein, were eluted in the second peak (V_e 235 ml) as well as traces of α_s- and κ-casein. When the fractionation was carried out at 25 °C (Plate 1a) no β-casein was detected in the latter peak, but as the temperature of fractionation was decreased to 5 °C approximately 58 % of the total β-casein was eluted with the whey proteins (Plate 1b). On further reduction in temperature to 0 °C, the percentage of β-casein eluted with the whey proteins decreased to about 17 % of the total, and over 80 % of the β-casein was eluted with the casein micelles. The percentage of the total β-casein eluted with the whey proteins (V_e 235 ml) at various temperatures from 0 to 25 °C is shown in Fig. 2b. The elution patterns of α_s- and κ-casein were only slightly affected

by changes in the temperature. At the lower temperatures, the α_s- and κ-casein content of the casein micelle peak decreased by about 5–10 % and small amounts of these caseins were detected in the troughs between the 2 protein peaks.
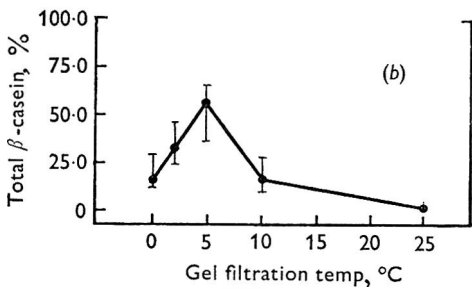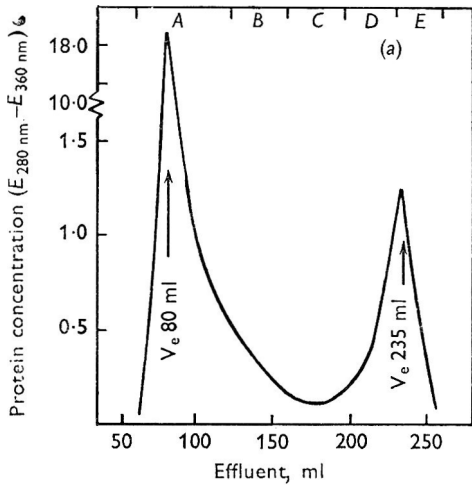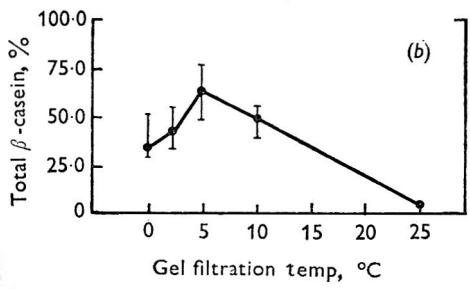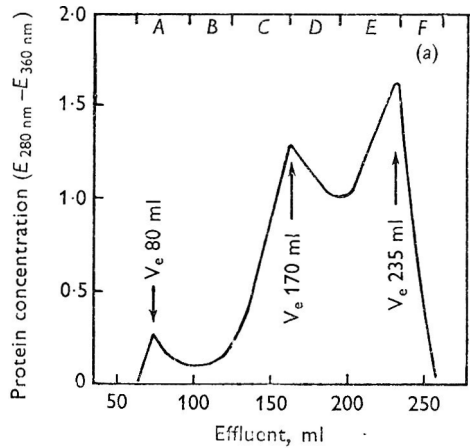

Fig. 2. Gel-filtration on Sepharose 2 B columns at various temperatures of 4 ml of skim-milk. Columns were equilibrated with synthetic milk serum. (a) Protein elution pattern and (b) percentage of total β-casein eluted in the whey protein peak (V_e 235 ml) at the various temperatures. At least 5 fractionations were carried out at each temperature and the points on the graph are average values. The range of values at each temperature is depicted. Electrophoretic analysis of the caseins in pooled fractions (A, B, C, D and E) obtained from columns at 5 and 25 °C is shown in Plate 1.

Fig. 3. Gel-filtration on Sepharose 2 B columns at various temperatures of 4 ml of colloidal-phosphate free milk. Columns were equilibrated with synthetic milk serum. (a) Protein elution pattern and (b) percentage of total β-casein eluted in the whey protein peak (V_e 235 ml) at the various temperatures. At least 5 fractionations were carried out at each temperature and the points on the graph are average values. The range of values at each temperature is also depicted. Electrophoretic analysis of the casein in pooled fractions (A, B, C, D, E and F) obtained from columns at 5 and 25 °C is shown in Plate 2.

Most of the proteins present in 4 ml of CPF milk were eluted (Fig. 3a) from Sepharose 2 B columns as 2 overlapping peaks (V_e 170 and 235 ml, respectively). The first of these peaks (V_e 170 ml) contained all the major casein fractions (Plate 2a, b), apparently as complexes of average mol.wt 2×10^6 (Downey & Murphy, 1970). The whey proteins were eluted in the other peak (V_e 235 ml) and as with skim-milk (Fig. 2b) the β-casein content of the 2 protein peaks was markedly influenced by changes in the fractionation temperature (Fig. 3b). At 25 °C (Plate 2a) very little

β-casein (c. 5% of the total) was eluted with the whey proteins; most of it emerged with the other caseins in the previous peak (V_e 170 ml). However, as the fractionation temperature was reduced the percentage of the total β-casein eluted with the whey proteins increased (Fig. 3b) to about 60% at 5 °C (Plate 2b). On further reduction in temperature to 0 °C the β-casein eluted with the whey proteins decreased to about 35% of the total (Fig. 3b) and this decrease was accompanied by a corresponding increase in the β-casein content of the previous peak (V_e 170 ml). The relatively small protein peak which was eluted (Fig. 3a) at the void volume (V_e 80 ml) appeared to contain some residual micellar casein. However, its protein content was too low to
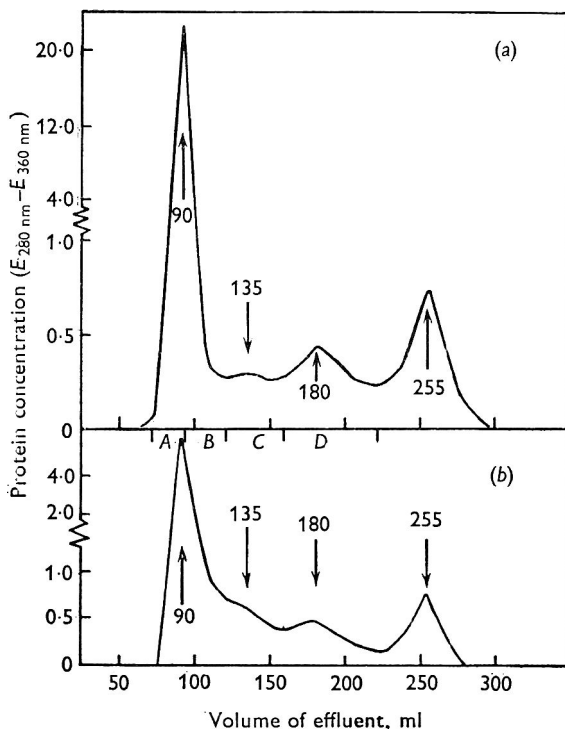

Fig. 4. Gel-filtration at 5 and 25 °C on Sephadex G-200 columns equilibrated with synthetic milk serum of 4 ml both of (a) skim-milk and (b) colloidal-phosphate free milk. Electrophoretic analysis of the casein in pooled fractions (A, B, C and D; Fig. 4b) obtained from columns at 5 °C is shown in Plate 3. On gel-filtration at 25 °C all the caseins were eluted at the void volume (V_e 90 ml).

permit electrophoretic identification of its constituent proteins. As with skim-milk, the elution patterns of the α_s- and κ-caseins were less affected by changes in fractionation temperatures.

On gel-filtration of 5 mg of purified β-casein on Sepharose 2B columns at 0 and 5 °C, the protein was eluted as a single peak in the same position as the whey protein peaks of Figs 2(a) and 3(a), and its elution volume (V_e 235 ml) was the same at both temperatures.

Sephadex G-200. Similar elution diagrams were obtained (Fig. 4a, b) on gel-filtration of skim-milk and CPF milks on Sephadex G-200. The casein micelles of milk and the casein complexes of CPF milk were eluted at the void volume (V_e 90 ml)

followed by 2 or 3 ill-defined peaks whose composition has been previously described (Downey & Andrews, 1966; Downey & Murphy, 1970). On gel-filtration (Fig. 4a) of skim-milk at 25 °C, the β-casein was eluted at the void volume (V_e 90 ml) together with the α_s- and κ-caseins, apparently as micelles. On reducing the temperature to 5 °C, approximately 5 % of the β-casein of skim-milk was eluted in the subsequent protein peak (V_e 135 ml), in the expected position for a globular protein of molecular weight *c.* 200000 (Downey & Murphy, 1970). No changes were detected in the elution patterns of the α_s- or κ-caseins.

On gel-filtration (Fig. 4b) of CPF milk at 25 °C, all the casein was eluted at the void volume (V_e 90 ml). In contrast, at 5 °C the second peak (V_e 135 ml) contained about 60 % of the β-casein, 15 % of the α_s-casein and 10 % of the κ-casein (Plate 3). In some instances a small amount (*c.* 5 %) of the total) of β-casein was also detected in the whey protein fraction (V_e 180 ml) but this may have been derived from the previous overlapping peak (V_e 135 ml).

Purified β-casein was eluted from Sephadex G-200 at 5 °C in the expected position for a globular protein of molecular weight approximately 130000 (Downey & Andrews, 1966).

DISCUSSION

In agreement with the results of other investigators (Bohren & Wenner, 1961; Rose, 1968) the soluble casein (non-sedimented casein) content of milks was increased by cooling (Tables 1, 2). In mid-lactation infection-free milks (from cows 1–11, Table 1) the percentage of the individual caseins soluble at 5 °C and in particular at 30 °C was considerably lower than that obtained at 35 °C by Rose (1968). Calculations based on the data of Schmidt (1969) for the relative α_s-, β- and κ-casein contents of milk (2·5 g total casein/100 ml of milk) suggest that in the milk employed by Rose (cf. Table 4, in Rose, 1968) approximately 43 % of the total casein was soluble at 5 °C and contained 25 % of the total α_s- and approximately 70 % both of the β- and κ-caseins. However, in both investigations β-casein was responsible for about half the increase in soluble casein obtained on cooling milk. The α_s- and κ-casein accounted for about equal portions of the remainder.

The dissociation of β-casein from the casein micelles of skim-milk or from soluble casein complexes (Downey & Murphy, 1970) of CPF milk, as the temperature is reduced to 5 °C, is analogous to the exothermic depolymerization observed (Payens & Markwijk, 1963) with pure β-casein. The decrease in the β-casein content of the whey protein peak (Figs 2b, 3b) on further reducing the fractionation temperature from 5 to 0 °C is attributed to a decreased dissociation of β-casein from the casein micelles and complexes at the lower temperatures. This phenomenon does not appear to be due to polymerization of the free β-casein released during the cooling of the samples, since purified β-casein was eluted in the same position from Sepharose 2B both at 0 and at 5 °C.

Purified β-casein exists in monomeric form at 4 °C (Payens & Markwijk, 1963). Similarly, Sullivan *et al.* (1955) considered that the β-casein which dissociates from casein micelles upon cooling milk is present as monomer. However, on gel-filtration of skim-milk or CPF milk on Sephadex G-200 at 5 °C, β-casein was not detected in the expected position for a typical globular protein similar in molecular weight to

monomeric β-casein (mol.wt 25000; Payens & Markwijk, 1963). The free β-casein of both preparations was eluted as aggregates of molecular weight approximately 200000 (Downey & Murphy, 1970). Similarly, when either 0·75 M-NaCl (Downey & Andrews, 1966) or 0·02 M sodium phosphate buffer of pH 7·0 (Yaguchi & Tarassuk, 1967) was used as column eluent, the non-micellar β-casein, apparently in aggregated form, emerged from Sephadex G-200 at 5 °C. Thus, it is suggested that the β-casein which dissociates from the casein micelles of skim-milk and from the soluble complexes of CPF milk exists as a polymer at 5 °C under conditions similar to those prevailing in milk. However, as a result of its highly disorganized structure (Noelken & Reibstein, 1968) β-casein may show atypical gel-filtration behaviour. In this context, it may be significant that the purified β-casein, at a concentration conducive to the formation of monomer (cf. Payens & Markwijk, 1963), was eluted from Sephadex G-200 at 5 °C in the expected position for a protein of molecular weight 130000.

Scheme 1. *Suggested equilibrium between β-casein, casein micelles and soluble-casein complexes*
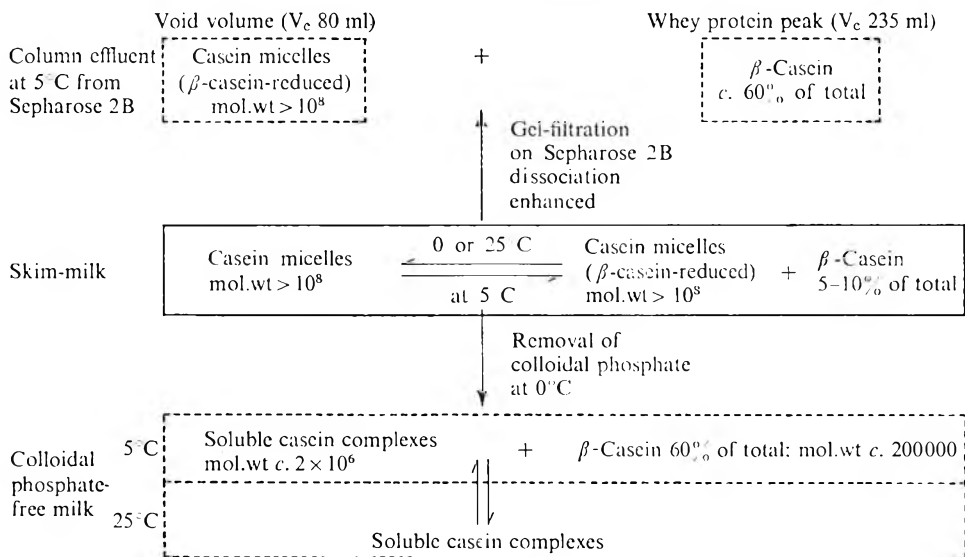
The native casein micelles of milk have molecular weights in the range 10^7–10^9 (Nitschmann, 1949; Carroll, Thompson & Nutting, 1968) and were eluted at 25 °C at the void volume of Sepharose 2B, which appears to have an upper exclusion limit greater than 10^8 (Downey & Murphy, 1970). Similarly at 5 °C, over 90 % of the α_s- and κ-casein was eluted together with the residual micellar β-casein (42 %) at the void volume of the columns, suggesting that the particle size of the β-casein-reduced micelles also exceeds 10^8. Furthermore, the opalescence ($E_{360\,nm}$) of the β-casein-reduced casein micelles, in the peak eluted at the void volume of Sepharose 2B columns at 5 °C, was approximately the same as that obtained at 25 °C with the native micelles. Thus, when 58 % of the β-casein was removed from micelles by gel-filtration at 5 °C, no evidence was obtained for any gross reduction in the size of micelles or for micellar disintegration similar to that observed (Downey & Murphy,

1970) on the removal of colloidal phosphate from milk. It appears that, if β-casein plays a structural role within casein micelles (Payens, 1966; Rose, 1969), then less than half of the total is sufficient for this function. Most (*c*. 60 %) of the β-casein appears to be loosely associated with the casein micelles of milk or with complexes of CPF milk, from which it dissociates as the temperature is reduced to 5 °C.

It has been suggested (McGann & Pyne, 1960) that colloidal calcium phosphate plays an integrating role in the association of β-casein with the other components of casein micelles. The larger amount (60 %) of free β-casein obtained from Sephadex G-200 columns on gel-filtration at 5 °C of CPF milk relative to that obtained from skim-milk (5 %) is consistent with this suggestion. On the other hand, no free β-casein was detected in CPF milk at 25 °C. Furthermore, colloidal phosphate did not prevent the release of β-casein from casein micelles during gel-filtration of skim-milk on Sepharose 2 B at 5 °C.

Rose (1969) indicated that the relation between micellar and serum casein is not a simple equilibrium dependent on the solubilities of the caseins or calcium casein-ates. The results now reported may be best explained by postulating that the β-casein which dissociates from casein micelles on cooling milk to 5 °C is in equilibrium with micellar casein (Scheme 1). On centrifugation, the micellar casein is sedimented and the equilibrium concentration of non-micellar β-casein retained in the super-natants. On Sephadex G-200 at 5 °C, the non-micellar β-casein is not completely separated from the micelles and the equilibrium is not sufficiently disturbed to cause appreciable dissociation of additional β-casein from the micelles. In contrast, during gel-filtration on Sepharose 2 B at 5 °C, the free β-casein enters the gel-matrix and is rapidly separated from the micellar casein which is excluded from the gel. Additional β-casein is consequently released from the micelles so as to re-establish equilibrium. Cheeseman (1968) also observed that the equilibrium between various casein com-plexes may be altered during gel-filtration. The apparent anomaly between results obtained on Sephadex G-200 and Sepharose 2 B was also encountered during studies on the association of lipases with micellar and soluble casein complexes (Downey & Andrews, 1966; Downey & Murphy, 1970).

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EXPLANATION OF PLATES

Plate 1

(a), (b) Starch-gel electrophoresis patterns for the caseins in pooled effluent fractions (A, B, C, D and E; Fig. 2a) obtained on gel-filtration of skim-milk on Sepharose 2B at 25 and 5 °C, respectively.

Plate 2

(a), (b) Starch-gel electrophoresis patterns for caseins in pooled effluent fractions (A, B, C, D, E and F; Fig. 3a) obtained on gel-filtration of colloidal-phosphate free milk on Sepharose 2B at 25 and 5 °C, respectively.

Plate 3

Starch-gel electrophoresis patterns for caseins in pooled effluent fractions (A, B, C and D; Fig. 4b) obtained on gel-filtration at 5 °C of colloidal-phosphate free milk on Sephadex G-200 columns.

(a)

$\oplus$

$\alpha_{s1} \longrightarrow$

$\alpha_{s2} \longrightarrow$
$\alpha_{s3} \longrightarrow$
$\beta \longrightarrow$

$\kappa \longrightarrow$

Origin $\longrightarrow$

A   B   C   D   E   F

$\ominus$

(b)

$\oplus$

$\alpha_{s1} \longrightarrow$

$\alpha_{s2} \longrightarrow$
$\alpha_{s3} \longrightarrow$
$\beta \longrightarrow$

$\kappa \longrightarrow$

Origin $\longrightarrow$

A   B   C   D   E   F

$\ominus$

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$\oplus$

$\alpha_{s1} \longrightarrow$

$\alpha_{s2} \longrightarrow$
$\alpha_{s3} \longrightarrow$

$\beta \longrightarrow$

$\kappa \longrightarrow$

Origin $\longrightarrow$

A   B   C   D

$\ominus$

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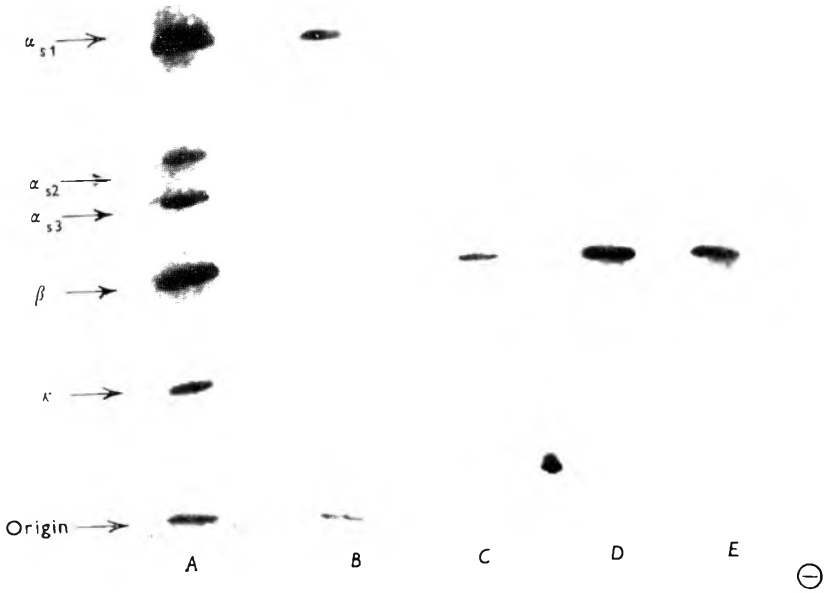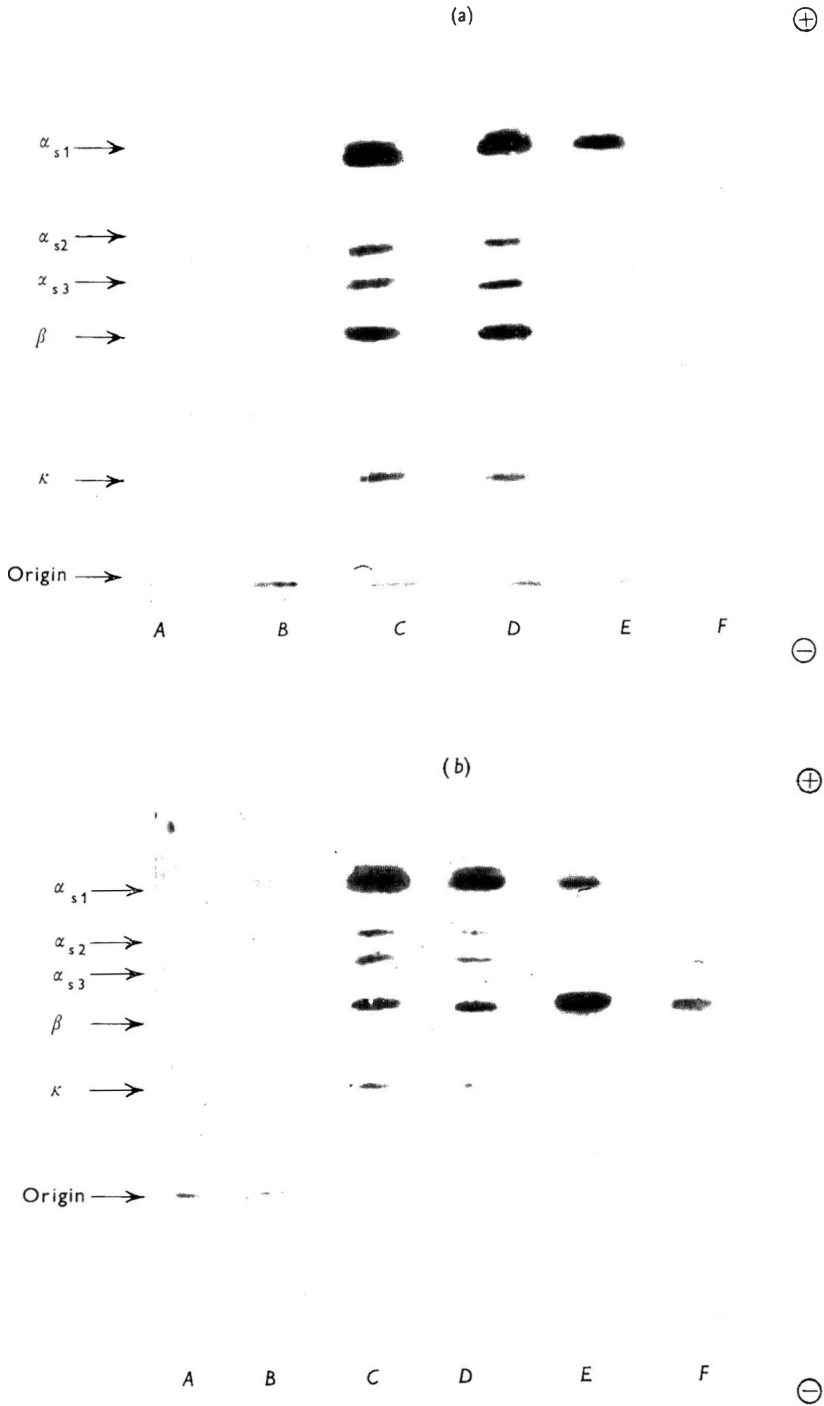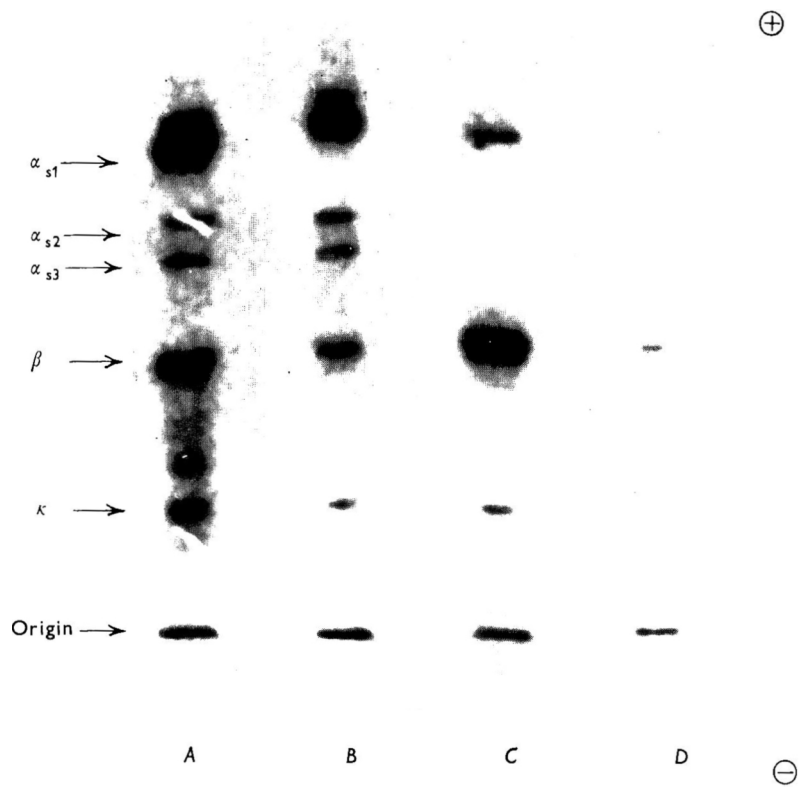
Metabolism of [1-¹⁴C]palmitate and [1-¹⁴C]oleate by the isolated perfused mammary gland of the sheep or goat

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SUMMARY. Lactating mammary glands of sheep and goats were perfused for several hours in the presence of [1-¹⁴C]palmitate or [1-¹⁴C]oleate. Adequate quantities of acetate, glucose, amino acids and chylomicrons were added to the perfusate.

The fall in specific activity of [1-¹⁴C]palmitic acid or [1-¹⁴C]oleic acid across the gland and the labelling of milk triglyceride fatty acids indicates an extensive transfer of radioactivity from plasma free fatty acids (FFA). The plasma triglycerides showed large arterio-venous differences in concentration. The small [¹⁴C] incorporation in plasma triglycerides decreased across the gland. In a control experiment triglycerides were also slightly labelled.

There were no significant arterio-venous differences in cholesterol esters and their fatty acid composition showed only slight changes during passage through the gland. Their specific activity showed a small rise across the gland.

In milk components, the [¹⁴C] was mainly localized in the triglycerides. An appreciable proportion of the palmitoleate is derived from palmitate by dehydrogenation within the gland, while there is no evidence for the hydrogenation of oleic acid to stearic acid. Elongation of palmitic acid to C_{18}-acids does not occur to any important extent. FFA are catabolized to a variable extent by the gland.

The role of FFA in labelling of milk and blood plasma fatty acid fraction is discussed.

The source of milk lipids has been and continues to be a subject of considerable interest. Acetate and β-hydroxybutyrate are known to be precursors of fatty acids $(C_4\text{-}C_{16})$ of milk fat. The circulating lipids supply a proportion of the C_{14} and C_{16} and most of the C_{18} acids of milk fat. The technique of perfusing the isolated mammary gland has been usefully applied in studies on milk fat synthesis and offers the advantage that any experimental observations obtained are easier to interpret. In early work the metabolism of [1-¹⁴C]stearate by the perfused cow udder was studied and substantial amounts of radioactivity were incorporated into milk triglyceride fatty acids (Lauryssens, Verbeke & Peeters, 1961). Stearate was dehydrogenated to a considerable extent giving rise to oleate. These results were later confirmed in experi-

ments with intact lactating goats (Annison, Linzell, Fazakerley & Nichols, 1967) and in experiments with the perfused goat udder (Linzell, Annison, Fazakerley & Leng, 1967). In the present experiments the incorporation into milk constituents and the metabolism of circulating free fatty acids (FFA), [1-^{14}C]palmitic acid and [1-^{14}C]-oleic acid by the isolated perfused mammary glands of sheep and goats have been studied.

EXPERIMENTAL

Mammary glands were taken from 2 sheep and 1 goat yielding 32–39 ml of milk per h per gland. Apart from some minor modifications, the technique described by Hardwick & Linzell (1960) and Hardwick, Linzell & Price (1961) for the perfusion of the isolated goat udder was used. The evening before the experiment feed was removed from the animals but water remained available. On the morning of the experiment the animal was milked out. The 2 halves of the udder were separated and removed from the animal under general and epidural anaesthesia (Verbeke, Peeters, Massart-Leën & Cocquyt, 1968) and perfused independently. During the removal of the gland the animal received 100 g glucose intravenously. The glands were perfused at 32–35 °C and a constant pressure of 140 cmH$_2$O. The sheep glands (expts 1, 2) were perfused with washed sheep blood erythrocytes suspended in an artificial plasma solution. The goat gland (expt 3) was perfused with heparinized goat blood to which was added 1 µg bromolysergic acid diethyl amide (BOL 148, Sandoz) per ml. Neomycin (10 µg/ml) and penicillin (0·1 units/ml) were used in the perfusion fluid and artificial kidney. The perfusate was oxygenated by a mixture of air + CO$_2$ (93:7) and O$_2$ + CO$_2$ (93:7). The glands were emptied by milking after intra-arterial injection of 0·1 i.u. oxytocin. Milking was performed at the start of perfusion, immediately before adding the labelled compounds and every hour thereafter. In expts 1–3 the glands were perfused respectively, for 11 h, 8 h, 25 min, and 13 h 30 min and milk of normal composition was collected throughout these periods but the yield of milk decreased gradually. The labelled precursors were added 110, 75 and 150 min, respectively, after the start of perfusion. From that time on a solution containing substrates (glucose, acetate, amino acids, phosphate and the labelled precursor) and another solution containing CaCl$_2$ and MgCl$_2$ were infused continuously at a rate appropriate to the rate of milk formation.

In order to maintain a normal fatty acid composition of the secreted milk fat, intestinal lymph was also run into the perfusion medium continuously at the rate of 2 vol. for 1 vol. of secreted milk in expt 2 and 1 vol. for 1 vol. in expts 1 and 3. The chyle was collected from the intestine of 2 other sheep (expts 1, 2) and 1 other goat (expt 3) by the technique of Lascelles & Morris (1961), for 2 days immediately before the perfusion experiment, and stored on ice in 100 ml polythene bottles containing 8 mg heparin, 1·5 mg neomycin and 10000 i.u. penicillin.

[1-^{14}C]Palmitic acid (expt 1) was obtained from C.E.A., Saclay, France, and [1-^{14}C]oleic acid (expts 2, 3) from The Radiochemical Centre, Amersham, U.K. [1-^{14}C]Palmitic acid (3 mg:50 µCi) and [1-^{14}C]oleic acid (5 mg:50 µCi) were purified and sodium salts formed according to the method of McBride & Korn (1964). The sodium salts were bound to plasma albumin as described by Fredrickson & Gordon (1958). In all experiments one-third (16·6 µCi) of the sodium salt solution was added

to 20 ml synthetic plasma and given via the artificial lung to the perfusate as a priming dose. The remaining two-thirds (33·4 μCi), dissolved in 50 ml synthetic plasma and transferred to 200 ml substrate solution, were infused continuously.

In a control experiment, 1000 ml oxygenated heparinized sheep blood was circulated through the apparatus for 8 h at 32 °C in the absence of a gland. [1-^{14}C]Palmitic acid (1·65 mg:27·5 μCi) as sodium salt bound to albumin was added to the blood at the onset.

Analytical methods. Casein, lactose and citric acid in milk were determined as described previously (Verbeke *et al.* 1968). The glyceride content of milk was determined colorimetrically (Blankenhorn, Rouser & Weimer, 1961).

In expt 1 the $^{14}CO_2$-production during the experiment was estimated according to Verbeke *et al.* (1968). In expts 2 and 3, the $^{14}CO_2$ content in arterial and venous blood samples was determined by a modification of the method of Cuppy & Crevasse (1963). Blood (1 ml) was added to a 25-ml Erlenmeyer flask containing a few drops of octyl alcohol. Hyamine hydroxide (Packard Instrument Company Inc.) solution (0·25 ml; 1 M, in methanol) was injected into a collection vial and the flask closed with a rubber stopper. Two ml 2 N-lactic acid were injected through the stopper into the blood mixture. The flasks were shaken at 37 °C for 2 h to ensure complete trapping of CO_2. The hyamine holder was transferred to a liquid scintillation vial and 15 ml of scintillator solution (containing 5 g PPO and 0·3 g dimethyl POPOP/l toluene) were added. The counting efficiency was about 75 %. All determinations were carried out in duplicate.

Milk fat, casein, citric acid and lactose were isolated according to the method described by Verbeke *et al.* (1968). Milk fat was separated into its lipid classes by the method of Carroll (1961).

Blood samples were immediately centrifuged (3000 g) at 0 °C; the separated plasma was lyophilized and the fat extracted by the method of Folch, Lees & Sloane Stanley (1957). The main lipid classes were separated by the method of Carroll (1961). In the first experiment the arterial FFA concentration during perfusion was determined by the method of Dole (1956).

Methyl esters of the different fat fractions were prepared by the method of De Francesco & Maglitto (1962), and separated and collected by GLC on an Aerograph Autoprep A 700 (Wilkens Instrument & Research Inc., Walnut Creek, California) equipped with a catharometer detector. The esters were separated by temperature programming on a 6-m coiled column (int. diam. 4 mm) packed with 15 % EGS (Appl. Sc. Lab. Inc., State College, Pa., U.S.A.) on Chromosorb P 60–80 mesh (Appl. Sc. Lab. Inc.). The different esters were trapped as separate fractions in collection flasks containing quartz sand and cooled in an ice bath. Appropriate corrections were made for response and collection efficiency. For an efficient separation of the saturated methyl esters from their unsaturated homologues a complementary purification was carried out by thin-layer chromatography on silica gel impregnated with $AgNO_3$ (De Vries & Jurriens, 1963). The zones containing the saturated or unsaturated fatty acid methyl esters were scraped off, extracted, rechromatographed by GLC and collected. All assays were carried out by liquid scintillation, using a Packard Tri-Carb Spectrometer, Model No. 3003.

The radioactivity of the water-soluble compounds was assayed as described by

Verbeke, Feteanu & Peeters (1967). Lipids were dissolved in toluene containing, per l, 5 g PPO and 0·3 g dimethyl-POPOP. The trapped methyl esters were washed into scintillation vials and their [^{14}C] content determined. Quenching corrections were carried out by the channel ratio method (Herberg, 1965). The specific activities were recorded as mμCi/mg C. The calculations were carried out with an IBM 360-30 computer. The program uses as data the heights and widths of the gas-chromatographic peaks and the numbers of counts obtained in 2 energy channels during liquid scintillation and calculates response corrections, collection efficiency corrections, and quench and background corrections. The results are given as weight per cent, moles per cent and C per cent, and the specific activities are expressed as pCi/μg acid, pCi/μmole or pCi/μg C.

<div align="center">RESULTS</div>

Milk yield and composition. In the experiments the milk yield obtained every hour during the perfusions was compared with the average hourly yield from the same gland during the week preceding the experiment (Table 1). The hourly milk yield on the living animal was taken to equal 100 % production. The fact that the animals had been fasted overnight may explain the low rate of milk secretion observed. However, this may have been caused by the perfusion technique used, which by present standards was not entirely perfect.

The protein and casein content of the milk in the different experiments was similar to that of the milk obtained from the living animal during the week preceding the experiment. The lactose content fell in expts 1 and 2 during the last hours of per-fusion while it remained constant in expt 3. In all 3 expts the citric acid content was somehow higher during perfusion. The milk fat content during each hour of the perfusion was maintained and rose at the end of the perfusion in the 3 expts (Table 1). The fatty acid composition showed practically no changes.

Synthesis of milk components. The specific activities of the different milk com-ponents during perfusion are given for the 3 expts in Table 2. It is apparent that practically no ^{14}C was transferred to lactose, citric acid and casein but an efficient ^{14}C incorporation was found in the milk fat fractions.

After fractionation of milk fat the specific activity of the triglyceride fraction was determined. The specific activity values are practically equal to those of the total fat in all samples examined. In all 3 expts the triglyceride content of milk fat was about 90–98 %. Maximum specific activity of milk fat was observed after 6 and 4 h of perfusion for expts 1 and 2, respectively. In expt 3 no steady state was reached at the end of perfusion. The component fatty acids were separated from milk fat triglyceride samples with highest specific activity and then isolated and assayed for radioactivity.

(*a*) Expt 1: [1-^{14}C]palmitic acid. The highest activity was localized in the pal-mitate residue, followed by palmitoleic acid, whose specific activity was about 23 % that of palmitic acid. It was calculated that 94 % of the activity of triglycerides was recovered in palmitic acid (Table 3). Stearic, oleic and the lower fatty acids were slightly labelled.

(*b*) Expts 2 and 3: [1-^{14}C]oleic acid. In both experiments oleic acid showed a very high ^{14}C incorporation (Table 3). The incorporation of oleic acid accounted for,

Time of perfusion, h

	Pre-experimental* ±s.d.	1	2	3	4	5	6	7	8	9	10	11
							Expt 1					
Milk production, % of yield from intact animal	·	64	31	20	23	20	11	7·5	5	2	—	—
Lactose, g/100 ml	6·8 ± 0·1	7·6	6·2	5·3	4·5	3·2	3·1	2·8	2·8	—	—	—
Triglycerides, g/100 ml	5·2 ± 0·4	—	5·7	5·2	4·4	4·1	4·4	4·9	4·7	—	—	—
Casein, g/100 ml	4·5 ± 0·1	3	2·5	2·2	2·4	2·5	3·3	4·0	5	—	—	—
Whey proteins, g/100 ml	0·80 ± 0·08	0·68	0·60	0·53	0·50	0·57	0·69	0·97	1·1	—	—	—
Citric acid, g/l	2·0 ± 0·4	2·9	3·6	4·1	3·9	3·4	2·3	2·2	2·2	—	—	—
							Expt 2					
Milk production, % of yield from intact animal	·	26	18	12	12	16	12	19	—	—	—	—
Lactose, g/100 ml	3·8 ± 0·3	3·7	4·3	4·1	4·1	3·4	3·1	—	—	—	—	—
Triglycerides, g/100 ml	4·8 ± 1·1	4·8	3·8	3·6	3·8	5·4	7·2	7·5	—	—	—	—
Casein, g/100 ml	3·5 ± 0·1	3·2	3·0	2·9	2·8	2·7	2·5	2·7	—	—	—	—
Whey proteins, g/100 ml	0·80 ± 0·15	0·7	0·7	0·7	0·9	1·1	1·3	1·2	—	—	—	—
Citric acid, g/l	1·5 ± 0·2	2·4	2·2	2·4	2·2	1·8	2·0	1·8	—	—	—	—
							Expt 3					
Milk production, % of yield from intact animal	·	19	15	10	6	6	6·5	6·5	7	6·5	8	7·5
Lactose, g/100 ml	3·7 ± 0·1	4·5	4·5	4·1	3·9	3·7	3·1	3·2	3·5	3·2	3·4	3·0
Triglycerides, g/100 ml	5·0 ± 0·2	5·6	6·0	5·8	5·8	6·9	6·9	7·0	7·0	11·3	10·0	9·1
Casein, g/100 ml	3·1 ± 0·1	2·9	2·6	2·7	2·7	2·7	2·9	3·0	2·6	2·9	3·3	2·9
Whey proteins, g/100 ml	0·88 ± 0·11	0·8	0·7	0·6	0·7	0·7	0·6	0·7	0·7	0·8	0·7	0·8
Citric acid, g/l	1·0 ± 0·2	0·8	1·0	1·3	1·0	1·3	1·5	1·0	1·2	1·5	—	—

* Average composition of milk taken from the same gland during the week preceding the experiment.
— Not determined.

Table 2. *Specific radioactivities (mμCi/mg of C) of the different milk components and of the expired CO_2 during perfusion of isolated glands with [1-14C]palmitic acid or [1-14C]oleic acid*

(The substrate mixture infused during perfusion contained [1-14C]palmitic acid (expt 1: sp.act. 22·20 μCi/mg C) or [1-14C]oleic acid (expts 2, 3: sp.act. 12·95 μCi/mg C).)

Time of perfusion, h after isotope addition

Expt		1	2	3	4	5	6	7	8	9	10	11
1	Milk fat triglycerides	—	0·014	0·047	0·096	0·11	0·34	0·34	0·28	0·29	—	—
	Casein	—	—	0·02	—	—	0·025	0·02	—	—	—	—
	Lactose	—	—	—	—	—	0·0004	—	—	—	—	—
	Citric acid	—	—	—	—	—	0·021	—	—	—	—	—
	Expired CO_2	—	1·10	0·50	0·17	0·36	—	0·30	0·27	—	—	—
2	Milk fat triglycerides	—	0·12	0·39	0·66	0·60	0·70	0·63	—	—	—	—
	Casein	—	0·002	0·003	0·004	0·008	0·005	0·009	—	—	—	—
	Lactose	—	—	—	—	—	0·0005	—	—	—	—	—
	Citric acid	—	—	—	—	—	0·049	—	—	—	—	—
	Expired CO_2	—	0·36	0·37	0·30	0·26	0·23	—	—	—	—	—
3	Milk fat triglycerides	—	—	—	—	—	0·15	0·24	0·29	0·40	0·42	0·49
	Casein	—	0·002	—	—	—	—	0·004	—	—	—	0·004
	Expired CO_2	0·12	0·13	0·43	0·047	0·081	< 0·05	< 0·05	< 0·05	< 0·05	< 0·05	< 0·05

— Not determined.

fat triglycerides during perfusion of isolated glands with [1-¹⁴C]palmitic acid or [1-¹⁴C]oleic acid

(The substrate mixture infused during perfusion contained [1-¹⁴C]palmitic acid (expt 1: sp.act. 22·20 μCi/mg C) or [1-¹⁴C]oleic acid (expts 2, 3: sp.act. 12·95 μCi/mg C).)

Time of perfusion, h after addition of isotope

Fatty acid	Expts 1, 6			Expts 2, 5			Expts 2, 6			Expts 3, 10		
	g, %	Sp.act.	¹⁴C* in FA, %	g, %	Sp.act.	¹⁴C in FA, %	g, %	Sp.act.	¹⁴C in FA, %	g, %	Sp.act.	¹⁴C in FA, %
6:0	2·1	0·20	0·9	0·2	0	0	0·8	—	0	0	0	0
8:0	3·2	0·090	0·7	1·3	0	0	0·9	0·08	0·06	1·7	0	0
10:0	8·7	0·029	0·6	6·2	0	0	5·3	0·06	0·05	5·8	0	0
12:0	7·3	0	0	4·0	0	0	3·6	0·02	0·01	5·2	0	0
13:0	0·1	0	0	0	0	0	0	0	0	0	0	0
13:1	—	0	0	0	0	0	0	0	0	0	0	0
14:0	11·4	0	0	7·1	0	0	7·4	0	0	10·3	0	0
14:1	0·3	0	0	0·3	0	0	0·3	0	0	0	0	0
15:0	0·8	0	0	0·7	0	0	0·7	0	0	0·8	0	0
15:1	0·2	0	0	0·3	0	0	0·3	0	0	0	0	0
16:0	21·3	1·60	94·4	23·1	0	0	23·0	0	0	27·8	0	0
16:1	2·3	0·37	2·3	2·9	0	0	2·8	0	0	2·0	0	0
17:0	0·6	0	0	0·49	0	0	0·50	0	0	0	0	0
17:1	0·3	0	0	0·3	0	0	0·2	0	0	0	0	0
18:0	11·3	0·02	0·6	9·8	0	0	8·9	0	0	11·1	0	0
18:1	26·8	0·004	0·3	40·8	1·45	100	42·6	1·70	99·8	35·2	1·20	100
18:2	1·5	0	0	2·1	0	0	2·5	0	0	0	0	0

* Per cent ¹⁴C of milk-fat triglyceride radioactivity recovered in fatty acid.

— Not determined.

respectively, 99 and 100 % of the [^{14}C] incorporation in the triglyceride fraction in these experiments. In expt 2 traces of radioactivity were found in the lower fatty acids. No detectable radioactivity was found in stearic acid.

Arterial and mammary venous FFA. Arterial concentrations of FFA in the [1-^{14}C]-palmitate experiment were, respectively, 0·10, 0·16, 0·38, 0·33 mmole/l plasma after 0, 2, 5 and 8 h perfusion. In the 3 expts, the FFA composition of the plasma was

Table 4. *Composition (g %) of the fatty acids of arterial and mammary venous plasma FFA during perfusion with [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid*

Mean values of samples taken during the perfusion are shown

Fatty acid	Expt 1		Expt 2		Expt 3	
	Arterial, %	Venous, %	Arterial, %	Venous, %	Arterial, %	Venous, %
10:0	—	—	0·3	0·3	0·4	0·4
12:0	—	—	0·5	0·5	0·6	0·4
Unidentified	—	—	2·8	4·1	4·3	3·1
14:0	—	—	2·0	2·5	2·4	2·1
15:0	—	—	1·5	1·7	2·0	1·4
16:0	33·9	34·6	29·2	33·9	34·8	30·0
16:1	2·7	3·4	8·1	8·7	8·9	9·0
16:2	—	—	0·4	0·3	—	—
17:0	—	—	0·6	0·7	0·7	0·6
17:1	—	—	1·0	1·8	2·2	1·0
18:0	17·8	18·7	12·3	11·9	10·7	11·0
18:1	36·0	36·0	33·5	32·2	30·5	32·1
18:2	6·9	6·9	5·3	5·0	4·9	5·9
18:3	—	—	2·2	0·4	0·4	—

— Not detected.

studied. As Table 4 indicates there were only small variations in fatty acid composition of the FFA fraction during passage through the gland during perfusion with [1-^{14}C]palmitic acid or with [1-^{14}C]oleic acid and the composition was normal as compared with values reported in the literature.

In expt 1 the only radioactive peak detected emerged at the retention volume of palmitic acid. In expts 2 and 3 the radioactivity of the FFA in all the blood samples examined was associated with a peak at the retention time of oleic acid. The minute amounts of the FFA did not allow complementary purification of the oleic and palmitic acids by thin-layer chromatography. Therefore, these activities are expressed as mμCi/unit area on the chromatogram.

The specific activities of the individual FFA in venous plasma were decidedly lower than those in the arterial plasma (Table 5).

Production of CO$_2$. The specific radioactivities of the expired CO$_2$ are given in Table 2. In expts 1, 2 and 3, respectively, 2·1, 1·2 and 0·04 % of the added palmitic acid and oleic acid were recovered as ^{14}CO$_2$.

Triglycerides in arterial and mammary venous plasma. In the different experiments there was usually a positive arterio-venous difference in concentration of the plasma triglycerides across the gland. The concentrations and the specific activities of the arterial and venous plasma triglycerides are given in Table 6. Addition of larger

Table 5. Specific activities (mμCi/unit area on chromatogram) of free palmitic acid (expt 1) and free oleic acid (expts 2, 3) in arterial and venous plasma during perfusion

Time of perfusion, h

		2	3	3½	4	5	6	6½	7	8	9	10
Expt 1	Arterial	—	—	1·30	—	—	—	1·06	—	1·53	—	—
	Venous	0·34	—	0·57	—	—	—	—	—	0·88	—	—
Expt 2	Arterial	0·72	—	—	0·40	0·52	—	—	—	—	—	—
	Venous	0·15	—	—	0·08	—	—	—	—	—	—	—
Expt 3	Arterial	—	—	—	—	0·40	0·44	—	0·35	—	—	0·56
	Venous	—	—	—	—	0·18	0·35	—	—	0·37	—	0·56

— Not determined.

Table 6. *Concentration (mg/100 ml plasma) and specific radioactivity (10^{-3} mμCi/mg C) of arterial and venous plasma triglycerides during the perfusion of isolated glands with [1-^{14}C]palmitic or [1-^{14}C]oleic acid*

Sp.act. (10^{-3} mμCi/mg C) of triglycerides of plasma in the control experiment

		Time of perfusion, h							
		0	2	4	5	6	7	8	10
					Expt 1				
Concentration	Arterial	—	—	—	53	—	—	67	—
	Venous	—	—	—	45	—	—	46	—
Sp. act.	Arterial	—	160	—	58	—	—	—	—
	Venous	—	135	—	52	—	—	—	—
					Expt 2				
Concentration	Arterial	—	102	227	289	—	—	—	—
	Venous	—	105	200	254	—	—	—	—
Sp.act.	Arterial	—	2·7	25	27	—	—	—	—
	Venous	—	1·8	2·7	10	—	—	—	—
					Expt 3				
Concentration	Arterial	16·9	15·1	21·7	—	33·1	—	34·3	36·8
	Venous	9·3	10·9	25·5	—	29·4	—	32·9	32·4
Sp.act.	Arterial	—	6·5	8·5	—	8·1	—	4·3	4·9
	Venous	—	2·2	8·1	—	7·4	—	5·1	6·0
				Control expt: [1-^{14}C]palmitic acid					
Sp.act.		—		219	253	250	270	180	—

Not determined

sterol esters during perfusion of isolated glands with [1-¹⁴C]*palmitic acid or* [1-¹⁴C]*oleic acid*

Sp.act. (10^{-3} mμCi/mg C) of cholesterol esters in the control experiment

Time of perfusion, h

		2	4	5	6	7	8	10
					Expt 1			
Concentration	Arterial	72	—	66		—	66	—
	Venous	99	—	48	—	—	70	—
Sp.act.	Arterial	7	—	10	—	—	—	—
	Venous	16	—	29	—	—	—	—
					Expt 2			
Concentration	Arterial	93	69	70		—	—	—
	Venous	106	61	76	—	—	—	—
Sp. act.	Arterial	4	1·6	4·3		—	—	—
	Venous	5	5	6	—	—	—	—
					Expt 3			
Concentration	Arterial	99·8	—	—	61·4	—	48	74
	Venous	99	—	—	61·8	—	50	63
Sp.act.	Arterial	—	—	—	4·7	—	22	10
	Vonous	—	—	—	3·7	—	59	27
				Control expt: [1-¹⁴C]palmitic acid				
Sp.act.		—	228	160	250	150	54	—

— Not determined.

Table 3. Composition (g/%) and specific radioactivities (μμc/mg of C) of the fatty acids of arterial and mammary venous plasma cholesterol esters during perfusion with [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid

Expt 1: time of perfusion, h

Fatty acid	Arterial (2 h) %	Arterial (2 h) Sp.act.	Venous (2 h) %	Venous (2 h) Sp.act.	Arterial (5 h) %	Arterial (5 h) Sp.act.	Venous (5 h) %	Venous (5 h) Sp.act.
16:0	17.5	} 0.07	24.0	} 0.11	25	} 0.10	28	} 0.28
16:1	7.2		9.8		10		10.8	
18:0	3.7	—	3.4	—	3.1	—	8.8	—
18:1	43.0	—	40.3	—	23	—	16.1	—

Expt 2: time of perfusion, h

Fatty acid	Arterial (2 h) %	Arterial (2 h) Sp.act.	Venous (2 h) %	Venous (2 h) Sp.act.	Arterial (4 h) %	Arterial (4 h) Sp.act.	Venous (4 h) %	Venous (4 h) Sp.act.
16:0	22.7		21.6		27		21.4	
16:1	6.0		7.8		3		6.6	
18:0	4.8	} 0.06	1.9	} 0.10	8.2	} 0.04	3.6	} 0.08
18:1	41.6		42.8		40.6		40.8	
18:2	24.8	—	23.3	—	13.6	—	27	—

Expt 3: time of perfusion, h

Fatty acid	Arterial (2 h) %	Arterial (2 h) Sp.act.	Venous (2 h) %	Venous (2 h) Sp.act.	Arterial (4 h) %	Arterial (4 h) Sp.act.	Venous (4 h) %	Venous (4 h) Sp.act.	Arterial (5 h) %	Arterial (5 h) Sp.act.	Venous (5 h) %	Venous (5 h) Sp.act.
16:0	27.4		22.8		17.9		18.0		21.4		31.6	
16:1	2.2		8.4		7.2		2.7		4.0		1.5	
18:0	3.5	} 0.03	4.6	} 0.02	3.1	} 0.01	3.3	} 0.06	5.3	} 0.13	1.3	} 0.35
18:1	48.3		46.7		41.4		50.9		43.0		50.0	

amounts of lymph to the perfusate in expt 2 explains the higher concentrations of triglycerides in arterial and venous plasma. The specific radioactivity of the plasma triglycerides remained very low during the whole perfusion in the 3 expts. The specific radioactivity of this lipid fraction was usually somewhat higher in the arterial than in the venous plasma. In the control experiment (without gland) the trigly-cerides of the blood became slightly labelled, as happened during the perfusions.

Cholesterol esters in arterial and mammary venous plasma. The concentrations and specific radioactivities of the arterial and venous cholesterol esters are shown in Table 7. In the different experiments there were no significant arterio-venous differences in concentration of the cholesterol esters across the mammary gland. The cholesterol esters were slightly labelled. Slight labelling occurred also in the control experiment. The specific activities of the cholesterol esters were mostly somewhat higher in the venous than in the arterial samples.

The fatty acid composition of plasma sterol esters showed only minor differences between arterial and venous samples. Among the fatty acids only palmitic acid was labelled in expt 1 while in expts 2 and 3 only oleic acid was labelled (Table 8).

<div align="center">DISCUSSION</div>

Most of the results obtained in the present experiments on the isolated perfused mammary gland are in agreement with those observed previously on the living animal. The substantial arterio-venous differences in plasma triglycerides during perfusion confirm the uptake observed in lactating goats (Barry, Bartley, Linzell & Robinson, 1963; Lascelles, Hardwick, Linzell & Mepham, 1964) and cows (Hartmann & Lascelles, 1964).

A very low but definite radioactivity was observed in the plasma triglycerides during the perfusion experiments. Since the plasma triglycerides acquired radio-activity in the control experiment, blood cells may play a role in the labelling of this fraction. In a perfusion on an isolated goat udder with [14C]stearic acid, Linzell *et al.* (1967) noted also some radioactivity in the triglyceride fraction of the plasma. In contrast, during infusion experiments with [14C]palmitic acid and [14C]oleic acid on living goats (Annison *et al.* 1967) the plasma triglycerides became appreciably labelled, probably through metabolism in the liver.

The specific activity of the FFA fraction of the plasma decreased considerably by passage through the udder. In accord with the results obtained by Annison *et al.* (1967), FFA composition did not change across the mammary gland. The FFA absorbed by the mammary gland was not estimated in the present experiments. However, most data in the literature point to an absence of a net uptake (Barry *et al.* 1963; Annison *et al.* 1967) or to a very small uptake (Hartmann & Lascelles, 1964) of this fraction in normally fed animals.

The considerable incorporation of [14C] in the milk fat triglycerides confirms the uptake of [14C]-labelled FFA from the blood since the other fatty acid fractions of the plasma were only labelled to a slight extent. The decrease in specific activity of the FFA fraction across the perfused gland and the labelling of the milk fat triglycerides may be explained by an uptake of labelled FFA from the blood stream and release of FFA by the mammary gland.

As to the origin of the FFA produced by the udder, most of the evidence favours a hydrolysis of plasma triglycerides (Patton & McCarthy, 1963; Dimick, McCarthy & Patton, 1966; West, Annison & Linzell, 1967). According to West et al. (1967) the plasma triglycerides taken up by the mammary gland are hydrolysed at the membrane surface of capillary cells and the liberated fatty acids would equilibrate with the plasma FFA before absorption by the mammary cell. Bishop, Davies, Glascock & Welch (1969), however, consider that an incomplete exchange takes place between FFA and plasma triglyceride fatty acids before absorption of the triglycerides.

Both hypotheses would lead to a decrease in specific activity of the FFA fraction of the plasma by passage through the udder. According to West et al. (1967) the specific activity of the plasma triglyceride fraction would pass unchanged through the mammary gland. In contrast, the hypothesis of Bishop et al. (1969) would lead to an increased specific activity in venous plasma triglyceride. The results of our experiments approximate to those to be expected from the first hypothesis. However, there was a marked decline in the specific radioactivity of the plasma triglycerides across the mammary gland.

No arterio-venous differences in the concentration of cholesterol ester were observed in these experiments, thus confirming findings in vivo (Hartmann & Lascelles, 1964; Annison et al. 1967). The fatty acid composition of this fraction showed no marked changes on passage through the udders. The cholesterol esters were found to be labelled in all the experiments, the activity being localized in the [^{14}C] fatty acid studied. The low but definite specific activities observed in this fraction suggest the existence of minor exchange processes between FFA and fatty acids of cholesterol esters. Red blood cells may be involved in these reactions. Relatively higher specific activities of the cholesterol esters were found in venous than in arterial blood, suggesting the operation of some minor small hydrolysis and exchange processes between cholesterol esters and fatty acids of the blood, during passage through the udder.

Most of the radioactivity in milk components was localized in the triglyceride fraction. The specific activities of palmitic acid and oleic acid of milk fat triglycerides support the view that substantial amounts of these acids are derived from blood lipid (Bishop et al. 1969). Extensive conversion of stearic acid to oleic acid by the isolated perfused udder was reported by Lauryssens et al. (1961) and by Linzell et al. (1967). Annison et al. (1967) reported similar findings for the gland on the living animal. The lack of appreciable activity in stearic acid of the milk fat triglycerides in the oleic acid experiments indicates that the reverse process—hydrogenation of oleic acid to stearic acid—does not occur in the udder. Dehydrogenation of palmitic acid to palmitoleic acid was shown in the udder. Comparison of the ratio of the specific activities of palmitoleic acid and palmitic acid in the milk fat triglycerides (present study) with that for oleic acid and stearic acid found by Lauryssens et al. (1961) suggests that the proportion of palmitoleic acid which is derived from palmitic acid is half as great as the proportion of oleic acid derived from stearic acid. The elongation of palmitic acid to stearic and oleic acid seems to occur to only a very slight extent.

The specific radioactivities of expired $^{14}CO_2$ indicate that the FFA are degraded to only a limited extent by the lactating udder. The low specific activities found in

citric acid, casein and lactose confirm this result. The $^{14}CO_2$ production obtained during the experiments was variable. In expts 1 and 2, the specific activities of the expired CO_2 were higher than those obtained by Annison *et al.* (1967) during infusion of [U-^{14}C]-labelled oleic acid or palmitic acid to lactating goats. The relatively higher $^{14}CO_2$ elimination observed in the present experiments may be partly ascribed to the use of [1-^{14}C]-labelled precursors, since adequate amounts of glucose and acetate were added to the perfusate.

In expt 3 the $^{14}CO_2$ production declined after 3 h of perfusion, although Van Slyke analyses indicated normal O_2 uptake and CO_2 production by the gland. The $^{14}CO_2$ elimination in this experiment was of the same order as that observed in perfusion experiments on the isolated goat udder in presence of [U-^{14}C]stearic acid (Linzell *et al.* 1967). As compared with expt 3 up to 50 times more $^{14}CO_2$ was recovered in expts 1 and 2. No explanation can be offered for the dissimilarity between these results, since adequate amounts of glucose and acetate were added to the perfusate. The results suggest that FFA may act as a substrate for the oxidative metabolism of the mammary gland.

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The primary phase of rennin action in heat-sterilized milk

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SUMMARY. The action of rennin in milk subjected to the ultra-high-temperature heat sterilization process has been studied. The primary phase of rennin action, as determined by the release of peptides soluble in trichloroacetic acid, was partially inhibited as a result of the heat treatment. This was largely due to a reduction in the release of non-carbohydrate-containing peptides from κ-casein. It is suggested that when whole milk is heated the formation of a complex between β-lactoglobulin and κ-casein proceeds more readily with the species of κ-casein which lacks carbohydrate. Evidence is presented which shows that there may be more than one type of carbohydrate moiety attached to κ-casein.

The coagulation of milk by the enzyme rennin (E.C. 3.4.4.3.) is considered to be a 2-stage process (Cherbuliez & Baudet, 1950). During the primary phase of rennin action, κ-casein is hydrolysed (Waugh & von Hippel, 1956; Wake, 1959), and then in the presence of Ca^{2+} ions the casein micelles are precipitated (Pyne, 1953).

Milk which has been heated and then immediately cooled shows an increase in rennet coagulation time (Powell & Palmer, 1935; Pyne, 1945). On standing, there is a further increase in the coagulation time.

When isolated β-lactoglobulin and isolated κ-casein are heated together a complex is formed between the 2 proteins (Long, Van Winkle & Gould, 1963; Sawyer, 1969). The formation of this complex causes a considerable increase in the rennin coagulation time of κ-casein (Zittle, Thompson, Custer & Cerbulis, 1962). Kannan & Jenness (1961) have obtained evidence which suggests that the primary phase of rennin action is inhibited by prior heat treatment of the milk and that this inhibition is largely dependent on the presence of β-lactoglobulin which may be complexing with the κ-casein. However, Tessier, Yaguchi & Rose (1969) consider that the formation in whole milk of a complex corresponding to that found in heated mixtures of β-lactoglobulin and κ-casein has not been demonstrated conclusively.

Milk which has been subjected to the heat-sterilization process does not normally form a clot with rennin and therefore we considered the possibility that the primary phase may be completely inhibited. However, when Ca^{2+} ions are added to the milk a clot is formed by rennin action showing that any inhibition of the primary phase is not complete or, alternatively, that the presence of additional Ca^{2+} ions allows the primary phase to proceed normally. To determine how the primary phase is affected, experiments have been carried out to study the release from κ-casein of peptides and glycopeptides by the action of rennin in heat-sterilized milk.

EXPERIMENTAL

Milk samples were supplied by Associated Dairies, Ltd, Accrington. The heat-sterilized milk sample was taken immediately after processing (137 °C for ~ 3 s preceded by holding at 80 °C for 7 min). The unheated control sample was taken from the same bulk milk. Rennin was supplied by Koch–Light Laboratories Ltd, Colnbrook, Bucks. and by Sigma Chemical Company Ltd, London.

A total of 3 experiments were performed in which the primary phase of rennin action in the heat-sterilized and unheated control milks was determined by the release of peptides and glycopeptides soluble in the trichloroacetic acid (TCA) filtrate of the milk. Portions (0·2 ml) of a solution of rennin were added to 20 ml samples of whole milk at 37 °C. The concentration of rennin was adjusted to give a clotting time of about 8 min for the control milk and the same rennin concentration was used for the corresponding heat-sterilized milk sample. At predetermined intervals after the addition of rennin, TCA was added to a final concentration of 2 or 12 % (w/v) to stop the rennin action and precipitate the milk proteins. The N content of the TCA filtrate was determined by micro-Kjeldahl analysis or with a Technicon Auto-Analyzer (Hindle & Wheelock, 1970). The carbohydrates attached to the glyco-peptides were determined by gas–liquid chromatography using the method of Sinkinson & Wheelock (1970).

RESULTS

Effect of the heat-sterilization process on the non-casein N and TCA filtrates of milk

The heat-sterilization process caused marked depressions in the non-casein N and in the N soluble in the 2 % TCA filtrate (Tables 1, 2). There was little change in the content of glycopeptides or glycoproteins containing N-acetyl neuraminic acid,

Table 1. *The effect of the heat-sterilization process on the non-casein N and on the non-protein N fractions of milk*

Sampling	Non-casein N, mg/100 ml milk		Non-protein N, mg/100 ml milk	
	Unheated	Sterilized	Unheated	Sterilized
1	107	41·6	26·5	24·6
2	106	42·1	23·4	22·8
3	96·9	29·7	25·9	28·8
4	103	46·5	26·3	30·3
5	105	29·3	18·9	19·9
6	85·5	31·7	16·6	17·1

Values were obtained from milk samples taken on 6 different occasions. The non-casein N was measured after precipitating the casein at pH 4·6. The non-protein N was measured after precipitating the protein with 12 % (w/v) trichloroacetic acid.

D-galactose or 2-acetamido-2-deoxy-D-galactose, but there was a very marked reduction in those containing D-mannose. The depression in the N soluble in the 2 % TCA was probably due to heat denaturation of β-lactoglobulin which is soluble at this concentration (Fox, Holsinger, Posati & Pallansch, 1967). The greater de-

Table 2. *The effect of the heat-sterilization process on the peptides and glycopeptides soluble in the trichloroacetic acid (TCA) filtrates of milk*

Expt	Milk	TCA used in precipitation of proteins, %	N-acetyl neuraminic acid, μmoles/ 100 ml milk	D-galactose, μmoles/ 100 ml milk	2-Acetamido- 2-deoxy- D-galactose, μmoles/ 100 ml milk	D-mannose, μmoles/ 100 ml milk	N, mg/100 ml m
1	Unheated	12	0·00	3·14	0·00	0·76	22·3
	Sterilized	12	0·00	2·43	0·00	0·55	23·4
	Unheated	2	1·14	4·86	8·65	1·53	51·0
	Sterilized	2	2·59	5·89	5·5	0·66	27·5
2	Unheated	12	0·72	4·52	1·41	0·98	31·3
	Sterilized	12	0·66	4·00	1·41	1·04	31·2
	Unheated	2	1·72	16·50	6·80	3·74	83·7
	Sterilized	2	5·10	14·10	3·10	1·25	40·3
3	Unheated	12	0·96	3·65	0·00	0·97	25·2
	Sterilized	12	0·40	3·49	0·00	0·87	26·7
	Unheated	2	3·31	8·17	1·41	4·47	78·8
	Sterilized	2	2·48	8·67	1·83	1·04	35·0
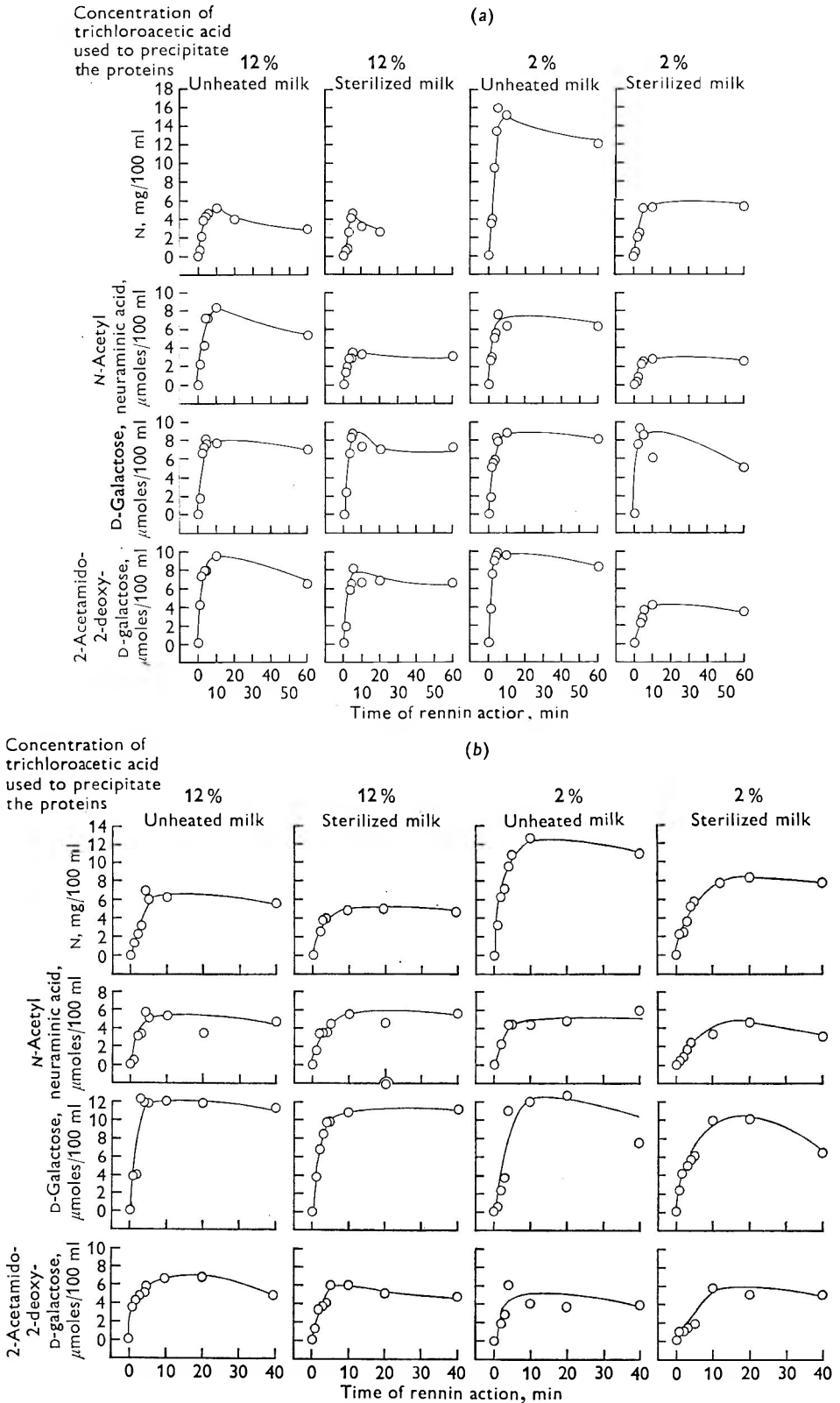
Fig. 1. For legend see opposite page.
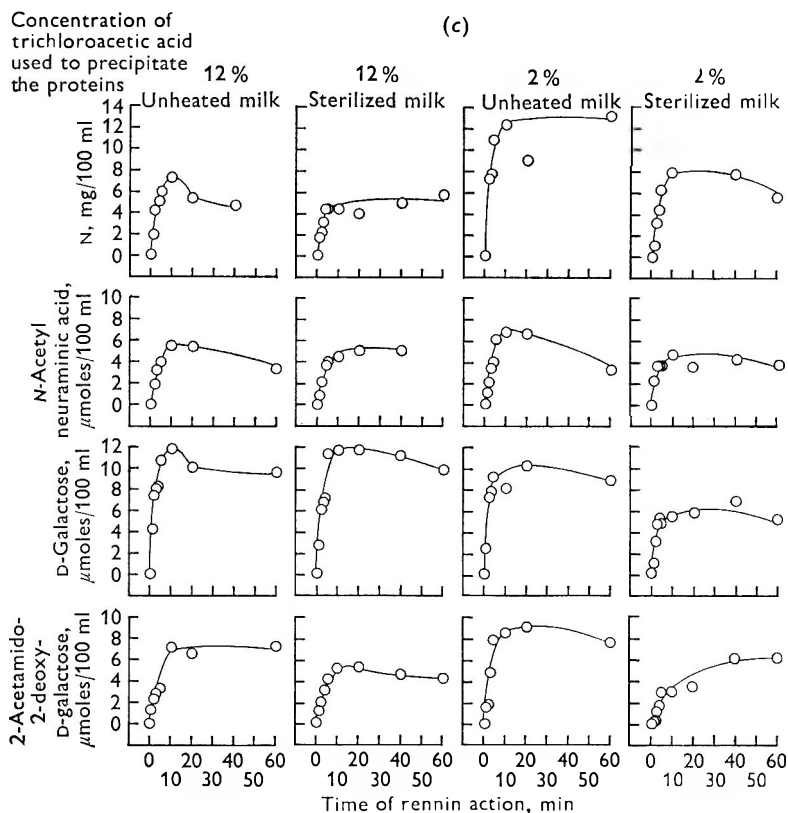
Fig. 1. Increase in N and in *N*-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose attached to glycopeptides soluble in 2 and 12 % trichloroacetic acid with time after the addition of rennin to the milk. (*a*), Expt 1; (*b*), expt 2; (*c*), expt 3.

pression in non-casein N shows that whey proteins other than β-lactoglobulin were also denatured by the sterilization process. The results for the sugars suggest that one of these proteins may contain D-mannose.

The differences in N and in the sugars attached to the glycopeptides soluble in the 12 % TCA filtrates of the unheated and sterilized milks were relatively small.

Release of peptides and glycopeptides by the action of rennin

The release by the action of rennin of peptides and glycopeptides soluble in 2 and 12 % TCA from heat-sterilized milk and from the corresponding unheated control milk is shown for the 3 different experiments in Fig. 1 (*a–c*). There was a rapid release of peptides, as determined by N, during the first few minutes of rennin action. The amount of peptides released reached a maximum after about 10 min and then decreased slightly or remained about constant for the remainder of the experiment. The rate of decrease was usually more pronounced in the control samples than in the sterilized samples. The maximum amount of peptides released was less with the sterilized milks than with the controls but this depression was much greater for those soluble in 2 than in 12 % TCA. The rate of release of peptides was slightly reduced by the heat processing.

In the control samples, the maximum amount of glycopeptides released as determined by their content of N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose, was about the same for the 2 and 12 % TCA filtrates. In expt 1, the maximum amount of galactose-containing glycopeptides released was about the same for all the filtrates but there was a reduction in N-acetyl neuraminic acid-containing glycopeptides for the sterilized milk samples. There was also a reduction in the release of 2-acetamido-2-deoxy-D-galactose-containing glycopeptides in the 2 % TCA filtrate from the heated milk but not in the 12 % TCA filtrate, whose content of this carbohydrate was similar to that in both the controls. In expt 2, the total release of glycopeptides soluble in 2 and 12 % TCA was not affected by the heat-sterilization process. In expt 3, the total release of N-acetyl neuraminic acid-containing glycopeptides was about the same for all the filtrates but there was a reduction in the 2-acetamido-2-deoxy-D-galactose-containing glycopeptides in both filtrates of the sterilized samples as compared with the corresponding controls. There was a reduction in D-galactose-containing glycopeptides in the 2 % TCA filtrates of the sterilized milk but the total amount released and soluble in 12 % TCA was the same in both 2 and 12 % TCA control filtrates.

DISCUSSION

The heterogeneity of κ-casein is due partly to differences in the amount of carbohydrate attached to the polypeptide chain of the protein (MacKinlay & Wake, 1965). During the primary phase of rennin action the κ-casein is split with the release of glycopeptides which contain the carbohydrates (Gibbons & Cheeseman, 1962) and so the differences in carbohydrate content are also present in the glycopeptides. The solubility of these glycopeptides in TCA increases with increase in their carbohydrate content (MacKinlay & Wake, 1965) and therefore in the present experiments the increase in N soluble in 12 % TCA after rennin action would be due to the release of glycopeptides which are rich in carbohydrates. On the other hand the 2 % TCA would contain those peptides, which have little or no carbohydrates, in addition to those which are soluble in 12 % TCA.

It is quite clear from our results that the primary phase of rennin action, as determined by the release of peptides soluble in TCA, is inhibited as a result of the heat-sterilization process. This finding is in agreement with the evidence of Kannan & Jenness (1961) who showed that the rate of rennet action at 16 °C was slower in milk that had been heated at 85 °C for 30 min than in raw skim-milk. In particular, our results demonstrate that the inhibition of the primary phase of rennin action affects mainly the release of those peptides which lack carbohydrate. The effects of the heat-sterilization process on the release of glycopeptides appear to be rather more complex and may be conveniently divided into 2 groups. In the first group there was a reduction in the release by rennin of certain glycopeptides. This is shown in expt 1 (Fig. 1 a) in a depression in the release of N-acetyl neuraminic acid-containing glycopeptides, and in expt 3 (Fig. 1 c) in a depression in the release of 2-acetamido-2-deoxy-D-galactose-containing glycopeptides soluble both in 2 and in 12 % TCA filtrates. In the second group the release of glycopeptides soluble in 12 % TCA was unaffected but certain of the glycopeptides were rendered insoluble in 2 % TCA. This

is shown by 2-acetamido-2-deoxy-D-galactose-containing glycopeptides in expt 1 (Fig. 1*a*) and by D-galactose-containing glycopeptides in expt 3 (Fig. 1*c*).

It has been established that the sugars *N*-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose are attached to the glycopeptides released from *κ*-casein by rennin action (Alais & Jollès, 1961; Wheelock & Sinkinson, 1969) and that the amount of carbohydrate attached to *κ*-casein may vary (MacKinlay & Wake, 1965). However, it has not been established how the structure of the sugar moiety changes to account for the variation in the amount of the carbohydrate attached to *κ*-casein. Jollès, Alais, Adam, Delfour & Jollès (1964) have proposed that the structure of the carbohydrate moiety of the glycopeptides released from *κ*-casein by rennin is as follows: protein:2-acetamido-2-deoxy-D-galactose:D-galactose:*N*-acetyl neuraminic acid. If this were the only type of sugar moiety to be attached to the glycopeptides of *κ*-casein then the ratio of the sugars would remain constant. Our results show that in certain cases, after heat treatment, the ratio of these sugars is altered. It is unlikely that there is any decomposition of sugars during the processing and therefore there are probably other sugar moiety structures in addition to that suggested by Jollès *et al.* (1964). On the basis of our results these sugar moieties would be expected to contain 1 or 2 monosaccharides. In this context it is of interest to note that Jollès, Alais, Delfour & Jollès (1963) have isolated a glycopeptide of *κ*-casein which contained only galactosamine. The presence of such a glycopeptide, the release of which is inhibited as a result of heat treatment, would explain the depression in the amount of 2-acetamido-2-deoxy-D-galactose-containing glycopeptides soluble in both 2 and 12 % TCA after rennin action in expt 3 (Fig. 1*c*).

Kannan & Jenness (1961) showed that the increase in the rennet coagulation time of artificial milk caseinate systems as a result of heat treatment was dependent on the presence of *β*-lactoglobulin during heating. There was also a slight decrease, caused by prior heat treatment, in the rate of release of peptides soluble in 12 % TCA when rennet acted on a mixture of casein and *β*-lactoglobulin (Morrissey, 1969). This evidence suggests that the *β*-lactoglobulin is involved in the inhibition of the primary phase of rennin action. It seems quite likely, therefore, that the inhibition of the primary phase in whole milk, as a result of prior heat treatment, is caused by the formation of a complex between *β*-lactoglobulin and *κ*-casein even though the complex formed is not necessarily identical to that formed when the isolated proteins are heated together.

The marked depression in the release of non-carbohydrate-containing peptides invariably obtained in our experiments suggests that, in general, the formation of any complex between *β*-lactoglobulin and *κ*-casein, when whole milk is heated, is likely to proceed much more readily with the *κ*-casein which lacks carbohydrate. This could mean that the presence of carbohydrate on *κ*-casein inhibits the formation of the complex or alternatively that the *κ*-casein which lacks carbohydrate is situated on the outside of the casein micelle and is therefore more readily accessible for complex formation. The slight reduction in the release by rennin of certain glycopeptides suggests that the presence of carbohydrate in *κ*-casein does not completely prevent complex formation. As this result was not consistent between experiments, the formation of such a complex may depend on the precise conditions of processing or on the composition of the milk.

It has been concluded (cf. Rose, 1962) that heat stability characteristics may be considerably affected by the formation of a complex between β-lactoglobulin and κ-casein. If the presence of carbohydrates inhibits the formation of this complex then the distribution of carbohydrates in κ-casein may be a factor in determining the heat stability characteristics of a particular milk.

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The release of peptides and glycopeptides by the action of heat on cow's milk

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SUMMARY. The release, as a result of heat treatment on cow's milk, of peptides and glycopeptides which are soluble in the 12 % trichloroacetic acid (TCA) filtrate of the milk has been studied. The results showed that the amount of peptides released increased with temperature and duration of the heat treatment. There were differences between milks in their response to a given heat treatment. Glycopeptides were released at temperatures as low as 50 °C but not at 37 °C. A comparison was made of the glycopeptides released by heat treatment and of those released from κ-casein by the action of rennin on the milk. This showed that N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose were invariably present in both groups of glycopeptides. D-Mannose was also present in the glycopeptides released by heat but was detected on the glycopeptides released by rennin in only one of 6 experiments. It is suggested that glycopeptides released by heat may be derived from κ-casein but that a definite conclusion cannot be reached at present because of lack of information on the carbohydrates attached to other milk proteins.

It has been shown by Alais, Kiger & Jollès (1967) that when isolated κ-casein is heated (120 °C for 20 min) a peptide is released which is similar to that released by the action of rennin on casein. It is therefore possible that the coagulation of milk as a result of heating may be partly due to the hydrolysis of κ-casein, so that its micelle stabilizing power is destroyed in the same way as in rennin coagulation. Glycopeptides released from κ-casein by the action of rennin are soluble in 12 % (w/v) TCA (Armstrong, Mackinlay, Hill & Wake, 1967) and contain the carbohydrates N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose (Alais & Jollès, 1961; Wheelock & Sinkinson, 1969), which may be used to characterize them. In the present investigation we have studied the release, by heat treatment of the milk, of glycopeptides and peptides which are soluble in the trichloracetic acid (TCA) filtrate.

EXPERIMENTAL

In all the experiments the milk used was from individual cows (cows 1–6), which were free of bacterial infections of the udder. The milk (80 ml) was heated under reflux at constant temperature with regular shaking. After a predetermined interval TCA was added to give a final concentration of 12 % (w/v). The precipitate was removed by filtering through a Whatman paper No. 42. N in the filtrate was deter-

mined by micro-Kjeldahl analysis. The carbohydrates attached to the glycopeptides in the filtrate were analysed by gas–liquid chromatography of their trimethyl silyl derivatives (Clamp, Dawson & Hough, 1967; Sinkinson & Wheelock, 1970a). The instrument used was a Pye Series 104 Gas Liquid Chromatograph (Pye-Unicam, Ltd, Cambridge). For comparison, the peptides and glycopeptides released by rennin (E.C. 3.4.4.3.) action and soluble in the 12 % (w/v) TCA filtrate were also analysed for the same constituents. In this case 0·8 ml of 1 % (w/v) rennin was incubated with 80 ml milk at 37 °C until just after clot formation and then the proteins were precipitated with 12 % TCA in the same way as with the heated samples. Rennin was supplied by Sigma Chemical Co. Ltd, London.
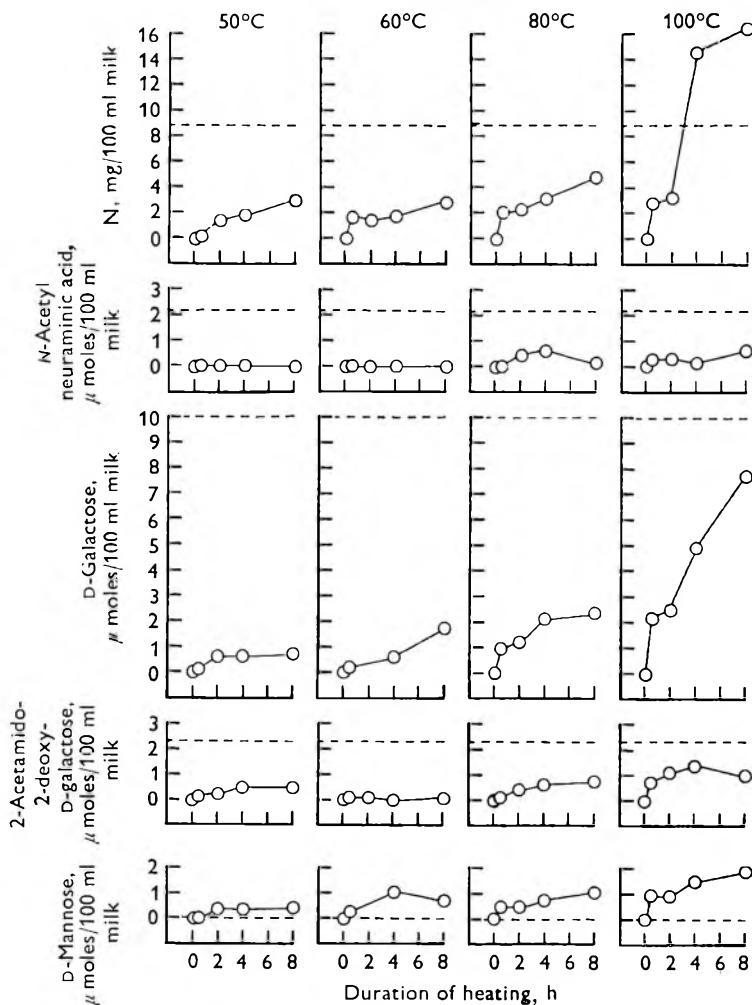
Fig. 1 (expt 1). The increase in N and in N-acetyl neuraminic acid, D-galactose, 2-acetamido-2-deoxy-D-galactose and D-mannose attached to glycopeptides soluble in the 12 % trichloro-acetic acid filtrate of whole milk after heat treatment. The dotted line indicates the corresponding values for the rennin treatment.

RESULTS

Three experiments were performed in which the effect of temperature on the release from milk of peptides and glycopeptides soluble in 12 % TCA was investigated. In the first 2 (expt 1, cow 1 and expt 2, cow 2) the temperatures were varied from 50 to 100 °C. In expt 3 a comparison was made of the release of peptides and glycopeptides
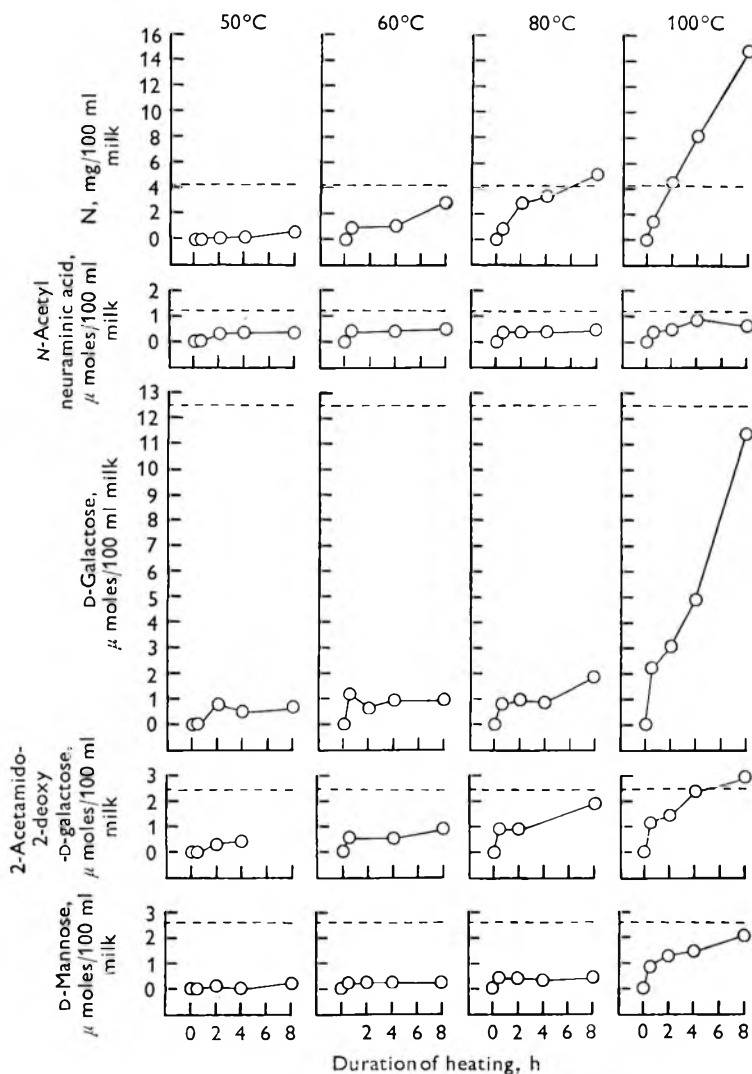
Fig. 2 (expt 2).The increase in N and in *N*-acetyl neuraminic acid, D-galactose, 2-acetamido-2-deoxy-D-galactose and D-mannose attached to the glycopeptides soluble in the 12 % trichloroacetic acid filtrate of whole milk after heat treatment. The dotted line indicates the corresponding values for the rennin treatment.

from the milk of different animals (cows 3–6) at temperatures of 80 and 100 °C. There was no release of peptides soluble in 12 % TCA with heating for up to 8 h at 37 °C. Therefore, the average values for N and for the carbohydrates attached to the peptides soluble in the 12 % TCA filtrate after heating the milk for varying intervals at 37 °C

have been used as a base-line in calculating the increase of peptides and glycopeptides soluble in 12 % TCA after heat treatment. The results are shown in Figs 1–3.

The total release of peptides, as determined by the increase in N soluble in the 12 % TCA filtrate of whole milk, shows quite clearly that the amount of peptides released increased with both the duration and the temperature of the heat treatment. In most of the experiments there was a characteristic pattern of a rapid release of peptides during the first 30 min of the heat treatment. The rate of release then decreased and remained approximately constant for the remainder of the heating period. In all the experiments the amounts of peptides released exceeded those
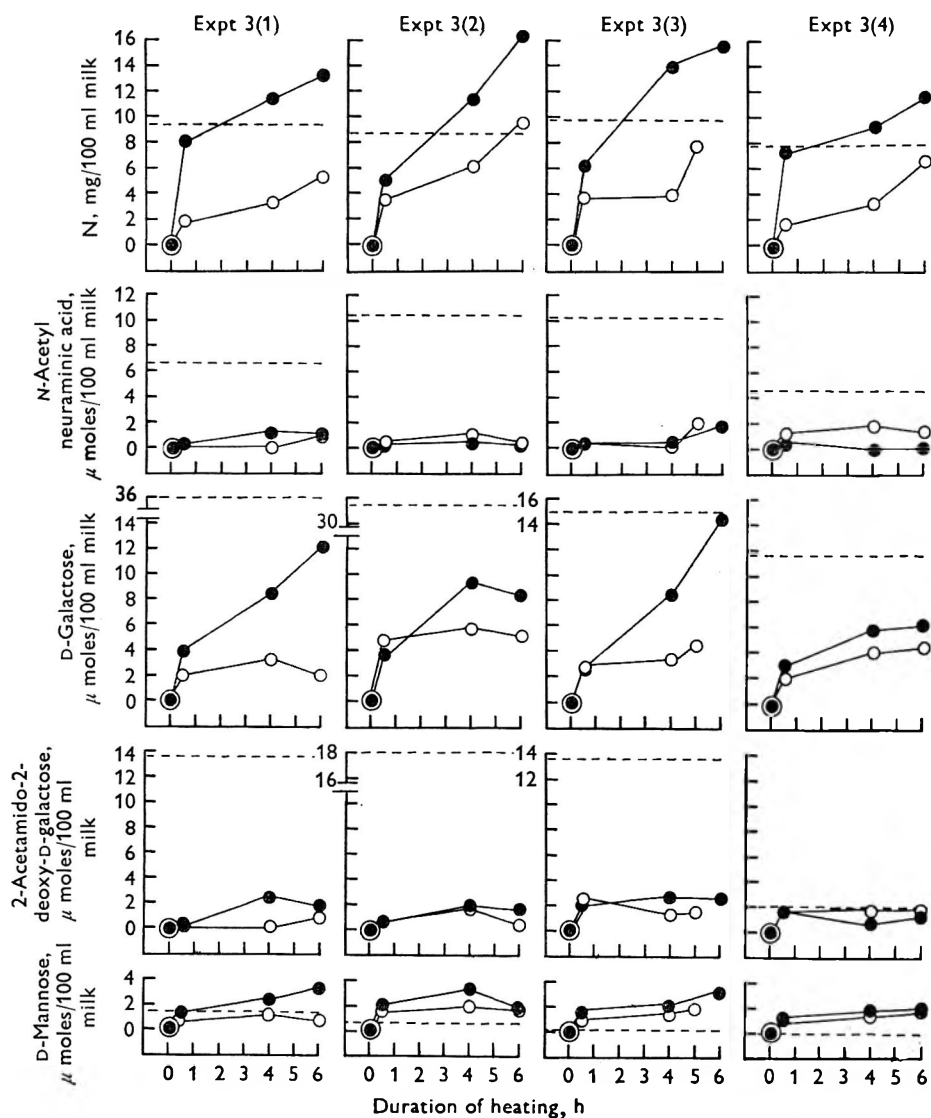
Fig. 3 (expt 3). The increase in N and in N-acetyl neuraminic acid, D-galactose, 2-acetamido-2-deoxy-D-galactose attached to the glycopeptides soluble in the 12 % trichloroacetic acid filtrate of whole milk after heat treatment. The dotted line indicates the corresponding values for the rennin treatment. $\bigcirc$, 80 °C; $\bullet$. 100 °C.

released by rennin action at some point during the 100 °C treatment. The results of expt 3 (Fig. 3) showed quite clearly that there are differences between milks in the amount and rate of release of peptides during heat treatment.

In all experiments at 80 and 100 °C there was an increase in the glycopeptides containing N-acetyl neuraminic acid, D-galactose, 2-acetamido-2-deoxy-D-galactose and D-mannose. In general, the amount of glycopeptides released increased with the temperature and duration of the heat treatment. There was some release of carbohydrate-containing peptides at temperatures as low as 50 and 60 °C (expt 1, Fig. 1; expt 2, Fig. 2) but the release of N-acetyl neuraminic acid-containing glycopeptides could not be detected at these temperatures in expt 1. For all the carbohydrate-containing peptides the general pattern was similar to that observed for N in that there was a rapid release during the first 30 min of all the heating periods. However, during the remainder of the heating period D-galactose- and D-mannose-containing glycopeptides continued to increase although at a slower rate, but there was little change in the glycopeptides containing N-acetyl neuraminic acid and 2-acetamido-2-deoxy-D-galactose.

With the exception of D-mannose, the amount of carbohydrates attached to glycopeptides released by heat was considerably less than those attached to the glycopeptides released by rennin action. The maximum release of N-acetyl neuraminic acid-containing glycopeptides was in expts 2 and 3 (4) which also had the least amount of N-acetyl neuraminic acid in the glycopeptides released by rennin. In expts 1, 3 (2) and 3 (4) the amount of N-acetyl neuraminic acid-containing glycopeptides released was apparently less during the 100 °C than during the 80 °C treatment.

Molar ratios of the carbohydrates attached to the glycopeptides released by heat treatment and by rennin action (Table 1)

The molar ratio is derived from 4 different experimental values and so a combination of experimental errors might result in ratios which are not entirely reliable for the shorter time intervals because the amount of glycopeptides released is comparatively small. Nevertheless the values do demonstrate certain trends. In general, the ratio of N-acetyl neuraminic acid: D-galactose is higher in the glycopeptides released at 80 °C than in those released at 100 °C and there is also a tendency for this ratio to decrease with duration of the heat treatment. A similar pattern was observed for the 2-acetamido-2-deoxy-D-galactose: D-galactose ratio. There was a clearly defined decrease in the ratios of D-mannose: D-galactose with duration of heating but the values were approximately the same for the glycopeptides released at 80 and 100 °C.

Expt 2 was the only experiment in which the molar ratios of the carbohydrates attached to the glycopeptides released by heating were similar to those of the glycopeptides released by rennin action. This was also the only experiment in which there was a substantial amount of D-mannose attached to the glycopeptides released by rennin. In all the other experiments the molar ratio of D-mannose: D-galactose in the glycopeptides released by rennin was close to zero whereas in the glycopeptides released by heating it varied from 0·2 to 0·5. The molar ratio of N-acetyl neuraminic acid: D-galactose in the glycopeptides released by heating was in general lower than in those released by rennin.

Table 1. *Molar ratios of the carbohydrates attached to the glycopeptides released by the action of heat and of rennin on whole milk*

Temperature of heat treatment		80 °C				100 °C			
Expt	Duration of heat treatment, h	N-acetyl neuraminic acid	D-Galactose	2-Acetamido-2-deoxy-D-galactose	D-Mannose	N-Acetyl neuraminic acid	D-Galactose	2-Acetamido-2-deoxy-D-galactose	D-Mannose
1	0·5	0	1·00	0·11	0·47	0·14	1·00	0·34	0·43
	2·0	0·39	1·00	0·37	0·39	0·12	1·00	0·44	0·38
	4·0	0·30	1·00	0·30	0·35	0·04	1·00	0·27	0·31
	8·0	0·06	1·00	0·31	0·44	0·08	1·00	0·11	0·25
	*	—	—	—	—	0·22	1·00	0·24	0
2	0·5	0·48	1·00	1·10	0·51	0·19	1·00	0·55	0·41
	2·0	0·46	1·00	1·34	0·51	0·16	1·00	0·47	0·42
	4·0	0·44	1·00	—	0·41	0·19	1·00	0·50	0·30
	8·0	0·24	1·00	1·03	0·23	0·06	1·00	0·26	0·18
	*	—	—	—	—	0·09	1·00	0·20	0·21
3 (1)	0·5	0·03	1·00	0	0·34	0·08	1·00	0·04	0·34
	4·0	0	1·00	0	0·33	0·14	1·00	0·31	0·29
	6·0	0·44	1·00	0·42	0·29	0·08	1·00	0·15	0·28
	*	—	—	—	—	0·18	1·00	0·38	0·04
3 (2)	0·5	0·10	1·00	0	0·30	0·07	1·00	0·17	0·57
	4·0	0·19	1·00	0·27	0·32	0·05	1·00	0·20	0·35
	6·0	0·07	1·00	0·08	0·31	0·04	1·00	0·23	0·22
	*	—	—	—	—	0·33	1·00	0·57	0·02
3 (3)	0·5	0	1·00	0·87	0·31	0·15	1·00	0·72	0·57
	4·0	0	1·00	0·38	0·43	0·06	1·00	0·31	0·26
	†	0·43	1·00	0·32	0·39	0·12	1·00	0·18	0·22
	*	—	—	—	—	0·69	1·00	0·91	0·01
3 (4)	0·5	0·63	1·00	0·81	0·45	0·15	1·00	0·53	0·41
	4·0	0·45	1·00	0·44	0·33	0	1·00	0·14	0·31
	6·0	0·32	1·00	0·41	0·39	0·03	1·00	0·22	0·33
	*	—	—	—	—	0·39	1·00	0·17	0

* Glycopeptides released by rennin.
† The 80 °C treatment was for 5 h and the 100 °C treatment for 6 h.

Ratio of carbohydrate:N in the glycopeptides released by heat treatment and by rennin action (Table 2)

On average, the ratio of carbohydrate:N was considerably lower in the glycopeptides released by heat treatment than in those released by rennin action. At 80 °C there was a tendency for all the carbohydrate:N ratios to decrease with the duration of the heat treatment. At 100 °C this tendency was not as marked except with N-acetyl neuraminic acid:N. The ratio of D-galactose:N remained about constant or showed a progressive increase and the D-mannose:N remained about constant in each experiment.

DISCUSSION

It is now well known that when milk is heated there is an increase in the non-protein N fraction of the milk (see e.g. Howat & Wright, 1934). Non-protein N is

released from isolated α_s-, κ- and β-caseins when subjected to temperatures of 100 °C or higher (Yoshino, Samuro, Yamauchi & Tsugo, 1964) but there is no evidence that the non-protein N released when whole milk is heated is derived from the caseins. In an attempt to obtain some information on the non-protein N when milk is heated we have analysed the carbohydrates attached to the peptides released, in addition

Table 2. *The ratios of the carbohydrates to N in the glycopeptides released by the action of heat and of rennin on whole milk*

	Temperature of heat treatment	80 °C				100 °C			
Expt	Duration of heat treatment, h	N-Acetyl neuraminic acid	D-Galactose	2-Acetamido-2-deoxy-D-galactose	D-Mannose	N-Acetyl neuraminic acid	D-Galactose	2-Acetamido-2-deoxy-D-galactose	D-Mannose
1	0·5	0·02	0·47	0·06	0·23	0·06	0·47	0·16	0·20
	2·0	0·21	0·57	0·20	0·21	0·06	0·48	0·21	0·18
	4·0	0·21	0·69	0·21	0·25	0·01	0·33	0·10	0·10
	8·0	0·04	0·50	0·16	0·22	0·04	0·47	0·06	0·12
	*	—	—	—	—	0·24	1·11	0·26	0
2	0·5	0·50	1·04	1·14	0·53	0·29	1·57	0·87	0·65
	2·0	0·13	0·28	0·37	0·14	0·11	0·68	0·32	0·29
	4·0	0·11	0·24	—	0·10	0·11	0·59	0·30	0·18
	8·0	0·09	0·37	0·38	0·09	0·04	0·77	0·20	0·14
	*	—	—	—	—	0·28	2·98	0·58	0·62
3 (1)	0·5	0·03	1·06	0	0·36	0·04	0·48	0·02	0·16
	4·0	0	1·02	0	0·34	0·11	0·73	0·23	0·21
	6·0	0·17	0·38	0·16	0·11	0·07	0·91	0·14	0·25
	*	—	—	—	—	0·70	3·84	1·45	0·14
3 (2)	0·5	0·13	1·36	0	0·42	0·05	0·68	0·11	0·38
	4·0	0·18	0·95	0·25	0·30	0·04	0·82	0·16	0·29
	6·0	0·04	0·54	0·04	0·17	0·02	0·50	0·12	0·11
	*	—	—	—	—	1·19	3·61	2·07	0·07
3 (3)	0·5	0	0·78	0·68	0·25	0·07	0·44	0·32	0·25
	4·0	0	0·88	0·34	0·38	0·04	0·60	0·19	0·16
	†	0·29	0·68	0·22	0·27	0·11	0·94	0·17	0·20
	*	—	—	—	—	1·05	1·52	1·38	0·01
3 (4)	0·5	0·74	1·18	0·96	0·53	0·06	0·42	0·23	0·17
	4·0	0·55	1·12	0·54	0·40	0	0·62	0·09	0·19
	6·0	0·21	0·65	0·27	0·25	0·02	0·53	0·12	0·18
	*	—	—	—	—	0·58	1·48	0·26	0

* Glycopeptides released by rennin.
† The 80 °C treatment was for 5 h and the 100 °C treatment for 6 h.

to estimating the total increase in peptides soluble in 12 % TCA. Our particular object was to see if the glycopeptides released by heat treatment were similar to those which are released from κ-casein by rennin action on whole milk. A comparison of the glycopeptides released by heat with those released by rennin shows that N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose were invariably attached to the glycopeptides released by rennin action, as was expected

from previous work (Alais & Jollès, 1961; Wheelock & Sinkinson, 1969). The molar ratios of these carbohydrates are in general agreement with results of other workers who obtained 1:1:1 (Jollès, Alais, Adam, Delfour & Jollès, 1964), 1:2:1 (Kuwata, Niki & Arima, 1969), and 2:2:1 (McCabe, 1967) for N-acetyl neuraminic acid:D-galactose:2-acetamido-2-deoxy-D-galactose on the κ-casein glycopeptides. The presence of D-mannose on these glycopeptides in expt 2 is in agreement with results obtained by Sinkinson & Wheelock (1970b). D-Mannose would not be detected by the colorimetric methods normally used for these analyses as they do not distinguish between different hexoses and total hexose is therefore assumed to consist entirely of D-galactose. These 4 carbohydrates were invariably attached to the glycopeptides released by heat treatment. The concentration of N-acetyl neuraminic acid, D-galactose and 2-acetamido-2-deoxy-D-galactose on the glycopeptides released by rennin is much higher than on the glycopeptides released by heat.

These findings show quite clearly that the glycopeptides and peptides released by rennin action are not the same as those released by heat treatment but do not eliminate the possibility that some of the peptides released by heat are derived from κ-casein.

The question arises whether the glycopeptides released by heat may have originated from a milk protein other than κ-casein. If the globulins in milk are similar to those in blood then they would probably contain 2-acetamido-2-deoxy-D-glucose (Rothfus & Smith, 1963; Dawson & Clamp, 1968; Spragg & Clamp, 1969). This carbohydrate was not detected on the glycopeptides released by heat treatment and so these proteins may be tentatively ruled out as the source of the glycopeptides. Although there are no reports of carbohydrates being attached to β-lactoglobulin or α-lactalbumin in the milk of Friesian cows (see McKenzie, 1967) it is possible that the glycopeptides could have originated from either or both these proteins if they do contain carbohydrates. N-acetyl neuraminic acid, D-galactose and hexosamine have been detected in α_s-casein (Reynolds, Henneberry & Baker, 1959) but their presence could have resulted from κ-casein contamination. Recently J. V. Wheelock & G. Sinkinson (unpublished) have shown that there are no carbohydrates attached to the residual α_s- and β-caseins left after the preparation of κ-casein by the gel filtration of acid casein (Yaguchi, Davies & Kim, 1968). Therefore, if the glycopeptides originate from the caseins, κ-casein must be the only source.

In our opinion there is a strong possibility that some of the glycopeptides released by heat originate from κ-casein. This is because the glycopeptides are soluble in 12 % TCA and with the exception of D-mannose, the carbohydrates attached to the glycopeptides released by heat treatment are qualitatively similar to those attached to the glycopeptides released by rennin action. In the present state of knowledge of carbohydrates attached to proteins in milk, there does not appear to be any other protein from which the glycopeptides released by heat could be derived.

There is now evidence that carbohydrates are attached to para-κ-casein (McCabe, 1967) and these have been identified by J. V. Wheelock & G. Sinkinson (unpublished) as 2-acetamido-2-deoxy-D-galactose, D-galactose and D-mannose. N-acetyl neuraminic acid has not been detected on para-κ-casein. The presence of D-mannose in the glycopeptides released by heat would be explained if κ-casein is split at a different peptide linkage to that split by rennin action so that the para-κ-casein carbohydrates

are included in the glycopeptides released. As there is little or no N-acetyl neuraminic acid attached to the para-κ-casein the ratio of N-acetyl neuraminic acid: N would therefore be lower in the glycopeptides released by heat than in those released by rennin action as observed in our experiments. This would not rule out the possibility of some release of free N-acetyl neuraminic acid as evidenced by the decrease in the ratio of N-acetyl neuraminic acid: D-galactose on the glycopeptides with increasing severity of the heat treatment (see also de Koning, Jenness & Wijnand, 1963). The results for expt 2 in which the carbohydrates on the glycopeptides released by heat are quantitatively similar to those released by rennin are quite consistent with this explanation as in this experiment para-κ-casein could be devoid of carbohydrates.

The low concentration of carbohydrates on the peptides released by heat as compared with those released by rennin action could be explained if some other peptides which completely lack carbohydrates are released from other milk proteins in addition to the glycopeptides.

The release of glycopeptides at temperatures as low as 50 °C is of some interest and suggests that there may be a release of glycopeptides during storage of heat-sterilized milk or milk products over a long period. If the glycopeptides are released from κ-casein then the degree of stabilization of the casein micelles would be reduced. This could provide an explanation of gelation during storage of milk and milk products which have been subjected to heat-sterilization temperatures (Burton, 1969; Schmidt, 1968).

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Low resolution nuclear magnetic resonance in the determination of moisture in cheese curd

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SUMMARY. Nuclear magnetic resonance was used to follow the decrease in moisture in curd cooled to solidify the fat as whey was exuded. The results did not correlate closely with those of moisture determinations by drying at 98 °C partly because of the relaxing effect of the solid curd structure when in close proximity to the whey. This effect was diminished as the whey left the curd, so that whey out of the curd was more readily saturated with radio-frequency energy and therefore absorbed less energy than whey in the curd when high intensity fields were used. The saturation effect could also be used to follow approximately the exudation of the whey.

In cheese-making it is essential to control the moisture, i.e. whey, content of the curd in relation to the rate of development of acidity. This is done by applying heat according to the cheesemaker's assessment of the curd moisture and he makes this assessment by feeling the firmness of the curd. The daily exercise of this art produces the requisite skill in those who have aptitude, but a rapid instrumental determination would have obvious advantages, which will be even more important when continuous methods of production are adopted. Nuclear magnetic resonance (NMR) affords a practically instantaneous determination of liquid content in certain mixtures, and of the moisture content of some foods, as shown, for example, by Shaw & Elsken (1955) and by Shaw, Elsken & Lundin (1960). It was therefore considered desirable to examine its possibilities in cheese-making.

METHODS

Determination of nuclear magnetic resonance signal

The Newport Quantity Analyser (Newport Instruments Ltd, Newport Pagnell, Bucks.) was used. It is designed to measure the absorption of radio-frequency (RF) energy by the nuclei of hydrogen atoms in compounds in the liquid condition. Thus, the meter reading per unit weight of any given hydrogen-containing compound or mixture can be used to determine the proportion of liquid phase by comparison with a suitable standard. The technique of using the instrument is adequately described

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in the manufacturer's literature, but a brief description of the operations required will serve to indicate the procedure.

The setting up of the instrument for a series of tests involved adjusting (1) the RF current level (40–500 μA), (2) RF tuning and 'gate' adjustment to obtain a satisfactory trace in the oscilloscope, and (3) the audio frequency gain to give the required meter reading with a standard (usually 100 with 100 % 'relaxed' water, the water being relaxed by adding 1·25 mg $MnCl_2.4H_2O$/ml). The term 'relaxed' as used here is fully explained on p. 414. For a series of similar samples containing less than 100 % water, no further adjustments were required. The samples were inserted into the coil former and 3 meter readings taken. These usually differed by < 1 % full scale deflexion. The mean was used in the calculations. The meter could be used either with a long (8 s) time constant or with a short one at the press of a button. The short-time circuit was operated to bring the meter quickly to the approximate value and the long-time circuit for diminishing the effect of 'noise'. Even so noise was sometimes troublesome and the use of a digital volt meter to integrate the signal for 20 s or more is recommended.

The signal obtained on the NMR meter is reduced when so-called unrelaxed water is present. This is due to saturation and it is overcome to some extent by the use of supplementary modulation, see p. 415. (Saturation and supplementary modulation are defined and considered in more detail on pp. 414 and 415.)

The reading of the NMR instrument depended on the size of the sample; the larger sample gave the larger reading, provided the whole sample was not too big to be totally within the volume of the homogeneous part of the RF and magnetic fields. The optimum sample size was obtained by using Nessler cylinders as containers and confining the sample to within 40 ml with the aid of special PTFE disks supplied. The weight or volume of the sample was always determined, and used to express the results as meter reading per g or per ml of sample. Comparisons on a weight basis were necessary when curd particles with air between them constituted the sample. To make the best use of the meter the instrument was often adjusted to give full scale deflexion with less than 100 % water. Thus, comparisons within sets of results are valid, but not comparisons between sets.

Curd samples

Ordinary curd was obtained from vats in the Experimental Dairy of this Institute during the course of normal cheese-making. As curd continues to exude whey, care was taken to make the whole sample as homogeneous as possible before removing any portion of it. When soft enough the curd was mashed until it was like porridge. Tougher curd was cut into fragments 2–3 mm across.

The curds used for the results reported in Tables 2–7 were made directly from milk + rennet in the Nessler cylinders used with the NMR instrument. The NMR signal is not very sensitive to temperature so that when curd-making was carried out at 30 °C (the cylinders being kept in a water bath) it was a sufficient precaution to leave the cylinders in the instrument for the minimum time only (say about 20 s) and then to return them to the water bath immediately. Samples which had been cooled in ice were treated similarly. It was essential to dry the outside of the Nessler cylinder

by wiping it rapidly with a dry cloth before insertion in the coil former of the instrument.

Determination of curd moisture

Approximately 10 g of purified sand were weighed into a stainless steel Petri dish lid together with a short glass stirring rod having a widened short end and a hooked handle to rest over the edge of the dish. The dish, glass rod and sand were placed in a drying oven at 99 ± 1 °C for at least 1 h. This had been found sufficient to ensure dryness. After cooling in a desiccator for 45 min the dish and its contents were weighed. Approximately 5 g of the curd sample was then accurately weighed into the dish and the glass rod was used to grind and mix the curd with the sand. The whole was then replaced in the drying oven for at least 4 h or overnight, as previous tests showed that these different times gave practically the same result. The dish and contents were then cooled in the desiccator again for 45 min and reweighed. The loss in weight was expressed as a percentage of the weight of the moist curd and is regarded as the moisture content. Determinations were made in duplicate.

RESULTS

The need for cooling

Since the low resolution NMR instrument responds to any liquid molecules containing hydrogen it was anticipated that in order to measure the water content of curd it would be necessary to solidify the fat by cooling. Table 1 confirms that

Table 1. *Lack of correlation between nuclear magnetic resonance (NMR) signal and moisture content*

Moisture content of curd, %	NMR signal, units/g at	
	30 °C	37 °C
95·1	2·10	1·83
85·6	2·14	1·87
74·2	2·20	1·98
55·2	2·24	2·16
39·0	2·15	2·18

measurements at vat temperature were useless. The almost constant level of NMR signal/g as moisture content decreased was probably due to the concomitant increase in fat content per unit of total weight.

When the fat was solidified by keeping the curd sample in ice for 1 h there was a steady decrease in the NMR signal as the moisture content decreased. Cooling periods shorter than 1 h tended to give erratic results, and longer periods (up to 3 days) gave no improvement over the results at 1 h.

It was considered possible that some of the fat globules might remain as a supercooled liquid at 0 °C. Two different curd samples were therefore cooled to -70 °C (at which temperature the NMR signal was zero), and then allowed to warm up to $+3$ °C. After determining the NMR signals the samples were heated to 50 °C and then again cooled to $+3$ °C. The NMR signals were virtually unchanged, showing that 3 °C was a low enough temperature to solidify all the fat.

Correlation between NMR readings and moisture content

In spite of the encouraging results reported above, repeated determinations with different curd samples on different days failed to give a constant relation between NMR signal per unit weight and moisture content. The amount of scatter is shown in Fig. 1, which represents the results obtained with curds from 6 different makings of Cheddar cheese.
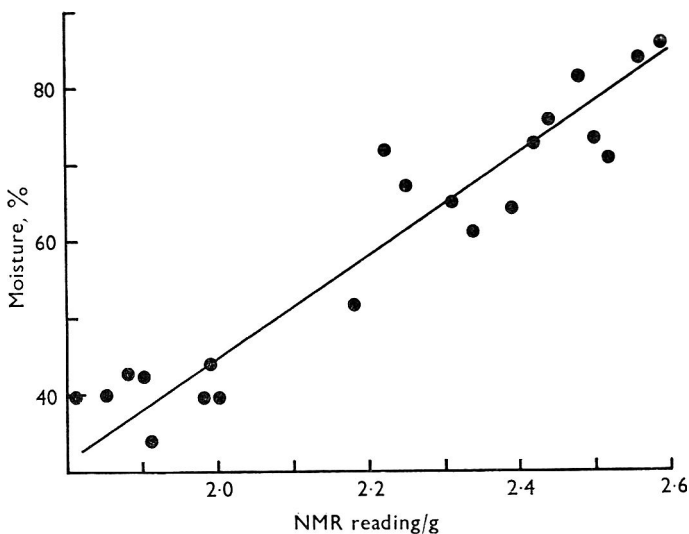
Fig. 1. Correlation between nuclear magnetic reasonance reading/g of curd after cooling in ice for 1 h and moisture content percentage.

Saturation effects in presence of whey

After considering possible sources of the scatter seen in Fig. 1 we felt it necessary to test the assumption that the same NMR signal would be given by free whey as by the same quantity of whey in curd. It was already known that the presence of particulate matter in water can reduce the saturation effect, and so produce, under certain conditions, a higher signal at high energy input than would be given by the same quantity of water in bulk (see Discussion). If this happens also in curd a gradual decrease in NMR signal is to be expected as whey exudes from curd when a high energy input is used. Table 2 shows that this decrease did occur as whey exuded from the cut curd. Separated milk was used to avoid possible complications due to fat. Supplementary modulation was accidentally retained, but did not completely mask the changes. While some decrease occurred with $200 \mu A$, a larger decrease was observed at $500 \mu A$, confirming that bulk whey is comparatively unrelaxed.

The results of an experiment to confirm the decrease in signal at high RF levels in curds and whey from full-cream milk as whey separates are recorded in Table 3. Sample 1 showed no apparent decrease in readings before scald whereas samples 2 and 3 fell by the amount expected from previous tests. The samples were as nearly identical as possible. They were from the same bulk of milk and the same quantity of rennet was used. Treatment was as constant as possible. Samples 1 and 2 were run concurrently, sample 2 being 5 min behind sample 1. Sample 3 was run about 60 min

later. The difference between sample 1 and samples 2 and 3 is possibly due to the difficulty of taking an accurate reading from a noisy signal (see Discussion). On the whole, however, Table 3 confirms the decrease due to whey separation.

Table 2. *Effect of exudation of whey from curd on nuclear magnetic resonance signal, using separated milk*

RF levels (with supplementary modulation)	Meter units/ml	
	200 μA	500 μA
Measured:		
After renneting	2·48	3·45
At firm clot	2·48	3·38
Immediately after cutting	2·43	3·30
After cutting, min: 5	2·32	3·14
10	2·26	2·95
15	2·23	2·92
25	2·21	2·73
40	2·17	2·69
55	2·15	2·67
100	2·16	2·46

Table 3. *Effect of exudation of whey from curd on nuclear magnetic resonance (NMR) signal/ml using whole milk (RF level 500 μA; no supplementary modulation)*

	NMR signal/ml		
	Sample 1	Sample 2	Sample 3
Measured:			
After renneting	1·46	1·43	1·49
At firm clot	0·96	0·89	0·81
After cutting, min: 1	1·46	1·41	1·46
5	1·45	1·31	1·38
20	1·45	1·24	1·29
After scald	1·24	1·13	1·15

Table 4. *The increasing saturation effect as whey exudes from curd and the absence of saturation at a low RF level with supplementary modulation, meter units/ml*

	RF levels			
	50 μA with supplementary modulation		500 μA without supplementary modulation	
Date	30. vi. 69	1. vii. 69	30. vi. 69	1. vii. 69
Measured:				
After renneting	2·44	2·36	1·40	1·40
After cutting, min: 5	2·40	2·36	1·32	1·20
15	2·39	2·40	1·29	1·13
30	2·37	2·36	1·20	1·10
After scald	2·40	2·36	1·17	1·00

It should be noted that in Table 2 no difference is indicated between the readings for renneted milk and a firm clot, whereas Table 3 shows a large decrease when the firm clot is formed. A number of repetitions failed to confirm this decrease.

When a low energy RF current was used, e.g. 50 μA, there was virtually no change in the signal (Table 4). Whole milk was used for this experiment also.

Effect of different concentrations of rennet

The addition of different concentrations of rennet to the milk had no effect on the NMR results; concentrations of 1:400, 1:4000 and 1:8000 parts rennet to parts of milk were used (Table 5).

Table 5. *Effect of different strengths of rennet and of different cutting on nuclear magnetic resonance signal/ml (RF level 500 μA ; no supplementary modulation)*

Rennet concentrations	1:400	1:8000	1:4000 CEV*	1:4000 CLG†
Measured:				
After renneting	1·36	1·40	1·36	1·39
After cutting, min: 5	1·27	1·27	1·27	1·20
15	1·18	1·19	1·18	1·15
After scald	1·09	1·06	1·06	1·03

* CEV = cutting curd early and vigorously.
† CLG = cutting curd late and gently.

Effect of different treatments of curd

If the curd was cut normally and stirred gently when it was firm (firming time allowed was twice the clotting time) the readings were slightly lower than if the curd was cut finely and stirred vigorously when it was soft (finishing time allowed was the same as the clotting time). The latter treatment produced many small curd particles that would be expected to induce relaxation of the whey near their surfaces and would thus cause more relaxation than the few larger pieces of curd produced by gentle cutting. On the other hand, the slower exudation of whey after gentle cutting would produce an opposite trend. Thus, the overall difference was small.

Effect of adding extra whey

The determination of free whey in a mixture of curds and whey is difficult because the rate of separation of the whey is sensitive to mechanical disturbance. Consequently, an independent determination of the volume of free whey was not attempted in the experiments described just above. Some quantitative results were, however, attempted by adding extra whey to mixtures of curds and whey at different stages during the shrinkage of the curd. The whey was prepared from the same milk just before the experiment.

In the first experiment of this type, 5, 10, 15 and 20 ml of whey were added to different identical 20-ml samples of curds and whey 1 min after cutting, and the NMR signals of the mixtures determined 5 and 20 min after cutting and also after scalding, using 500 μA radio frequency current and no supplementary modulation. The signals decreased during whey separation as anticipated from earlier results but the individual decrements due to the 5 ml increments of free whey were too small to be accurately measured. The mean value over the whole experiment for the addition of 5 ml of whey was a decrease in meter reading of 0·04 ml.

It was felt necessary to confirm the assumption that the change in signal on the addition of whey would be constant at different stages during syneresis. Four identical samples of 20 ml of curds and whey were therefore processed, one being a control. Whey (10 ml) was added to the second about 1 min after cutting, to the third

6–7 min after cutting, and to the fourth just before scalding. The NMR results are shown in Table 6 and differences from the control are given in Table 7. Samples below the lines contained the added whey. It can be seen that as the experiment progressed there was a slight downward drift in the results for the samples, which differed from the control only in being processed slightly later. The average downward drift per reading was 0·05 while the average decrease in reading due to 10 ml of extra whey was 0·13. The difference between these is 0·08, which compares well with the previous value of 0·04 for 5 ml of whey, and the results suggest that the effect of added whey is not dependent on the stage of the process at which it is added.

Table 6. *Effect of adding* 10 *ml whey to* 20 *ml curds-and-whey on nuclear magnetic resonance signals/ml*

	Sample no.			
	1	2	3	4
Measured:				
After renneting	1·60	1·60	1·53	1·48
After cutting, min: 1		W*		
5	1·30	1·20	1·23	1·30
6–7			W*	
20	1·28	1·12	1··5	1·23
25–30				W*
After scald, cutting + 30 min	1·20	1·09	1·09	1·04

W* 10 ml of whey added to sample at time shown in left-hand column.

Table 7. *Differences between sample* 1 *and samples* 2, 3 *and* 4 *of Table* 6. *Values below the lines refer to measurements taken after the addition of whey*

	Samples		
	1–2	1–3	1–4
Measured:			
After renneting	0·00	0·07	0·12
After cutting, min: 5	0·10	0·07	0·00
20	0·16	0·13	0·05
After scald	0·11	0·11	0·16

DISCUSSION

A disquieting feature of the results for curd samples taken at different stages throughout the shrinkage of curd during cheese-making on different days was the poor correlation between moisture content and NMR readings (Fig. 1).

A possible source of error in these results was the treatment of the curd after sampling. Curd continues to exude whey at a rate which depends on its temperature and on the physical and mechanical treatment it receives. This means that curds prepared on different days, and which might have been identical at sampling, could have contained different proportions of free whey at the time of measurement. It was assumed at the time that the same NMR signal would be generated by a given quantity of water whether it was free or within the (assumed) pores of the curd. Therefore, no attempt was made to keep the treatment of curd samples uniform. They were finely cut and mixed as convenient (within 20 min), then a subsample was

taken into the Nessler cylinder and cooled in ice as already described. It was sub-sequently found, however, that the NMR signal from free whey was smaller than that from whey still in the curd when measured in the Quantity Analyser. This was due to RF saturation effects resulting from different degrees of relaxation, which are explicable as follows.

<div align="center">

Relaxation and saturation effects

</div>

When protons are placed in a steady magnetic field, there are 2 energy states in which they can exist, one being higher than the other. By applying a radio-frequency field of the right frequency at right angles to the main magnetic field, protons in the low energy state may be excited by resonance into the high energy condition. When this occurs, energy is withdrawn from the RF coil, and it is this absorption of energy that is detected and amplified as the signal in the Quantity Analyser. Thus, the size of the signal is proportional to the number of protons in the low energy state, and this is, in turn, proportional to the absolute temperature and the total number of protons present in the sample. Once a proton has been excited to the high energy level, it must shed this quantum of energy it has obtained, and return to the low energy state before it can be detected again.

A frequent repetition of this process thus leads to a steady loss from the RF coil which can then be translated into a steady electrical current. If, however, there is some delay in the shedding of energy by the excess of high energy protons the re-peated application of resonant RF energy causes the population at the 2 energy states to become permanently altered, i.e. the higher energy state becomes more populated, and the low energy state less populated. As a result the number of protons being excited upwards diminishes, and hence the signal decreases. These conditions are therefore brought about when the time between resonance conditions being set up is short compared to the time it takes the nuclei to shed their excess energy.

Now a proton may shed this energy in 2 ways. The excited protons may be con-sidered to be at a higher temperature than their surroundings (the lattice) and hence a thermal equilibration process is set up whereby the excited protons shed heat to the surroundings, raising the lattice temperature a minute amount. This is brought about by normal thermal collisions in the liquid state and it has an exponential time factor. This is the spin-lattice relaxation time, t_1. The proton may also shed its energy by spin-coupling with adjacent nuclei in the lattice. This mode of energy dissipation is of more importance when adjacent nuclei are fixed with respect to one another, and hence experience a local field which is several gauss different from the applied field. In this case the energy levels are broadened and energy is passed from one proton to another by in-phase coupling of the magnetic spins. The lifetime or 'phase-memory time' of the nuclear spin state is the spin–spin relaxation time, t_2. It is equal to the inverse of the line-width of the NMR absorption.

Now the magnitude of the signal in the Quantity Analyser (S) is given by

$$S = \frac{kNH_1}{T\,(1+\gamma^2 H_1^2 t_1 t_2)},$$

where

N = number of nuclei/cm³,

H_1 = value of RF field = 40×10^{-3} G,

T = absolute temperature,

γ = gyromagnetic ratio of the nucleus,

k = a constant.

Thus, for any one sample at constant RF level and constant temperature

$$S = \frac{K}{(1+\gamma^2 H_1^2 t_1 t_2)^{\frac{1}{2}}}.$$

Now γ for the proton is $4 \cdot 2559 \times 10^3 \, Hz/G$ and as the H_1 field is 40×10^{-3} G

$$\gamma^2 H_1^2 = 28980 \cdot 0.$$

On the assumption that the proximity of protein and fat molecules to the water molecules in unshrunk curd reduces the frequency of normal thermal collisions in the liquid state, a rather long spin-lattice relaxation time may be anticipated (t_1 = approx. 1 s). On the other hand, the varying magnetic fields next to protein (and fat) molecules would facilitate the dissipation of energy by spin–spin coupling, shortening this component of the relaxation time (t_2 = approx. 10^{-3} s).

Putting these values of t_1 and t_2 in the equation for the magnitude of the NMR signal we obtain

$$S = \frac{K}{(1+28980.10^{-3})^{\frac{1}{2}}} = 0 \cdot 18264 \, K.$$

With the separation of the water from the curd t_1 would be shortened and t_2 lengthened. Assuming both approximate to 10^{-1} s we obtain

$$S = \frac{K}{(1+28980.10^{-2})^{\frac{1}{2}}} = 0 \cdot 05864 \, K.$$

Hence, it can be seen that the decrease in the NMR signal from a sample of curds and whey as the whey exudes from curd could be of the order of 60 %. This, however, assumes that all the whey comes out of the curd, whereas 50 % may be retained in the wettest samples. The actual measurements taken indicate the signal to fall by 20–30 % in 30 min after cutting. Thus, there is quite good agreement between calculated and observed values.

RF saturation is worse at higher RF levels as more energy is being offered to the sample and more protons are being excited at each resonance; thus, the decrease in signal as whey exudes from the curd is likely to be highest at high RF levels. This was found to be so (see Tables 2, 3).

Similar observations have been used by Hall, Lawrence & Simpson (1967) to follow the rehydration of dried materials.

Supplementary modulation

Supplementary modulation is an electronic technique (devised by Newport Instruments Ltd) which recovers some of the signal lost in RF saturation. Saturation occurs when all the variables have settled into steady conditions, and supplementary modulation achieves its purpose by varying the time interval between the setting

up of resonance conditions in the sample. Thus, by continuously changing this time variable some signal is regained. Hence, one would expect the signal decrease in a sample of curds and whey to be smaller when supplementary modulation is used. This was confirmed by experience.

Reproducibility

Although meter readings with a standard mixture containing manganese chloride were of adequate reproducibility it was sometimes difficult to obtain reproducible results with curds and whey. It seems likely that a digital volt-meter which enabled the signal to be integrated over a longer period would have given more reproducible results.

General

The results suggest that with suitable precautions it should be possible to determine moisture content in separated cooled curd by NMR. It would, however, be advantageous to be able to carry out the determination without first separating the curds from the whey, especially since the exudation of whey is accelerated as soon as curd is compressed by its own weight instead of being supported in the whey. In this respect the measurement of saturation effects seemed promising. However, the results depended to some extent on the amount of settling of the curd during the measurements. Attempts to keep the curd mixed by inverting the sample tube a few seconds before the measurement led to very erratic results.

A sampling difficulty also appeared during the course of the work. If the whey is to be drained from the curd a sample may be taken from the vat with a perforated vessel in the normal manner, but a representative sample of curds and whey cannot be obtained merely by dipping because the normal stirring is only just sufficient to prevent permanent settling of the curd, and the concentration of curd particles is different in different parts of the vat. More rapid stirring incurs the risk of breaking the curd particles.

An examination of the detailed results leaves one with the impression that there is a gap in our knowledge of the changes going on in curd which could influence the NMR signal. For example, there is the temporary change which sometimes occurs when a firm clot is first formed. There are also sometimes differences between the readings for a curd sample after 1 h at 0 °C and 1 or 2 days at 0–4 °C. These differences are considerably greater than the uncertainties in determining the NMR signal. Sometimes, however, they do not occur. They could represent the sum of a number of unrecognized errors or they could be the result of phenomena not yet understood.

With reference to practical cheese-making it seems that low-resolution NMR offers promise as a method for moisture determination in cheese curd but that further study is required before it can be recommended.

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The influence of various treatments on the drainage of continuously made curd

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SUMMARY. The effect of layer thickness and orientation, and of pressure, temperature, and disturbance on the drainage rates of continuously made curd were determined. The effects of heat treatment of the milk and adjustments of its pH were briefly examined. An increase in temperature of the curd caused the expected increase in drainage rate as did also the application of pressure. Vertical layers of curd drained more quickly than almost horizontal layers, and keeping curd in whey retarded its drainage. The effects of pH change over the narrow range of interest (6·1–6·5) were relatively small. Pasteurization of the milk had no effect. Disturbance of the curd had the most pronounced effect in increasing its rate of drainage.

In the manufacture of cheese from continuously made curd it will be advantageous to achieve the maximum rate of syneresis or drainage in order to obtain the maximum output from the equipment, provided this can be done without a deleterious effect on the quality of the cheese. Experiments already reported (Berridge & Scurlock, 1969) have indicated that there is no need to wait for starter development at intermediate stages as is done in the usual process but that all the starter growth can take place in the finished (but not salted) curd. It follows that conditions (other than excessively high temperatures) should be chosen to secure the quickest removal of whey. One of these is the immediate separation of whey from the newly formed curd. The experiments now reported were carried out to define some of the other conditions necessary for the most rapid drainage.

There is also need for more fundamental knowledge of the processes of curd formation and shrinkage and the experiments were planned in the hope of obtaining such knowledge. It was felt for example that as continuously produced curd does not need to be cut and as it can be allowed to commence drainage in air almost as soon as it is formed it might, therefore, drain in a more reproducible manner than curd formed by the usual method. The results, however, indicated that continuously made curd was very sensitive to the conditions prevailing during its formation. These conditions presumably include rates of flow, temperature differences across the membrane and composition of the heating fluid, and it is still not possible to decide exactly what these conditions should be. Nevertheless, the results serve to show the drainage rates which may be expected when some of the more easily controlled conditions are imposed.

METHODS

Continuous curd-making was carried out in the single membrane apparatus already described (Berridge, 1968), and determinations of drainage rate were made by weighing the curd in a large balance case maintained at a constant temperature and at
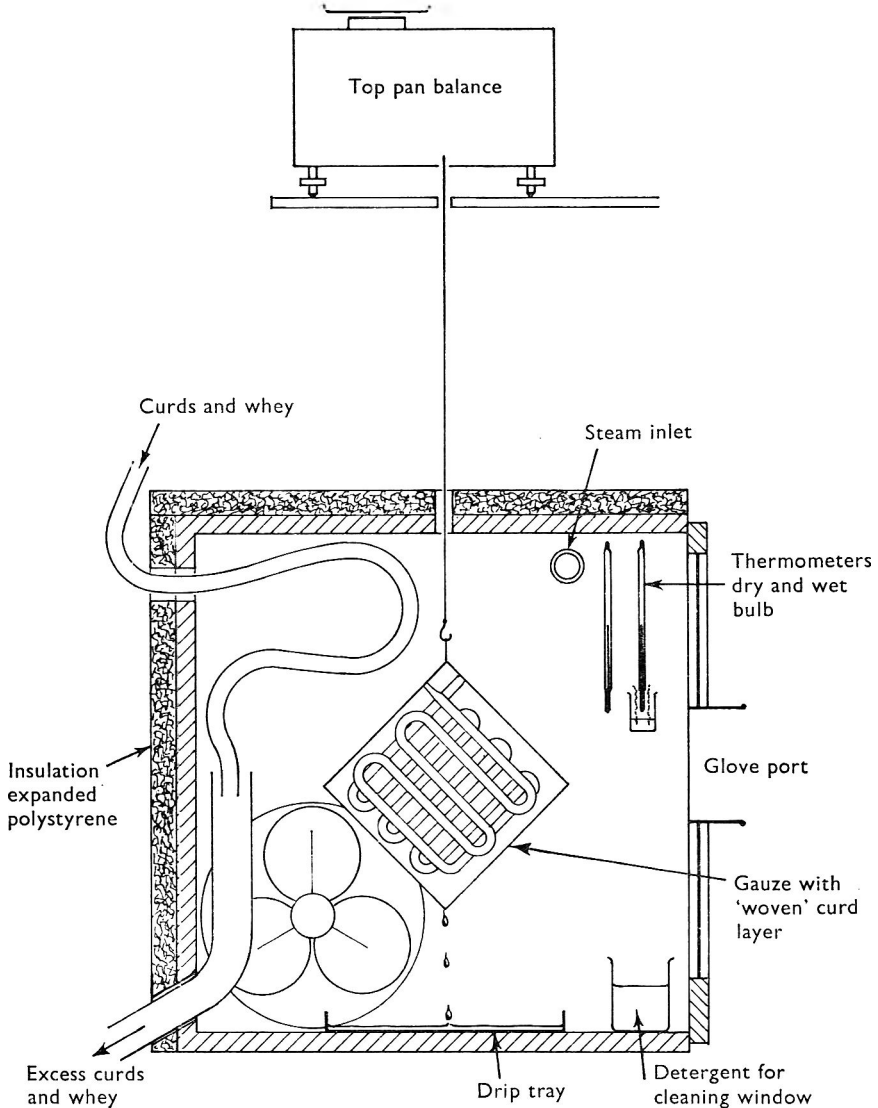
Fig. 1. Apparatus for measuring drainage of curd. (Thermostat omitted.)

5–100 % relative humidity. The curd was led through a PVC tube of the same nternal diameter as the membrane into the balance case which contained a 500 W heater and was thermostatically controlled. The case contained also a fan, an inlet for steam, wet and dry bulb thermometers, a drip tray, an outlet tube for unwanted curd, a container of detergent and a stainless steel gauze. The arrangement of the

apparatus is shown in Fig. 1. The 2 glove ports, of which only one is shown in the figure, were fitted with rubber gauntlets so that manipulation could be made without opening the case. The atmosphere within the case was kept as near as possible to a relative humidity of 95% to avoid 2 sources of error. The first—cooling of the curd by evaporation—was shown in preliminary experiments to be quite large. The second source of error was a loss of moisture by evaporation. The humidity was maintained by passing steam into the case from an electrically heated flask controlled with a 'Sun-vic' energy regulator. It was easy to set the regulator so that sufficient steam entered the cabinet to keep the reading of the wet-bulb thermometer not more than 1 deg C below that of the dry bulb. Detergent was used to clear the windows of droplets whenever condensation became troublesome.

Each experiment was begun with the curd-maker functioning continuously at steady levels of rate and temperature. (These levels were normally 80 ml of curd and whey/min at 48 °C.) The end of the PVC inlet tube was kept in the wider outlet tube until needed so that unwanted curd flowed continuously out of the balance case. When the temperature and humidity had remained at the required values long enough to show that a steady state had been reached the curd layer was formed on the gauze. This was a skilled operation that required some practice. The gauze was unhooked from the suspension wire and held horizontally in the left hand, while the end of the curd inlet tube was brought with the right hand to a position a few millimetres above the gauze and then passed backwards and forwards with a weaving motion equal in linear speed to the rate of exit of the curd (5 cm/s), so as to form a zig-zag pattern of straight strips of curd spaced from one another by a distance equal to their own width. This was continued for 30 s. The direction of 'weaving' was then changed by 90° and continued for the same length of time to produce a criss-cross pattern. The gauze was maintained horizontal for a further minute to ensure adhesion of the curd and then hung in its drainage position, usually vertical, on the suspension wire. Time and weight readings were taken at approximately minute or half-minute intervals with the fan switched off. At the end of the drainage period, usually 6–8 min, the gauze was removed from the balance case and the curd layer carefully rolled off and placed in a sample jar which was tightly closed until the moisture content of the curd could be determined. Owing to the weaving technique of spreading the curd it was usually a simple matter to roll up the layer in spite of its thinness and to transfer it quantitatively. The gauze was reweighed immediately to give the final weight of the curd by difference. The initial tare of the gauze could not be used since at the end some of the interstices held whey.

In the descriptions which follow, the curd, spread as just described, is referred to as a single layer since its average thickness was that of curd strips laid side by side and just touching. The description is of the ideal. In practice it was difficult to keep the speed of movement of the inlet tube and the spacings accurate. Occasional breaks occurred in the strip of curd issuing from the apparatus. Then for a few seconds whey only would come out and as far as possible the tube had to be held steady until the next strip of curd began.

Multiple layers were formed by repeating the spreading process a sufficient number of times, remembering that 6 layers, for example, meant 12 half-layers woven at right angles to one another. The timing of each half-layer together with the total time

served as a check on the number of layers. With multiple layers the spreading time of each half-layer was reduced as necessary to limit the total weight (e.g. 10 s were used for each half-layer when 6 layers were to be spread instead of 30 s when 1 layer was required). Multiple layers also needed to be allowed to remain longer in the horizontal position to cause them to adhere to the gauze. At least 2 min were necessary for 6 layers.

Although the curd was produced as a cylinder 6 mm in diam. it shrank so rapidly at the high temperatures used that after a few minutes on the gauze a single layer appeared at its thickest to be only 1–2 mm thick.

If the weight of the curd on the gauze was plotted against time a curve was obtained, but if the moisture content of the curd was plotted against time a straight line was usually obtained (between about 80 and 60 % moisture). This enabled most of the results to be reported briefly as time required to reach 70 % moisture and rate of moisture loss in percentage/min. Some of the thick layers were exceptional in that they drained relatively rapidly to about 74 % moisture content. The drainage rate then became comparable with that for other thick layers and then below 64 % it diminished slightly. However, between 74 and 64 % the rate was constant enough to enable meaningful comparisons to be made. The moisture contents at the moment of each weighing were calculated from the total weights, the final moisture content and the weight of whey lost, assuming 7 % total solids in the whey.

Final moisture contents were determined at first by drying at 98 °C as in earlier papers of this series, but later infra-red heating by means of a 250 W reflector lamp was used and the sample remained on the scale of a top-pan balance. The balance was shielded with aluminium foil. By spreading a 3-g sample of curd over a circle of about 8 cm diam. on a dried filter paper, a drying time of 5–7 min could be achieved. Curd samples, which of course contained lactose, became black by the time a constant weight had been reached, but the differences in apparent moisture content between the value obtained by oven drying at 98 °C and that for the charred curd amounted to less than 2 %.

The fat content of whey samples was determined by means of the Milko-tester (Murphy & McGann, 1967) but since the values were so low they were out of the normal range for that instrument and must be regarded as approximate.

EXPERIMENTS AND RESULTS

Time course of the whey drainage

Fig. 2 shows the moisture content of several samples of curd plotted against the time which had elapsed since curd formation. Within the range of interest the graphs are rectilinear. This was confirmed by nearly all the other results obtained. The linearity of the graphs permitted interpolation and occasionally extrapolation to determine the time required for the curd to reach a given moisture content (e.g. 70 %). It was also possible to give a single figure for the rate of drainage as percentage decline in moisture content/min. These 2 values were therefore chosen as a brief way of expressing the results.
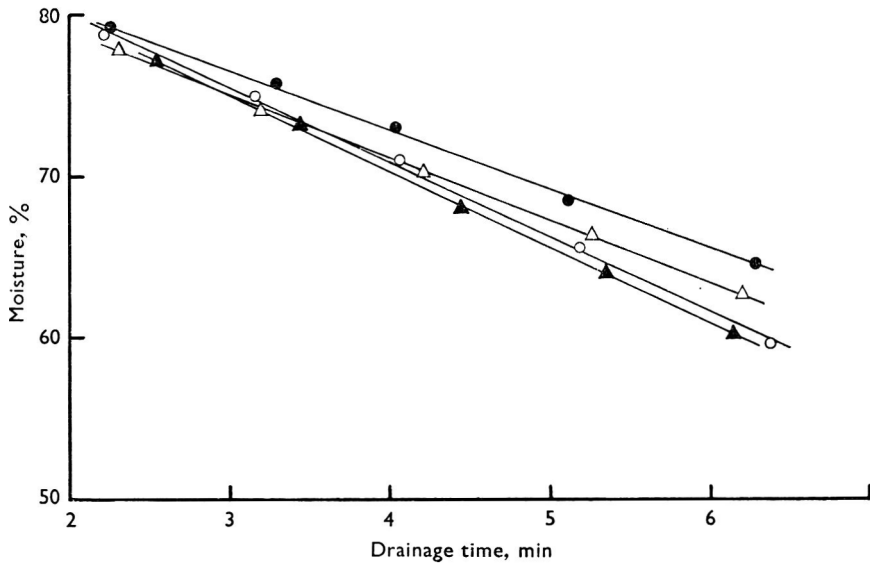
Fig. 2. The rate of drainage of curd from milk of different pH values. ◯, pH 6·1 by addition of HCl; ▲, pH 6·1 by addition of H_3PO_4; ●, pH 6·3 by addition cf H_3PO_4; △, natural pH (6·62).

Reproducibility

Experiments were not usually duplicated on the same day but this was done occasionally to check the reliability of the technique. Since, as shown in Fig. 1, the graph of moisture content (%) versus time was approximately rectilinear, differences between replicates could be indicated by the differences in time required to reach any given moisture content. The figures were as follows: on the first occasion the maximum difference between triplicates was 28s and the minimum 6s; some days later the maximum difference between triplicates was 9 s and the minimum 3 s; on another occasion duplicates differed by 10s. The total times in all the experiments were about 5 min.

Temperature

Some indication of the effect of temperature had already been gained in previous work (Berridge & Scurlock, 1969) and it was necessary only to show that no great difference would arise in the effect of temperature as a result of draining the curd on gauze. Consequently, only a few observations were made and these mostly during experiments on the effect of different thicknesses of the curd layer. The results are shown in Table 1. Owing to the long period of time required for the cabinet to settle to a constant humidity as well as to a constant temperature it was not easy to work with different temperatures on the same day. A comparison of the results at 44 °C on 15 May with those on 19 May shows that large differences could occur from day to day. Consequently, comparisons between different temperatures on different days must be made with caution. On 16 May the cabinet was used at a low humidity. As expected the moisture loss from the curd was greater on that day than on 13 May when the temperature was the same but the humidity was higher.

Table 1. *The effect of temperature, humidity and layer thickness on curd drainage*

Date	Temperature of cabinet, °C			Thickness of curd, no. of layers	Time to reach 70% moisture, min	Time (min) ÷ no. of layers	Rate of drainage, decrease in % moisture content/min
	Nominal	Actual					
		Wet bulb	Dry bulb				
13 May	48	47·5–47·8	48·0–48·2	½	3·4	6·8	5·6
		47·5	48·0	1	5·1	5·1	3·2
		48·5	49·2–49·0	2	6·2	3·1	2·8
		48·0	49·0–48·5	3	6·9	2·3	2·3
15 May	53	52·3–52·5	53·2	½	2·8	5·6	7·3
		52·3–52·0	53·0–52·5	1	4·2	4·2	4·7
		52·8–53·2	53·2	2	5·4	2·7	3·8
	44	44·2–44·8	45·0–44·5	2	10·6	5·3	1·6
		43·5–43·8	44·0	3	13·0	4·3	1·2
16 May	48	48·0–49·1	66·0	½	2·7	5·4	6·4
		48·0–50·5	63·0–64·0	1	4·0	4·0	4·3
		47·2–51·0	68·0–69·0	2	5·5	2·8	3·3
19 May	44	43·3–44·1	44·0–44·5	1	5·3	5·3	3·2
		44·0–45·0	44·8–45·0	2	6·1	3·0	2·6
		43·8–45·2	44·0–45·6	4	10·1	2·5	1·6
		43·0–43·5	44·0–44·5	6	10·4	1·7	1·6
17 Oct.	42	42·0–42·8	42·8–43·2	1	5·6	5·6	3·8
	46	46·0–47·5	46·2–47·5	1	5·2	5·2	5·0

The experiments on 17 October were done with curd produced at approximately the temperature of the cabinet, i.e. 42, 46 and 51 °C, but the earlier observations were made with curd produced at a constant temperature of 48 °C and as some of the cabinet temperatures were different it was possible that the time needed for the curd to reach cabinet temperature was a significant proportion of the total drainage time. The time course of the temperature in curd layers of different thickness was therefore plotted, using a thermocouple and recording milliammeter. When more than one layer was used the thermocouple was placed on the top of half the layers and the rest were deposited over the thermocouple, so that it was in the centre of the curd thickness. The times found were therefore maxima. The results are given in Table 2. The times to get within 2 deg C of the cabinet temperatures were much less than those usually needed to reach 70 % moisture. It is, therefore, likely that time lag was not a major factor, though it could have caused some of the differences between rates of drainage at different temperatures to be a little smaller than they would otherwise have been (see Table 1).

Table 2. *Rate of approach of curd temperature to cabinet temperature*

Curd temperature 46 °C throughout

Cabinet temperature, °C		No. of curd layers	Minimum temperature recorded in curd	Time to reach minimum, min	Time to reach within 2 °C of minimum, min	Time for other curd of same thickness to reach 70 % moisture (from Table 1) at 44 °C, min
Wet bulb	Dry bulb					
41·2	42·2	1	41·1	2	< 1	5
41·0	42·0	2	41·1	4	2	8
41·2	42·0	2	41·1	5	< 2	8
41·2	42·0	4	41·1	5	< 2	10
41·1	42·0	6	41·1	8	3	11
40·0	41·3	2	39·2	5	2	8
39·3	40·5	2	39·2	4	2	8
39·8	40·9	6	39·2	7	< 3	11

Layer thickness

The layer thickness of curd has been expressed as the number of superimposed single layers. The formation of the layers has been described under Methods. The effect of layer thickness on the time required to reach 70 % moisture is shown in Table 1. The slopes of the lines relating percentage moisture to time (i.e. rate of drainage in %/min) are also shown. The values for time divided by the number of layers decreased as the number increased, showing that in a given apparatus more curd could be processed in a given time with thick than with thin curd. Additional results relating to curd thickness are recorded in Tables 3 and 4, and the subject is referred to again when the effects of curd disturbance are discussed (p. 428).

pH

Fig. 2 shows the effect on the rate of drainage of acidifying the milk before renneting. The results demonstrate an increase in the rate when the pH had been adjusted to 6·1 with HCl and a further slight increase when H_3PO_4 was used instead

of HCl. It is remarkable that at pH 6·3 the rate was less than at pH 6·6. This was observed on several occasions. Other results not included in the figure showed that the rate at pH 6·5 was indistinguishable from that at pH 6·6. Further, when a sample that had been renneted at pH 6·1 was adjusted to pH 6·5 its rate of drainage was similar to that of curd from the original renneted milk still at pH 6·1 (4 points out of 5 were actually on the pH 6·1 line). This was tested because curd for Cheddar is normally formed when little or no acidity has been developed. The result suggests that it would be satisfactory to accelerate the action of rennet by adjusting the pH to 6·1 followed by readjusting the pH to 6·5 for curd formation.

Time and pH

Experience had suggested that curd properties improved when renneting was done in the cold for times in excess of the minimum required for rapid clotting at 48 °C. The rates of syneresis of curd after prolonged treatment of the cold milk with rennet

Table 3. *The effect of prolonged cold-renneting time on the subsequent drainage of the curd*

Time of rennet action at low temperature,* min			Time to reach 70% moisture, min		Slope, loss of water as % in 1 min		Approximate fat content of whey, %	
Milk 1†	Milk 2	Milk 3	1 layer	6 layers‡	1 layer	6 layers	1 layer	6 layers
45	—	—	4·2	—	4·1	—	0·05	—
—	85	—	—	9·9	—	1·1	—	0·23
165	—	—	3·9	—	4·3	—	0·03	—
—	170	—	—	8·5	—	1·2	—	0·20
—	—	210	—	11·0	—	1·2	—	0·18
235	—	—	4·5	—	3·6	—	0·08	—
320	—	—	4·6	—	3·7	—	0·04	—
—	320	—	—	16·0	—	0·7	—	0·20
—	—	330	—	9·7	—	1·3	—	0·20
~ 1400	—	—	4·2	—	4·1	—	0·06	—
—	~ 1400	—	—	10·6	—	1·2	—	0·28
—	—	~ 1400	—	10·1	—	1·4	—	0·18

* Minimum time to give a clot immediately on heating was 35 min.
† Bulk milk from one herd on 28 Aug., 2 Sept. and 3 Sept. was used.
‡ A cabinet temperature of 44 °C was used for the 6 layers.

were therefore measured. These are expressed in Table 3, as times required to reach 70 % moisture and also as the slopes of the graphs of moisture versus time. Renneting (2 ml/gal) was done at pH 6·1, milk samples then being adjusted to pH 6·5 just before use. After treatment with rennet at 4–6 °C for 35 min, the milk immediately clotted on being heated.

Further results, also shown in Table 3, were obtained in another experiment in which the rates of drainage of 6 layers at 44 °C were measured after different periods with rennet. The results shown in Table 3 do not indicate a regular change either in the time required to reach 70 % moisture or in the rate of drainage as the time of rennet action is increased.

Since milk is not a system in chemical equilibrium and is therefore changing during

storage, it was necessary to determine whether keeping cold-renneted milk at pH 6·5 would change its clotting properties. Accordingly, milk renneted at pH 6·1 overnight was adjusted to pH 6·5 and curd drainage measurements were made from time to time. The results are given in Table 4. Again, the differences between the treatments were not significant and the results were confirmed using 6 layers at 44 °C.

Table 4. *The effect of keeping cold-renneted milk at pH 6·5 on the subsequent drainage rate of the curd*

Time at pH 6·5, min		Time to reach 70 % moisture, min		Slopes, loss of water as % in 1 min		Approximate fat content of whey, %	
Milk 1	Milk 2	1 layer	6 layers	1 layer	6 layers	1 layer	6 layers
20	—	4·6	—	5·0	—	0·09	—
—	20	—	10·0	—	1·6	—	0·24
160	—	4·1	—	5·2	—	0·06	—
—	175	—	10·7	—	1·4	—	0·28
275	—	4·1	—	5·0	—	0·05	—
1440	—	4·0	—	4·8	—	0·05	—

Effect of heat treatment of the milk

Fully pasteurized milk (71·5 °C for 17 s) was used for the experiments on 19 May (Table 1). Comparison with the results obtained on 15 May failed to show the expected deleterious effect of heat treatment on curd shrinkage; in fact the pasteurized milk drained more quickly than the raw milk. A comparison was therefore made using a sample of raw milk and a sample of the same milk that had been pasteurized, all other conditions being the same (single layer at 48 °C). The results showed no significant difference due to pasteurization; the times required for duplicate raw milk curds to reach 70 % moisture content were 4·7 min and 4·9 min and that for the pasteurized milk curd was 5 min. Similarly in another experiment, curd from raw milk reached 70 % moisture content in 6·1 min, compared with 5·8 min for curd from a sample of the same milk after it had been pasteurized. These were double thickness layers at 44 °C. Again the difference was small.

Orientation of curd layer during drainage

It was already known that disturbance of curd as it is being formed accelerates drainage (Cheeseman & Chapman, 1966) and it seemed possible that the curd newly deposited on the gauze might still be 'young' enough to be susceptible to a disturbance such as might occur as a result of slight flow when the gauze was moved from a horizontal to a vertical orientation. Experiments were done in which the movements were repeated by inverting the vertical gauze several times. The results were compared with others in which the curd remained stationary on the almost horizontal gauze. (The gauze was arranged at about 15° from the horizontal to cause steady drainage.) It was expected that effects of flow would be more marked in a thick layer, so a layer of 6 times the normal thickness (i.e. 6 normal layers superimposed on one another) was used in addition to the normal thickness. The 6 layers required 3 min to collect, and when the gauze was to be hung vertically a further 1·5–2 min were needed in the horizontal position to prevent the curd peeling off

when the gauze was vertical. A lower cabinet temperature was also used, namely wet bulb 44 ± 1 °C, because in cheese-making the longer drainage times at a higher temperature would kill the starter. It was found that repeated inversion of the gauze impeded drainage slightly just as it would from the surface of a simple solid body and that the rate of drainage in the vertical orientation was 40–50 % greater than that in the almost horizontal orientation. The single vertical layer reached 70 % moisture 2 min earlier than the horizontal one, and with 6 layers the difference in time was 3 min.

Effect of pressure on the curd layer

Pressure is used to assist the drainage of normal curd during cheddaring but there is reason to think that care must be exercised in dealing with freshly formed curd. Some approximate indications had already been obtained regarding the effects of

Table 5. *The effect of rolling with and without folding on curd moisture content*

Treatment	Moisture content after $6\cdot5 \pm 0\cdot1$ min (total), %
None	61
Rolled immediately, hung	57
Hung 2 min, rolled, hung	58
Hung 4 min, rolled, hung	58
Hung 2 min, folded once, rolled, hung	56
Hung 2 min folded twice, rolled, hung	52

rolling and different pressures (Berridge, Scurlock & Aston, 1969). It was thus desirable to collect more evidence under the stricter control of the cabinet environment.

One means of exerting a pressure was to cover the layer of curd with glass beads. More than one layer of beads was added as soon as the curd had been collected on the gauze and the excess of beads was 'poured' off leaving a single layer with the beads touching one another. The weight of the beads was 46·4 g and the area of the layer was about 100 cm². A double layer of curd was used at 44–45 °C cabinet temperature (wet and dry bulbs) and drainage was continued at about 15° from the horizontal. The time required to reach 70 % moisture content was 4·4 min, and that for the control *drained vertically* was 6·1 min, but the rate of drainage at about 70 % moisture was nearly the same, namely 3·0–3·1 %/min, showing that the beads caused a rapid loss of whey at an early stage.

With continuous processing in mind further experiments were done in which a roller was used. For these experiments, the gauze with the curd was supported horizontally on a wooden block. A polythene bottle of 500 ml capacity weighing 150 g was used as a roller and rolling consisted of a single pass of the roller. A single layer of curd was used. All experiments were done at a cabinet temperature of 47·0–48·1 °C (wet bulb) 48·0–49·9 °C (dry bulb). The curd was collected for 1 min with the gauze held horizontally; it was then not rolled, or rolled at once, or rolled after 2 or 4 min of hanging in the vertical position. The curd was rehung to drain until a total time of 6·5 min had elapsed since the beginning of curd collection. The resulting 4 samples all had moisture contents of 63 ± 1 %. The light rolling had made no difference. The experiment was repeated the following day with the roller containing enough water to give a total weight of 550 g. Two additional measurements were

carried out with curd which had been allowed to drain vertically for 2 min. In one, the curd was folded once (making 2 layers) and in the other it was folded twice (making 4 layers) before rolling. The results are shown in Table 5. Rolling reduced the curd moisture but folding and rolling reduced it more.

Effect of disturbance

This was seen qualitatively in an experiment in which increasing disturbance of the curd was produced by progressively restricting the outlet from the curd-maker. The time required by the curd to reach 70 % moisture content fell from 5·1 to 3·5 min.

Since disturbed curd releases more fat into the whey than does normal curd, a measure of the disturbance can be obtained by determining the fat content of the whey. This was done in a further experiment, in which the disturbance was such that the whey contained 0·92 % fat, normal values being about 0·06 % for curd produced in the membrane (see immediately below). The disturbed curd reached 70 % moisture content in 2·3 min and was then draining at the rate of 3·2 %/min, the corresponding values for the undisturbed control being 5·8 min and 2·8 %/min. These curds were both double layers drained at 44 °C (wet and dry bulbs).

The appearance of fragments broken from 6-layer curds suggested that they had suffered some disturbance which might explain their relatively rapid drainage. Therefore, in the experiments described on p. 424 under the heading 'Time and pH', all the whey draining from the curds was collected separately and the fat contents determined. The results, which are recorded in Tables 3 and 4, show the higher fat content in the whey from 6-layer curds and they indicate that there is a small amount of disturbance when 6 layers are deposited.

Effect of keeping the curd in the whey

The removal of curd from its whey accelerates the drainage (Thomé, Axelsson & Liljegren, 1958). This was confirmed in a single experiment in which curd was collected on the gauze beneath the whey and allowed to remain there for 3 min. Since the normal procedure is to allow curd 1 min of drainage horizontally to make it adhere to the gauze the curd in this experiment was raised out of the whey after 3 min and kept horizontal for 1 min. It was then hung vertically as usual. It reached 70 % moisture after a total time of 6·9 min and was then draining at the rate of 3·7 %/min. The corresponding values for the control were 5·5 min and 4·0 %/min. It is to be noted that the control was drained vertically for the whole period, after the normal 1 min in the horizontal position. Thus, a shorter drainage time was required for the curd which had been separated immediately from the whey.

DISCUSSION

The results show that the factor having the greatest effect on drainage rate is disturbance of the newly formed curd. Moderate to severe disturbance can be detected and the disturbance can be defined and measured by the quantity of fat released into the whey if the conditions, e.g. pretreatment of the milk, are kept constant. The effect of the treatment of the milk on the amount of fat released as a result of disturbance was investigated by Mabbitt & Cheeseman (1967) and Cheeseman

& Mabbitt (1968). However, since the rate of drainage is so sensitive to disturbance it seems possible that slight disturbance may affect the drainage rate without greatly changing the fat content of the whey. Such disturbances could occur as a result of different flow patterns in the tubular membrane of the curd-maker. The course of curd formation in the curd-maker follows an irregular rhythm because part of the newly formed curd sometimes adheres to the membrane for periods varying between a few seconds and about half a minute. These variations could cause small changes in curd structure which would affect its rate of drainage, and would be responsible for some of the differences for which no other explanation is apparent.

It was expected that thin layers would drain more rapidly than layers of normal thickness and that higher temperatures and shorter times could therefore be used and a greater output would thereby be obtained. When it was found that the saving of time with a thin layer was not proportional to the thinness, thicker layers were tried. It was surprising to find that the thick layers drained more rapidly than would be expected according to simple proportion, and it was not until they were carefully examined that the cause was found to be the slight disturbance caused in the lower layers of delicate newly formed curd by the weight of the superimposed layers. Since the resulting level of fat in the whey is still quite low and not much different from the levels now obtained in conventional processes, it might be economical to use thick layers for the sake of a greater output per unit of equipment.

Our findings suggest that repeated folding and rolling of a continuous layer of curd might prove a useful procedure in commercial cheese-making and would be worth investigation.

The effect of disturbance on the structure of the clot and on its drainage rate has not hitherto received the attention it deserves. A suggestion as to how flow may affect the way in which long molecules could link to form a gel arises from the diagrams relating to fine and coarse fibrin clots published by Ferry (1948). The coarse clot which exhibits rapid syneresis was thought to contain many chains in side-to-side association, leaving relatively large voids between the bundles. Although with fibrin the side-to-side association was not ascribed to flow, it is known that flow does induce a parallel alignment of long molecules, as is shown by streaming birefringence. A similar alignment could take place when milk clots under conditions where flow may occur. Cheeseman & Chapman (1966) were the first to show the dramatic effect of disturbance on the drainage rate of curd. This was done in an attempt to explain how continuous curd-making machinery then in use could achieve such a rapid throughput. It is possible, however, that disturbance may be important in conventional cheese-making since a residual movement of the milk after stirring has ceased may continue until clotting occurs.

The results for the effect of different temperatures are not sufficient to permit the drawing of any firm conclusions. The results for October 17 (Table 1) suggest that the change from 46 to 52 °C had more effect on the very early stages of whey separation and less effect on subsequent drainage, whereas the increase from 42 to 46 °C had more effect on the later drainage. The factors which accelerate curd formation are probably different from those which accelerate drainage, and it is likely that the temperature coefficient of drainage is quite different from that of curd formation. If the suggestion from these results that the temperature coefficient of drainage

decreases at temperatures above about 46 °C is confirmed, the phenomenon would merit further study.

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431

Composition of the bound lipids in caseins and in ripening cheeses

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SUMMARY. Neutral lipids and phospholipids, firmly bound to rennet- or acid-precipitated casein and also to α-, β- and κ-casein, have been detected by histochemical means. On sections of the caseins they appeared as areas of variable shape and size, uniformly distributed. In cheeses they partially disappeared, probably owing to the action of the milk and bacterial lipases. This led to new patterns of distribution of the lipids in the sections.

Lecithin, cephalin, sphingomyelin, cerebrosides (2 spots), ceramides (1 spot), phosphatidylserine and phosphatidylinositol were identified in the phospholipid fraction of the casein by 2-dimensional thin-layer chromatography. In the bound phospholipid fraction of 3 cheeses the same phospholipids were found together with 1 new unidentified spot.

Cerbulis & Zittle (1965) and Cerbulis (1967) found that the lipids associated with acid-precipitated casein are not completely extractable by any single solvent. Even a chloroform–methanol (2:1) mixture did not remove all casein-bound lipids, some of which were removed only by saponification. Cerbulis (1967) studied the distribution of lipids in casein, skim-milk, whey, cream and separator slime, and defines as 'free lipid' the fraction extractable with petroleum ether, and as 'bound lipid' the fraction extractable with chloroform–methanol mixture. Neutral lipids were found both free and bound, while phospholipids were found only in the 'bound' fraction.

In the present paper we report on 2 histochemical reactions that permit detection of the lipids bound to acid- and rennet-precipitated casein, and to α-, β- and κ-casein. The quantities of free and bound lipids and phospholipids in each of the casein fractions were determined. At the same time the histochemical and chemical transformations that phospholipids undergo during the ripening of the curd were studied in 34 cheeses.

MATERIALS AND METHODS

Preparation and staining of the sections. Fresh uncooled raw morning milk collected from the university herd was used for preparation of the caseins. Acid-precipitated casein and α- and β-caseins were prepared according to Hipp, Groves, Custer & McMeekin (1952) and κ-casein by the procedure of Zittle & Custer (1963). Rennet-precipitated casein was prepared from the same bulk of defatted milk at 37 °C using

300 mg/l of a commercial rennet. Sections of the caseins 15 μm thick were cut in a Terzano–Leitz cryostat at -20 °C. The sections were transferred to slides, left to air-dry to ensure adequate fixation before staining, immersed for 12 h in petroleum ether to eliminate the free lipids and then stained for total bound lipids and phospholipids by the propylene glycol–Sudan IV method of Chiffelle & Putt (Pearse, 1960, p. 855). The method was modified by fixing the sections for 90 min in calcium–formol instead of formaldehyde and then staining them in the propylene glycol–Sudan IV solution for 6–10 h at 37 °C. The excess of colour was removed by washing for 30–60 s in 85 % propylene glycol and again for the same length of time in 50 % propylene glycol. Counterstaining in Mayer's haemalum was optional. Lipids and phospholipids appeared orange coloured.

For staining the phospholipids, Okamoto's mercury diphenylcarbazone method after Ueda was used (Pearse, 1960, p. 852); it gives a violet colour with phospholipids and cerebrosides. In order to separate 2 lipid classes, Okamoto et al. (Pearse, 1960, p. 852) proposed a cold pyridine extraction performed after staining but their claim that this removed only cerebrosides was not substantiated by Edgar & Donker (1957). This method has not been widely applied in histochemistry because it gives inconsistent results and also because it gives a positive reaction not only with phospholipids and cerebrosides but also with certain other non-lipid materials. With casein, Okamoto's techniques presented some difficulties which were solved principally by fixing the sections in calcium–formol instead of formalin for 45–60 min at 2–4 °C and immersing them in $MgCl_2$–acetone solution for only 24 instead of 48 h. The subsequent steps of this method were not modified. After staining, the slides were immersed for 48 h in pyridine at 4 °C and observed twice a day under the microscope. The fact that some stained areas disappeared rapidly and others only slowly led us to examine, by chemical methods, whether cerebrosides were also present in the lipids bound to the caseins.

Extraction and separation of lipids. Free lipids were extracted from the freeze-dried caseins with petroleum ether and the bound lipids with chloroform–methanol (2:1, v/v) as described by Cerbulis (1967). From the bound lipid fraction some non-lipid contaminants were removed as described by Folch, Lees & Sloane Stanley (1957); the lipid mixture was then separated into neutral lipids and phospholipids by silicic acid chromatography (Abramson & Blecher, 1965). The phospholipid fraction was analysed by TLC on silica gel G using a 2-dimensional technique described by Rouser, Kritchevsky & Yamamoto (1967, p. 147). The reference compounds were L-α-lecithin (Fluka AG, Buchs, Switzerland), cephalin (BDH, Poole, England) and sphingomyelin, cerebrosides, ceramides, phosphatidyl-L-serine and phosphatidylinositol (Koch–Light Laboratories Ltd, Colnbrook, Bucks). The detection reagent, which was sensitive and gave relatively stable spots, was a sulphuric solution of potassium dichromate (Rouser et al. 1967, p. 147). To identify the carbohydrates bound to the cerebrosides, the phospholipid fraction, which contained no free carbohydrates, was hydrolysed in a sealed tube with 2 N-H_2SO_4 for 3 h at 100 °C (Schwarz, Dreisbach, Barrionuevo, Kleschik & Kostyk, 1961) to free the sugar moiety of the cerebrosides. The hydrolysate, previously neutralized with solid $BaCO_3$, filtered, lyophilized and then dissolved in a small amount of distilled water, was chromatographed on Whatman No.1 paper (Partridge, 1948) together with glucose and galactose standards,

using ammoniacal silver nitrate as the colour reagent (Trevelyan, Procter & Harrison, 1950). Phosphorus was determined by the method of Fiske & Subbarow (1925).

RESULTS AND CONCLUSION

Table 1 shows the amounts of free and bound neutral lipids and phospholipids found in the casein preparations. The data for iso-electric casein agree with those found by Cerbulis (1967). α-Casein appeared to have the highest content of bound phospholipids.

Table 1. *Lipid content of the caseins and cheeses*

	Free lipids, %	Bound lipids, g %	
		Neutral	Phospholipids
Acid-precipitated casein	0·880	0·640	0·150
α-Casein	0·450	0·550	0·140
β-Casein	0·620	0·490	0·310
κ-Casein	0·430	0·110	0·160
Rennet-precipitated casein from defatted milk	0·780	0·501	0·166
Parmesan cheese	30·020	0·840	0·310
Emmental cheese	43·060	0·810	0·340
Provolone cheese	43·840	0·850	0·320

The 2-dimensional chromatograms on silica gel G of the phospholipid fractions of the rennet-precipitated and iso-electric caseins gave 8 spots which were identified by means of the reference compounds as lecithin, cephalin, sphingomyelin, cerebrosides (2 spots), ceramides (1 spot), phosphatidylserine and phosphatidylinositol. The cerebrosides contained galactose. Cerebrosides have been identified in spray-dried buttermilk (Smith & Freeman, 1959; Smith & Lowry, 1962) and in milk fats (Parsons & Patton, 1967). Cholesterol was not present in this fraction; it was present in the free and bound fractions only. The free lipids did not contain organic phosphorus.

The phospholipids were uniformly distributed on the acid-precipitated casein as can be observed in Fig. 1*a*, which shows a section stained by Okamoto's method for phospholipids. The stained areas appeared both in round (1–15 μm diam.) and ir-regular forms. Small- and medium-sized forms predominated. Much the same was found for the sections coloured with Sudan IV (Fig. 1*b*) except that the stained areas were greater in number compared with those stained by Okamoto's method. Sudan IV is in fact a general dye for all lipids and therefore it also made visible the neutral lipids. The distribution, shape and size of the areas stained with Sudan IV or by Okamoto's method in sections of α-, β-, κ- and rennet-caseins were substantially the same as those observed in sections of the acid-precipitated casein. The sections of the caseins did not take up any stain if they were first immersed in the chloroform–methanol mixture. It is possible that the lipids on the caseins are derived from low density lipoproteins or from chylomicrons. The lipoprotein may itself be bound to the casein or it may transfer its lipid moiety to the casein. It is of interest that the lipids and phospholipids observed in sections of the caseins and cheeses are very similar to sudanophilic and osmiophilic particles observed in the liver under conditions that

stimulate lipoprotein synthesis. These lipid particles, measuring 50–2500 Å, could fuse to form large spots of 2 μm (Ashworth, Wrightsman, Cooper & Di Luzio, 1965). Our particles also are osmiophilic, turning black in an aqueous 1 % solution of osmium tetroxide.
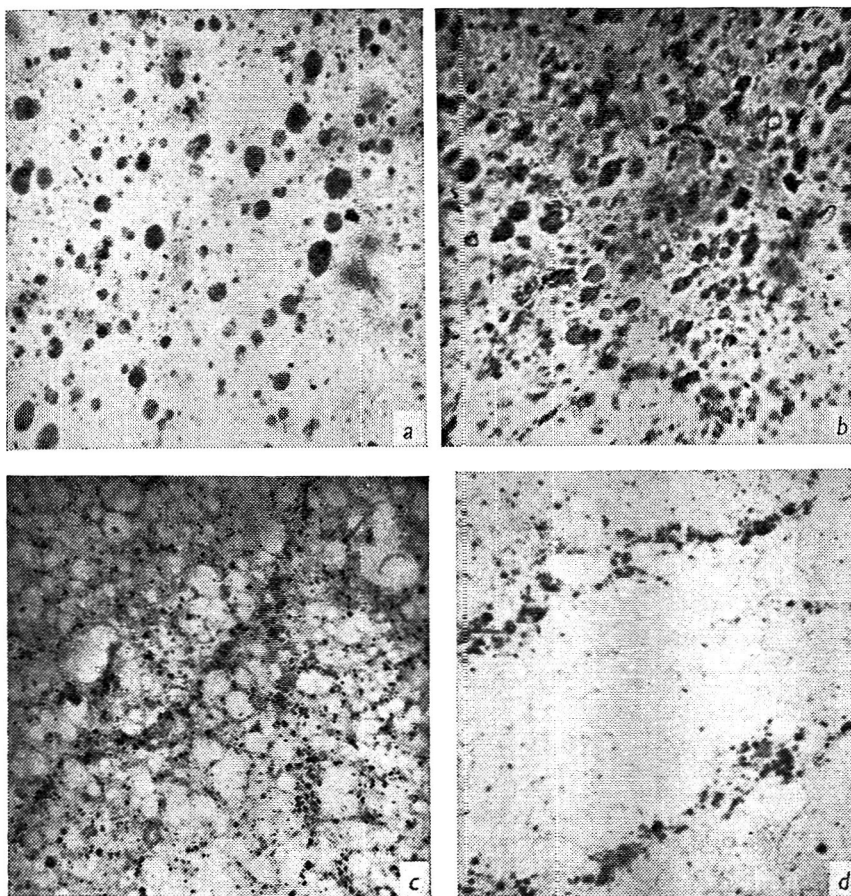
Fig. 1. The lipids bound to caseins and their histochemical changes during the ripening of the cheeses. (a) Spots of phospholipids in a section of acid-precipitated casein. Okamoto's stain ($\times$ 320). (b) Spots of lipids and phospholipids in a section of acid-precipitated casein. Sudan IV stain. ($\times$ 320). (c) Seams of spots of phospholipids in a section of Provolone cheese. Okamoto's stain ($\times \sim$ 70). (d) Parallel seams of spots of phospholipids in a section of Parmesan cheese. Okamoto's stain ($\times \sim$ 70).

In preceding papers (Bolcato & Spettoli, 1969a, b) we have described particulate 'granules' of lipoproteins that form during the ripening of hard and semihard cheeses. We think that these granules derive from the lipid part of the casein by a mechanism already suggested (Bolcato, Spettoli & Grinzato, 1969). Of the 34 cheeses histo-chemically studied only 3 have been analysed for free and bound lipids (Table 1). Emmental and Provolone cheeses had a higher content of free lipids than Parmesan cheese, which may be expected as whole milk is used in their manufacture whereas the Parmesan cheese is made from partially defatted milk. The free lipid fraction did not contain organic phosphorus, thus demonstrating the absence of the free phospholipids

Table 2. *Distribution of phospholipids on the sections of cheeses,*
as revealed with Okamoto's stain

(Sudan IV gave similar results.)

Distribution pattern	Cheeses
(1) Sections dotted with small and medium-sized spots. Their concentration was reduced in comparison with those in the rennet-casein section.	Sardinian Pecorino. Camembert. St Paulin. Brie. Provolone A and B. Taleggio A. Cacciocavallo. Curd 15 days old (in environmental room at 18 °C with a relative humidity of 80–85%)
(2) On a background similar to that described above, more or less parallel narrow bands appeared in which the spots were more densely concentrated.	Swiss Emmental A and B. Fontina A. Taleggio B. Parmesan. Valmouse. Dutch.
(3) As (2) above, but with wider bands.	Asiago A. Fontina B. Gorgonzola A. Panerone. Emmental C. Roquefort. Roman Pecorino.
(4) As above, with bands of varying width, criss-crossing to form zones of densely concentrated spots.	Gorgonzola B. Parmesan of 4, 6, 8 and 10 months ago. Asiago B. Atypical Parmesan. Provolone C.
(5) Background as (1) above with more or less wide zones denser in spots superimposed.	Tuscan Pecorino. Gorgonzola C.
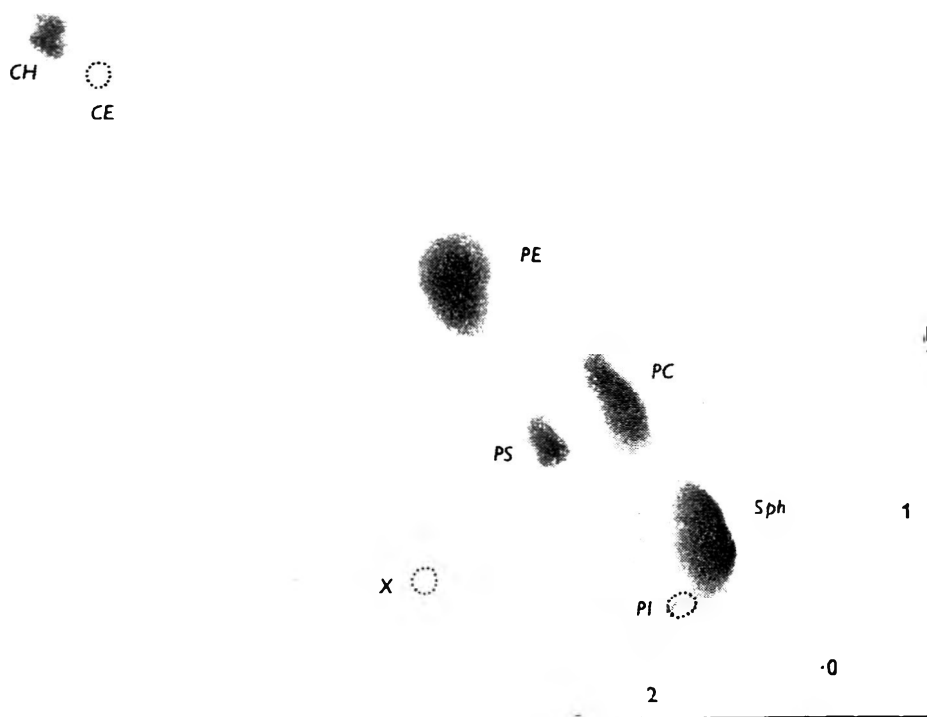
Fig. 2. 2-dimensional TLC of phospholipids extracted from the casein of Parmesan cheese. Solvents: first direction, chloroform–methanol–28% aqueous ammonia 65:35:5. Second direction, chloroform–acetone–methanol–acetic acid–water 5:2:1:1:0·5. O, origin; *PI*, phosphatidyl inositol; *Sph*, sphingomyelin; *PS*, phosphatidylserine; *PC*, lecithin (phosphatidylcholine); *PE*, cephalin (phosphatidylethanolamine); *CE*, ceramide; *CH* cerebrosides.

that are soluble in petroleum ether. The content of bound lipids (neutral and phospholipids) was greater in the cheeses which contained the lipoproteins of the fat globule membranes than in the rennet casein. The bound lipid content of the Parmesan cheese was approximately the same as that of the Emmental and Provolone cheeses although the milks used in the preparation of the 3 cheeses contained different quantities of fats. Probably this was due to differences in the rates of enzymic degradation of bound lipids by milk and bacterial lipases during the ripening of the cheeses— more extensive degradation in the Emmental and Provolone cheese than in the Parmesan. Enzymic degradation of these lipids was also demonstrated histochemically in the distribution of the lipid spots on sections of the 34 cheeses examined. The spots did not appear to be uniformly distributed on the sections as with the rennet-casein but occurred in new patterns as briefly described in Table 2. In general, on a background in which the spots were small and infrequent, parallel or crossed bands or zones were superimposed in which the distribution, concentration and size of the spots resembled those in the curd. We think it probable that the partial disappearance of the spots in some parts of the sections may be ascribed to the action of lipases. The distribution of the spots on the sections was not specific for a cheese or for a group of similar cheeses, as appears from the Table 2; it is shown for Parmesan and Provolone cheeses in Figs 1c and d.

In the 2-dimensional chromatogram (Fig. 2) of the bound phospholipid fractions of the Parmesan, Emmental and Provolone cheeses 9 spots were visible, 8 of which were identified as the same phospholipids found in acid- and rennet-precipitated casein (see above). Apart from the new unknown phospholipid, there was no substantial qualitative difference between the bound phospholipids of the curd and those of the 3 cheeses examined.

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The tertiary phase of rennin action on α_s- and β-caseins

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SUMMARY. Casein, whole α_s-casein and β-casein were incubated for 3 and 14 h with crystalline rennin, at pH 6·60 and 36 °C, both in phosphate buffer and in milk dialysate. Products obtained from both systems, comprising 30–83 % calcium-sensitive (Ca_s) components, gave similar patterns on starch gel electrophoresis. Whole casein and whole α_s-casein were not so soluble in milk dialysate as in phosphate buffer. No significant differences in composition were observed between the Ca_s and the calcium-insensitive (Ca_i) products from the same source.

The α_{s_1}-component of the Ca_s product from rennin-treated whole α_s-casein had faster gel mobility in comparison to the α_{s_1}-component in the Ca_s product from untreated whole α_s-casein. Also, α_{s_1}-casein yielded one faster-moving degradation product, while $\alpha_{s_{2,3,4}}$ appeared unaltered after 14 h. The Ca_s product of rennin-treated β-casein also had faster mobility than untreated β-casein and yielded one faster degradation product and several minor ones of slower mobility. Arginine was the only N-terminal amino acid found in the Ca_s product of both rennin-treated and untreated α_s- and β-caseins. The arginine content increased from 3·48 and 4·98 moles/ 10^5 g to 5·12 and 6·38 moles/10^5 g in the Ca_s products from rennin-treated β- and α_s-caseins, respectively.

The primary and secondary phases of crystalline rennin action on casein and κ-casein were reported by El-Negoumy (1968): κ-casein was the only component attacked during 50 min of rennin action on whole casein. No other specific electrophoretic changes accompanied the appearance of coagulum characterizing the secondary phase. The tertiary phase of rennin action involves the degradation of α_s- and β-caseins (Lindqvist, 1963). The present report deals with the types and composition of degradation products resulting from crystalline rennin action on whole casein, α_s-casein (comprising $\alpha_{s_{1,2,3,4}}$) and β-caseins.

Alais, Mocquot, Nitschmann & Zahler (1953) were the first to demonstrate the difference between the primary phase and the concurrent tertiary phase of rennin action. Several reports dealt with rennin action on whole casein (Nitschmann & Lehman, 1947; Masayoshi, 1956; Tsugo & Yamauchi, 1956), while others (Nitschmann & Keller, 1955; Cerbulis, Custer & Zittle, 1959; Lindqvist & Storgårds, 1959; Lahav & Babad, 1964) were concerned with the degradation of α-casein which included κ-casein.

Despite several reports to the contrary (Cherbuliez & Baudet, 1950; Alais *et al.* 1953; Nitschmann & Keller, 1955), Hagberg & Sullivan (1953) and Lindqvist & Storgårds (1960, 1962) demonstrated that β-casein is hydrolysed to a considerable extent by rennin. Lahav & Babad (1964) isolated 3 calcium-insensitive (Ca_i) products from crystalline rennin-treated α-, β- and γ-caseins, which they designated $K\alpha$, $K\beta$ and $K\gamma$. These products protected their parent caseins against precipitation with ionic calcium.

In all the above studies either free boundary or paper electrophoresis were applied.

Three objectives were sought in the present work: (1) to determine the composition and origin of the compounds produced during the tertiary phase, using high resolution starch gel electrophoresis (SGE); (2) to investigate the effect of the reaction medium (phosphate buffer or milk dialysate) on the nature and course of degradation; (3) to examine the composition of the Ca_i fractions isolated from whole α_s- and β-caseins by the technique used by Lahav & Babad (1964).

MATERIALS

Casein

Whole casein was prepared by precipitation at the iso-electric point from fresh raw skim-milk, washed exhaustively with distilled water and extracted with acetic acid (McMeekin, Hipp & Groves, 1959) to remove any milk protease. The final product was again washed with distilled water and then lyophilized.

α_s- plus β-casein fractions

The κ-casein was separated from whole casein by gel filtration through a 4.5×95 cm column of Sephadex G-150 equilibrated and eluted with 0.005 M tris–citrate buffer of pH 8.6 containing 6 M urea. The technique was essentially that of Yaguchi, Davies & Kim (1968). κ-Casein was eluted in the void volume. The effluent fractions following the void volume were combined, exhaustively dialysed, concentrated and lyophilized. From several runs, 24 g of α_s- + β-casein were prepared.

Whole α_s- and β-caseins

α_s- and β-caseins were each prepared from the α_s- + β-casein mixture by an urea-extraction technique (El-Negoumy, 1966). The κ-casein content of a mixture of 125 mg each of purified whole α_s- and β-casein was determined by column chromatography on DEAE cellulose. The caseins were alkylated and fractionated on a $1.5 \times$ 20-cm column DEAE cellulose (0.90 m-equiv./g), Distillation Products Industries, Rochester, 3, New York, by the procedure of Rose, Davies & Yaguchi (1969). The fractions containing κ-casein were pooled, dialysed and lyophilized.

Crystalline rennin. Crystalline rennin was prepared as previously reported (El-Negoumy, 1968).

Reaction solutions. Milk dialysate was prepared by dialysing 500 ml of 5 % α-lactose against 37.9 l of fresh skim-milk for 48 h. Phosphate buffer of pH 6.60 contained 4.60 g NaH_2PO_4 and 2.60 g Na_2HPO_4/l.

Whole casein (4%), whole α_s- and β-caseins (3%) were dissolved in reaction medium. The solutions were heated to 80 °C for 10 min to destroy any micro-

organisms or enzymes, cooled and supplemented with 1% toluene (Lindqvist & Storgårds, 1959). Crystalline rennin (0·02 mg/100 ml) dissolved in sterile distilled water was added and the reaction solution was held at 36 °C for 3 or 14 h after adjustment to pH 6·60. This treatment was followed by heating to 88 °C to inactivate the rennin.

Control solutions in milk dialysate containing 3 and 6% of whole α_s- or β-caseins were treated exactly as above, except for the omission of rennin.

Ca$_s$ and Ca$_l$ products. These were prepared from whole casein, α_s- and β-caseins, before and after rennin action, as described by Lahav & Babad (1964).

METHODS

Phosphorus, nitrogen and starch gel electrophoresis (SGE). These were carried out as previously reported (El-Negoumy, 1966).

N-*acetyl neuraminic (sialic) acid.* Sialic acid was determined by the thiobarbituric-acid method of Warren (1959), using a reference sample prepared from casein according to Ribadeau Dumas & Alais (1961).

N-*terminal amino acids.* The N-terminal amino acids of the Ca$_s$ products from α_s- and β-casein were determined before and after 14 h of rennin action. Treatment of the protein solutions, their dinitrophenylation and hydrolysis of the DNP proteins was as described by Manson (1961). The DNP-amino acids were chromatographed on paper, together with authentic samples of DNP-amino acids, using the technique and solvent system described by Levy (1954). DNP-arginine was the only N-terminal amino acid identified in the hydrolysates from DNP-, α_s- and β-caseins; its presence was confirmed using a modified Sakaguchi reagent (Block, 1960).

Quantitative estimation of DNP-arginine was made on triplicate samples of protein hydrolysates. The results were corrected for losses during hydrolysis, chromatography and electrophoresis as described by Fraenkel-Conrat, Harris & Levy (1955).

RESULTS

Composition of whole, α_s- *and* β-*casein controls.* The κ-casein content of the α_s- and β-casein preparations was estimated chromatographically using DEAE cellulose. The elution patterns of a mixture of 125 mg each of α- and β-casein and 125 mg of κ-casein standard obtained using tris–citrate–urea (TCU) buffers is shown in Plate 1(a).

The κ-casein standard was eluted in 100–400 ml with a recovery of about 98·6%. The equivalent amount of eluant from the α_s- and β-casein mixture contained about 0·57 mg (0·23%) indicating a very low content of κ-casein. No κ-casein was detected by SGE in α_s- or β-caseins at 6% concentration (see pattern 1, 3, Plate 1(b) for β- and α_s-casein controls, respectively).

The Ca$_s$ and Ca$_l$ products from each of the α_s- and β-casein controls were more or less identical in their P/N ratios (Table 1) and in their electrophoretic composition (see patterns 1, 2, Plate 3(a, b) for α_s-casein and patterns 1, 2, 3, 4, Plate 4(b) for β-casein). The sialic-acid content of Ca$_s$ α_s-casein (0·022%) and β-casein (0·015%) indicated the presence of only traces of κ-casein (Schmidt & Payens, 1963; Garnier,

Ribadeau Dumas & Mocquot, 1964). DNP-arginine was the only N-terminal amino acid found in either α_s- or β-caseins. This is in agreement with published results for α_s-casein (Manson, 1961) and for β-casein (Kalan, Thompson, Greenberg & Pepper, 1965). Whole α_s-casein contained 3·48 moles of N-terminal arginine, while β-casein contained 4·92 moles/10^5 g protein (See Table 1).

Table 1. *Composition of products from whole α_s- and β-caseins before and after 14 h rennin action in milk dialysate*

| | Untreated controls | | | | Rennin-treated | | | |
| | Ca-sensitive | | Ca-insensitive | | Ca-sensitive | | Ca-insensitive | |
	From α_s-	From β-	From α_s-	From β-	From α_s-	From β-	From α_s-	From β-
P, %	1·11	0·62	1·19	0·58	0·83	0·25	0·92	0·19
N, %	14·96	15·28	15·14	15·32	15·23	15·50	15·44	15·68
P/N ratio	0·076	0·040	0·078	0·037	0·054	0·016	0·059	0·012
N-acetyl nouraminic (sialic) acid, % (mean of 3 analyses)	0·022	0·015	—	—	—	—	—	—
DNP-N-terminal arginine, moles/10^5 g protein	3·48	4·92	—	—	5·12	6·38	—	—

Composition of products from rennin-treated whole casein, whole α_s- and β-caseins

Whole casein

As rennin action was extended from 3 to 14 h, the yields of unsoluble Ca_s and Ca_l products decreased by 40 and 58 % and 6 and 30 %, respectively, depending on the composition of the reaction medium (see Table 2). This extensive degradation was also confirmed by the increase in the percentage of whole casein solubilized.

All major components of the Ca_s whole casein were degraded after 3 h of rennin action. This is evident by comparing in Plate 2 (*a*) patterns 2 and 5 for rennin-treated casein with 1 and 4 for the controls. Differences in the composition of the reaction medium did not cause any compositional differences (compare pattern 4 for phosphate buffer with pattern 5 for milk dialysate). The α_s- and β-casein components had increased mobility on SGE and were accompanied by degradation products of faster mobilities. The degradation products of κ-casein had decreased mobility on SGE, similar to those previously reported (El-Negoumy, 1968). The Ca_l products from phosphate buffer and from milk dialysate (patterns 3 and 6, respectively) gave similar patterns on SGE. β-Casein with increased mobility on SGE was the main component and was accompanied by the degradation products of other casein components.

After 14 h of rennin action, the Ca_s fractions (Plate 2 (*b*), patterns 2 and 4, for phosphate buffer and milk dialysate, respectively), showed very little resemblance to the controls (patterns 1 and 6). These products showed much lower affinity for Amido Black as compared with similar products obtained after 3 h of rennin action. The Ca_l product from milk dialysate (pattern 5) contained mainly β-casein, while that from phosphate buffer (pattern 3) contained low concentrations of some minor components.

Table 2. *Yields of Ca-sensitive* (Ca_s) *and Ca-insensitive* (Ca_i) *products and percentage solubilized during treatment of whole-, α_s- and β-caseins with rennin in phosphate buffer and in milk dialysate for 3 and 14 h*

	Reaction medium							
	Phosphate buffer + rennin				Milk dialysate + rennin			
	g Precipitated/ 100 g after reaction time of		g Solubilized/ 100 g* after reaction time of		g Precipitated/ 100 g after reaction time of		g Solubilized/ 100 g* after reaction time of	
	3 h	14 h	3 h	14 h	3 h	14 h	3 h	14 h
Whole casein								
Ca_s	76·13	30·80	21·35	68·45	82·21	47·53	8·30	51·92
Ca_i	2·52	0·75	—	—	8·86	0·55	—	—
Whole α_s-casein								
Ca_s	77·54	56·10	20·23	43·90	83·10	62·34	8·92	35·93
Ca_i	2·23	Traces	—	—	7·98	1·73	—	—
β-casein								
Ca_s	47·80	42·38	45·80	54·03	50·66	48·29	48·81	51·36
Ca_i	6·42	3·59	—	—	0·53	0·35	—	—

* Grams solubilized/100 g = (100 g whole, or α_s- or β-casein)—(g Ca_s + g Ca_i product from 100 g).

Whole α_s-casein

After 14 h of rennin action, α_s-casein was degraded much less extensively than was whole casein (see Table 2). After 3 h, rennin action on Ca_s α_s-casein in phosphate buffer gave more than twice as much of the soluble degradation products as was found in milk dialysate. Both the Ca_s and Ca_i products contained much less phosphorus than did the comparable products from α_s-casein controls (see Table 1). This gave a decrease in the P/N ratio from 0·076 for untreated Ca_s α_s-casein to 0·054, after 14 h of rennin action. The content of DNP-arginine, the only N-terminal amino acid found, increased by 1·64 moles/10^5 g Ca_s α_s-casein over that in the untreated product (see Table 1).

After 3 h of rennin action on the α_{s1}-component of Ca_s α_s-casein, 2 derivatives were obtained. The major component had a mobility only slightly faster than the parent α_{s1}-casein; the other was markedly faster moving. No differences in electrophoretic composition resulted from differences in the nature of the reaction medium (Plate 3(a), cf. patterns 5 and 7 for phosphate buffer and milk dialysate, respectively). Except for the increase in gel mobility of the product derived from α_{s1}-casein, no further changes in the composition of Ca_s α_s-casein resulted from 14 h of rennin action (Plate 3 b). The $\alpha_{s2,3,4}$ components showed no change during rennin treatment for 14 h (cf. patterns 5 and 7 for rennin-treated with patterns 1 and 3 for untreated Ca_s α_s-casein).

The Ca_i fractions obtained after 3 and 14 h of rennin action in phosphate buffer (Plate 3(a, b) pattern 6) differed electrophoretically from those obtained from milk dialysate (pattern 8). While the former contained mainly the components $\alpha_{s2,3,4}$, the latter contained the degradation products of α_{s1}-casein.

β-Casein

From 45 to 48 % of β-casein was solubilized during 3 h of rennin action (Table 2). Thus, β-casein was much more extensively degraded during this period than was either whole- or α_s-casein, but subsequent breakdown was much less. In contrast to the untreated controls the phosphorus content and the P/N ratio decreased very significantly in the Ca_s and Ca_l products from rennin-treated β-casein, but the N-terminal DNP-arginine increased by 1·48 moles/10^5 g Ca_s β-casein (Table 1).

The Ca_s product obtained after 3 h of rennin action on β-casein (Plate 4(a), patterns 6 and 8 for phosphate buffer and milk dialysate, respectively), gave similar patterns on SGE to those obtained after 14 h (Plate 4(b), patterns 6 and 8). The action of the enzyme on β-casein gave rise to 2 products. One of these had a mobility slightly faster than the parent β-casein, the other had a substantially faster mobility (Plate 4b).

The Ca_l fractions obtained after 3 h of rennin action (Plate 4(a), patterns 7 and 9) are composed mainly of a spreading zone of β-casein, plus a trace of a faster moving minor component. After 14 h of rennin action the Ca_l fractions (Plate 4(b), patterns 7 and 9) had an electrophoretic composition similar to that of the Ca_s products (patterns 6 and 8) except that the concentration of the faster moving major degradation product of β-casein was much lower.

DISCUSSION

The results presented in this investigation confirm the proteolytic nature of crystalline rennin reported by some investigators (Cerbulis *et al.* 1959, 1960; Lindqvist & Storgårds, 1962; Lahav & Babad, 1964). One of the disadvantages of some of these investigations, however, is the heterogeneous nature of the casein fractions used. When α-casein, prepared by urea fractionation, is used (Nitschmann & Keller, 1955; Lindqvist & Storgårds, 1959; Cerbulis *et al.* 1959; Lahav & Babad, 1964), the degradation products include those obtained from α_s- and κ-caseins. El-Negoumy (1968) has shown that the action of rennin on κ-casein produces several degradation components, with starch gel mobilities as high as or even higher than those of the α_s-casein components. Overlapping of these products would make it difficult to determine their origins. The problem was simplified by the use of more homogeneous casein fractions (α_s- and β-caseins) which were shown to be practically free from κ-casein and the application of high resolution starch gel-electrophoresis.

Our results indicate that the α_{s_1}-component of whole α_s-casein and β-casein give specific products whose patterns on SGE do not alter when rennin action is extended from 3 to 14 h. Such products were not observed when α_s- and β-caseins were incubated in milk dialysate devoid of rennin for 14 h. The resistance of $\alpha_{s_{2,3,4}}$-casein to rennin attack for 14 h is difficult to explain and must await further research. The degradation of β-casein by prolonged rennin action is contrary to results obtained by some investigators (Cherbuliez & Baudet, 1950; Nitschmann & Keller, 1955), although consistent with the results obtained by others (Hagberg & Sullivan, 1953; Lindqvist & Storgårds, 1960; Lahav & Babad, 1964). Changes in the composition of the reaction medium, although influencing the rate of rennin action, appeared to have no effect on the composition of the reaction products.

The Ca_1 products obtained from α_s- and β-caseins before and after rennin action may represent monomeric forms of the Ca_s product, which did not aggregate in the presence of Ca^{2+}. This is supported by the observation that their electrophoretic composition was similar to that of the Ca_s product in part or in whole. The Ca_1 products were prepared by a procedure identical to that used by Lahav & Babad (1964) to isolate similar fractions from α-, β- and γ-caseins. However, our results do not confirm their conclusion that the Ca_1 products from rennin-treated casein fractions have mobilities on SGE different from those of the Ca_s products.

The significant decrease in the P/N ratio in both the Ca_s and Ca_1 products from rennin-treated α_s- and β-caseins (Table 1) suggests that the peptides split by rennin action may be in the form of phosphopeptones but the confirmation of this must await future research. The increase in the molar concentration of N-terminal arginine in α_s- and β-caseins suggests that some peptide bonds are broken during rennin action, thus setting free some new N-terminal arginine. It is also possible that the action of rennin exposes N-terminal arginine residues that are not available in the untreated material.

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EXPLANATION OF PLATES

PLATE 1

(a) Elution curves for a mixture of 125 mg each of whole α- and β-casein preparations and for 125 mg of purified κ-casein. See the text for details of chromatography. - - -, κ-Casein; —, whole α_s- + β-casein. (b) Electrophoresis patterns of whole α_s- ($\alpha_{s1,2,3,4}$) and β-casein controls (rennin-free) at 3 and 6% concentration. Conditions: Starch gels contained 14% hydrolysed starch, 4·5 M urea, 0·20% 2-mercaptoethanol in pH 8·60 tris–citrate buffer. Sodium borate (pH 8·50) as electrode buffer. Power: 10 V and 5 mA for 3 h followed by 175 V and 25 mA for 16 h.

PLATE 2

Degradation products of rennin action on whole casein after 3 h (a) and 14 h (b). Conditions for electrophoresis: Same as in Plate 1.

PLATE 3

Degradation products after 3 h (a) and 14 h (b) of rennin action on whole α_s-casein ($\alpha_{s1,2,3,4}$). Conditions for electrophoresis: Same as in Plate 1.

PLATE 4

Degradation products after 3 h (a) and 14 h (b) of rennin action on β-casein. Conditions for electrophoresis: Same as in Plate 1.

(b)

(a)
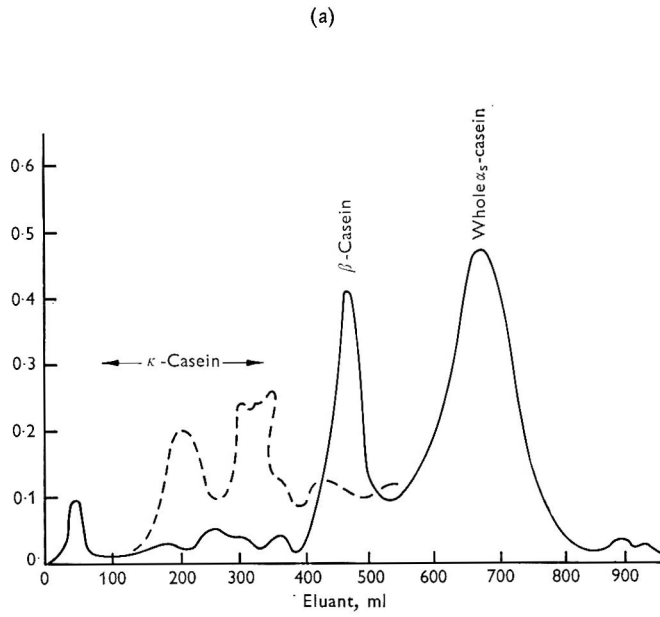
Controls. Incubated for 14 h in milk
dialysate devoid of rennin

A. M. EL-NEGOUMY

α_s- +Rennin

Untreated α_s- (controls)

Milk dialysate

Phosph. buffer

Milk dialysate

Phosph. buffer

Ca_i Ca_s Ca_i Ca_s Ca_i Ca_s Ca_i Ca_s

Degradation products from
α_{s1} - casein

α_{s1} - Casein

α_{s2}
α_{s3}
α_{s4}

α_{s1}
α_{s2}
α_{s3}
α_{s4}

8 7 6 5 4 3 2 1

(b)

Rennin action. 14 h

Untreated α_s- (controls)

α_s- + Rennin

Phosph. buffer

Milk dialysate

Phosph. buffer

Milk dialysate

Ca_s Ca_i Ca_s Ca_i

Ca_s Ca_i Ca_s Ca_i

Degradation products from α_{s1}-casein

α_{s1}-Casein

1   7   6   5   4   3   2   1

(a)

Rennin action, 3 h

(b)

Rennin action, 14 h

β-Casein+rennin

Milk dialysate

Phosph. buffer

Ca$_s$ Ca$_i$ Ca$_s$ Ca$_i$

Fast moving degradation
product from β-casein

Major degradation
product (Ca-
sensitive fraction)

β-Casein

β-Casein

Minor degradation
products from
β-casein

9   8   7   6

(a)

Rennin action, 3 h

The effect of fatty acids on the metabolism of pyruvate in lactic acid streptococci

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SUMMARY. The metabolism of pyruvate by resting whole-cell suspensions of Group N streptococci was studied over the pH range 4·0–7·0, in the presence and absence of oleic acid. In the absence of oleic acid pyruvate was utilized maximally at pH 4·5 with the formation of acetate (volatile acid), acetoin + diacetyl and carbon dioxide. The formation of acetate took precedence over the formation of acetoin + diacetyl. In the presence of oleic acid the utilization of pyruvate was maximal at pH 6·5 and completely inhibited at pH 4·5. The only products detected at pH 6·5 were acetoin + diacetyl and carbon dioxide. This effect of oleic acid on the metabolism of pyruvate was also obtained after treating the cells with acetone. The mechanism of action of oleic acid on cells of Group N streptococci and its possible influence on the formation of flavour compounds in cultured dairy products is discussed.

Oleic acid can both stimulate and inhibit the growth of some lactic acid bacteria, notably lactobacilli. This dual effect is concentration-dependent, stimulation occurring only at the lower concentrations of oleic acid. At concentrations above 10 μg/ml oleic acid can strongly inhibit the growth of some organisms (see review by Nieman, 1954). The observation that the growth of *Streptococcus cremoris* strain C 13 is inhibited by the presence of relatively low concentrations of oleic acid in the growth medium (Anders & Jago, 1964 *a*, *b*) suggested that the loss of viability of this strain in Cheddar cheese early in the ripening period (Dawson & Feagan, 1957; Perry, 1961) may be due to the accumulation of this fatty acid.

Studies carried out by Baker, Northcote & Peters (1962) on pigeon breast mito-chondria, by Dalgarno & Birt (1963) on carrot tissue, and by Coles & Lichstein (1963) on *Lactobacillus arabinosus*, have shown that the activity of some dehydrogenases is inhibited by fatty acids.

There are 2 dehydrogenase systems associated with the metabolism of pyruvate in Group N streptococci, the activities of which appear to be essential for the growth of these organisms. These are the lactate dehydrogenase and the pyruvate dehydro-genase systems. Lactate dehydrogenase activity, which couples the reduction of pyruvate (to form lactate) to the reoxidation of reduced NAD, is essential for con-tinued energy gain from the glycolytic pathway. The results of Reed, DeBusk, Johnston & Getzendaner (1951), Jago (1957) and Reiter & Oram (1962) suggest that Group N streptococci would not initiate growth under aerobic conditions in the

absence of added acetate, without the activity of the lipoic acid-dependent pyruvate dehydrogenase system.

As pyruvate is a precursor of several end-products produced by starter organisms, inhibition by fatty acids of dehydrogenase systems coupled to the metabolism of pyruvate might well effect a change in the end-products produced and thereby influence the flavour of cultured dairy products.

The present paper reports a study of the effect of fatty acids on the metabolism of pyruvate by Group N streptococci.

MATERIALS AND METHODS

Micro-organisms. The starter organisms used were *Str. lactis* strains C6 and C10 and *Str. cremoris* strains C13 and HP. For the preparation of inocula, each strain was grown in sterile skim-milk for 16 h at 30 °C.

Quantities of bacterial cells were prepared by growth in a medium of pH 6·5 containing per l: tryptone (Difco), 30 g; yeast extract (Difco), 10 g; lactose, 5 g; K_2HPO_4, 5 g; and meat extract, 2 g. The medium was filtered and sterilized by autoclaving at 115 °C for 20 min. The cells were harvested by centrifugation and washed twice with 0·9 % NaCl, and resuspended in this medium (10 mg dry wt/ml) before use.

Acetone-dried cells were prepared as described by Gunsalus (1955).

Studies on respiration. The carbon dioxide released and the oxygen taken up by suspensions of whole cells were measured in a conventional Warburg apparatus. The reaction mixture contained: 300 μmoles of Na_2HPO_4 adjusted to the desired pH with 0·1 M-citric acid; 10 μmoles of $MnSO_4$; 60 μmoles of sodium pyruvate (unless stated otherwise) and 1 ml of the bacterial suspension, in a total volume of 3 ml. The reaction was started by addition of the pyruvate from the side-arm of the reaction flask. For anaerobic studies the air in the flasks was replaced with oxygen-free nitrogen.

The reaction was stopped by placing the flasks in an ice–salt bath. The contents of each flask were centrifuged and the supernatant stored at − 20 °C until analysed for residual pyruvate and for products arising from the pyruvate metabolized.

Estimation of pyruvate. Pyruvate was estimated by the method of Friedemann & Haugen (1943). Benzene was used in the initial extraction of the dinitrophenyl-hydrazine derivative of pyruvate.

Estimation of acetoin + diacetyl. Acetoin + diacetyl was estimated as diacetyl by the method of Westerfeld (1945).

Estimation of lactic acid. Lactic acid was estimated by the method of Hullin & Noble (1953).

Estimation of acetic acid. Acetic acid was estimated as volatile acid by the following procedure: 2 ml of the supernatant obtained from the incubation mixture were added to a Markham still, together with 3 g of $MgSO_4$, 1 ml of 10 % orthophosphoric acid and 3 ml of distilled water. Steam was passed continuously through the solution in the still until 50 ml of distillate was collected. A 15 ml portion of the distillate was titrated against 0·01 M-NaOH, which was delivered from an Agla micro-syringe while the solution was flushed continuously with carbon dioxide-free air. The end-point was obtained by following the titration with a Radiometer pH meter.

Estimation of lactate dehydrogenase activities. NAD-dependent lactate dehydrogenase activity was estimated from the change in absorbance at 340 nm of a reaction mixture which contained in a total volume of 3 ml: 250 μmoles of triethanolamine adjusted to pH 7·0 with acetic acid; 20 μmoles of sodium pyruvate; 0·4 μmoles of reduced NAD, and 0·1 ml of bacterial suspension.

Preparation of fatty acids. Fatty acids were added to the reaction mixtures in solution in the buffer. These solutions were prepared by adding an equivalent of concentrated NaOH to the fatty acid. The salt formed was dissolved in the basic component of the buffer, the pH of which was adjusted with the acidic component immediately before use.

RESULTS

Effect of oleic acid on the metabolism of pyruvate. Warburg manometry showed that suspensions of whole cells of *Str. cremoris* strain C13 readily metabolized pyruvate at pH 7·0. The addition of oleic acid to the reaction mixture (final concentration 5 mM) had a marked effect on the respiration. As shown in Fig. 1 there was an inhibition of the uptake of oxygen and an increase in the carbon dioxide evolved.
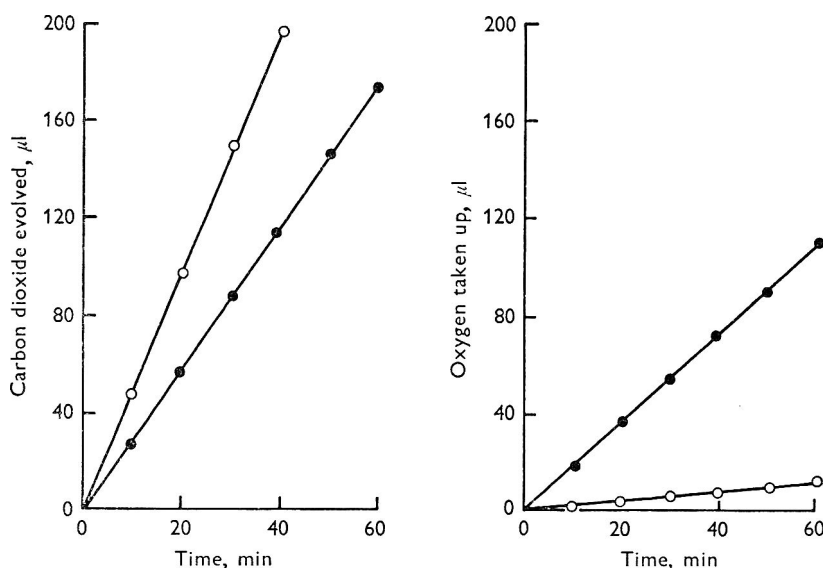
Fig. 1. Uptake of oxygen and evolution of carbon dioxide by suspensions of whole cells of *Streptococcus cremoris* strain C13 in absence (●) and in presence (○) of oleic acid. The reaction mixture contained 300 μmoles of Na_2HPO_4 adjusted to pH 7·0 with 0·1 M-citric acid; 10 μmoles of $MnSO_4$; 60 μmoles of sodium pyruvate and 1 ml of bacterial suspension (10 mg dry wt) in a total volume of 3 ml. All incubations were at 30 °C. Values for the evolution of carbon dioxide were corrected for any retention of carbon dioxide in solution as bicarbonate.

The results plotted in Fig. 2 show that the rate at which pyruvate was utilized by whole cells of strain C13 was maximal at pH 4·5. The amount of carbon dioxide evolved showed the same variation with pH as did the amount of pyruvate utilized. Over the whole pH range studied the molar ratio of carbon dioxide evolved to pyruvate utilized was approximately 1:1.

Within the pH range 5·5–7·0, for each mole of carbon dioxide evolved there was a mole of acetate formed. However. the increased utilization of pyruvate and evolution of carbon dioxide between pH 5·5 and 4·5 was not associated with an increased formation of acetate but was explained partly by the formation of acetoin + diacetyl.
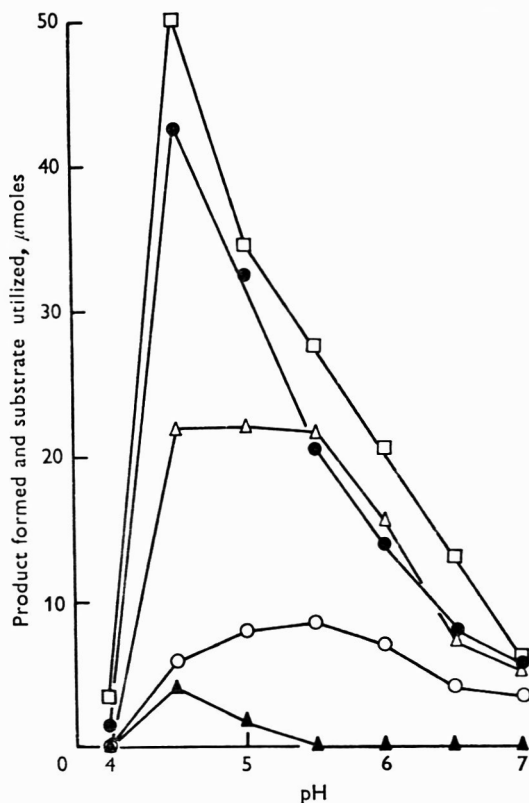
Fig. 2. Effect of pH on the metabolism of pyruvate by suspensions of whole cells of *Strepto-coccus cremoris* strain C13. Details of the reaction are given in Fig. 1. □, Pyruvate utilized; ●. carbon dioxide evolved; ○, oxygen uptake; △, acetate formed; ▲, acetoin + diacetyl formed.

Within the pH range 5·5–7·0 the uptake of oxygen was proportional to the amount of acetate formed. However, at pH 4·5 and 5·0 the uptake of oxygen was proportion-ally less (see Fig. 2)

The effect of oleic acid on the metabolism of pyruvate over the pH range 4·0–7·0 can be seen by comparing Fig. 2 with Fig. 3. At pH values from 4·5 to 5·5 the utiliza-tion of pyruvate and the evolution of carbon dioxide were markedly inhibited. while above pH 6·0 both were stimulated. The uptake of oxygen and the formation of acetate were inhibited by oleic acid over the whole pH range studied. Whereas acetate and carbon dioxide were the only products detected in the absence of oleic acid at pH 6·0 (Fig. 2), acetoin + diacetyl and carbon dioxide were the only products found in the presence of oleic acid at the same pH (Fig. 3).

The effect of anaerobiosis on the metabolism of pyruvate by strain C13 is shown in Figs 4(a, b). In the absence of oxygen the formation of acetate was inhibited so that carbon dioxide and acetoin + diacetyl were the only products formed in significant

amounts. As before, the rate of formation of carbon dioxide and acetoin + diacetyl was highest at pH 4·5 in the absence of oleic acid, and highest at pH 6·5–7·0 in the presence of oleic acid·

In the above studies oleic acid was added in a final concentration of 5 mM. Table 1 shows the effect of lower concentrations of oleic acid on the metabolism of pyruvate. The same effect was observed with 0·5 mM as with 5·0 mM oleic acid. However, at a concentration of 0·1 mM, oleic acid had no effect.
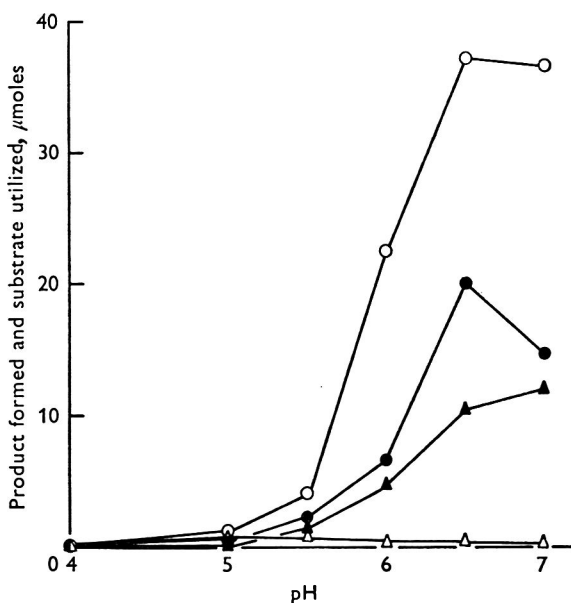
Fig. 3. Effect of pH on the metabolism of pyruvate by suspensions of whole cells of *Streptococcus cremoris* strain C 13, in the presence of oleic acid. Details of the reaction are given in Fig. 1 except for the inclusion of 15 μmoles of oleic acid in the reaction mixture. ○, Pyruvate utilized; ●, carbon dioxide evolved; △, acetate formed; ▲, acetoin + diacetyl formed.

As shown in Table 2, oleic acid appeared to affect the metabolism of pyruvate by other strains of Group N streptococci in a manner similar to that observed for *Str. cremoris* strain C 13.

Because of the high cost of pure oleic acid, the material used in the experiments was of Laboratory Reagent grade, supplied by British Drug Houses Ltd, and its oleic acid content was only 81 %, as determined by gas–liquid chromatography. The remaining 19 % was made up by the following fatty acids, %: myristic, 3·42; myristoleic, 0·88; palmitic, 5·87; palmitoleic, 4·86; stearic, 0·71; others, 4·01. Substitution of the BDH reagent by chromatographically pure oleic acid (obtained from Applied Science Laboratories Inc.) produced the same effect on the metabolism of pyruvate by Group N streptococci.

Evidence supporting a purely physiochemical effect of oleic acid was gained by comparing the long-chain fatty acid composition of the reaction mixture before and after incubation. The fatty acid composition was identical in both cases with that given above. These results indicated that there was no specific utilization of oleic acid by the bacterial cells during the incubation period.
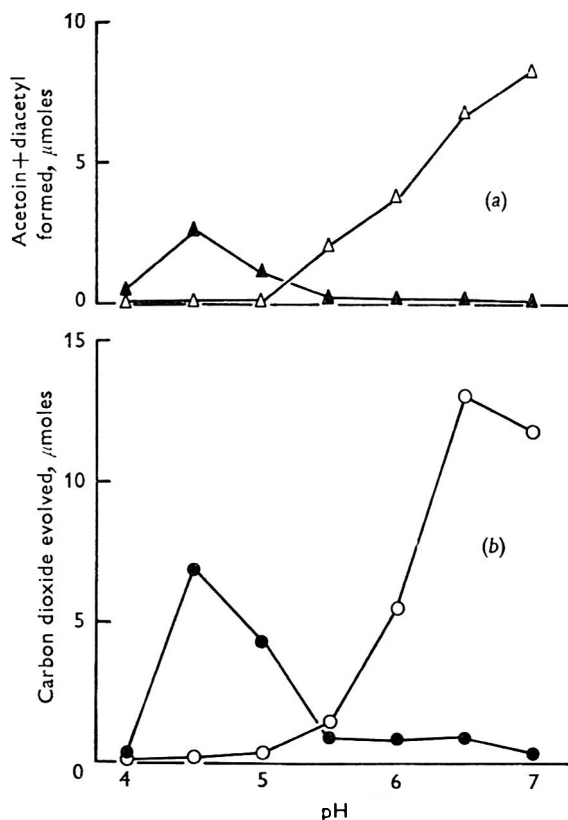
Fig. 4. Effect of oleic acid on the metabolism of pyruvate by *Streptococcus cremoris* strain C13 under anaerobic conditions. Details of the reaction are given in Fig. 1. The atmosphere in the flasks was nitrogen. Acetoin+diacetyl formed in absence (▲) and in presence (△) of 5 mM oleic acid. Carbon dioxide evolved in absence (●) and in presence (○) of 5 mM oleic acid.

Table 1. *Effect of oleic acid concentration on the metabolism of pyruvate in* Streptococcus cremoris *strain C 13*

Concentration of oleic acid, mM	O_2 taken up, μmoles		CO_2 evolved, μmoles		Acetoin + diacetyl formed, μmoles	
	pH: 5·0	6·5	5·0	6·5	5·0	6·5
0·0	6·5	4·0	42·0	6·5	4·0	0·0
0·1	8·2	5·5	38·0	8·1	3·8	0·0
0·5	3·6	2·4	9·4	15·0	0·8	7·0
5·0	0·0	0·4	0·0	14·2	0·0	9·8

Reactions were carried out for 1 h at 30 °C in a conventional Warburg apparatus, under aerobic conditions. The reaction mixture contained 300 μmoles of Na_2HPO_4 adjusted to the appropriate pH with 0·1 M citric acid; 10 μmoles of $MnSO_4$; 60 μmoles of sodium pyruvate and 1 ml of a suspension of whole cells of *Str. cremoris* strain C13 (10 mg dry wt), in a total volume of 3 ml. Analytical procedures were as described under Methods.

Conditions which simulate the effect of oleic acid

Drying of cells with acetone. When cells of *Str. cremoris* strain C13 were dried in acetone and incubated with pyruvate there was no uptake of oxygen and no forma-tion of acetate. The only products formed were carbon dioxide and acetoin + diacetyl

(Table 3). The ratio of moles of carbon dioxide evolved to moles of acetoin + diacetyl formed was approximately 2 over the pH range 6·0–7·0. The formation of acetoin + diacetyl by cells treated either with acetone or with oleic acid showed the same dependence on pH (see Fig. 5).

Table 2. *Effect of oleic acid on the metabolism of pyruvate by*
group N streptococci

| | | Acetoin + diacetyl formed, μmoles | | | |
| | | pH 4·5 | | pH 6·5 | |
Species	Strain	− Oleic	+ Oleic	− Oleic	+ Oleic
Streptococcus cremoris	C13*	2·9	0·0	0·1	6·5
Str. cremoris	HP	6·3	0·8	0·2	2·5
Streptococcus lactis	C10	11·3	0·0	0·4	5·0
Str. lactis	C6	7·4	0·1	0·2	9·7

Reactions were carried out as described in Table 1. The concentration of oleic acid used was 1 mM, and that of the bacterial cells was 10 mg (dry wt) in a total volume of 3 ml.
* Oleic acid-resistant variant (Anders & Jago, 1964b).

Table 3. *Products of the metabolism of pyruvate by acetone-dried cells*
of Streptococcus cremoris *strain* C 13

pH	CO_2 evolved, μmoles	Acetoin + diacetyl formed, μmoles
5·0	0·4	—
6·0	8·8	4·0
6·5	12·3	6·0
7·0	12·1	6·4

Reactions were carried out as described in Table 1 except that the incubation period was 30 min.

Presence of arsenite. The addition of arsenite to the reaction mixture (final concentration 1 mM) resulted in a change in the metabolism of pyruvate by strain C13 which resembled that produced by the addition of oleic acid. As shown in Table 4 the addition of arsenite at pH 6·5 partially inhibited the uptake of oxygen and the formation of acetate, whereas the formation of acetoin + diacetyl was stimulated. However, the addition of arsenite at pH 5·0 and 4·5 completely inhibited the uptake of oxygen and the formation of acetate, whereas the formation of acetoin + diacetyl was only partially inhibited.

High concentration of pyruvate. As the concentration of pyruvate in the incubation mixture was increased from 10 to 60 mM there was only a relatively small increase in the uptake of oxygen and in the amount of acetate formed. There was, however, a marked increase in the amount of acetoin + diacetyl formed (Table 5). Only a trace amount of acetoin + diacetyl was detected when the concentration of pyruvate in the reaction mixture was 10 mM. At the 2 highest concentrations of pyruvate used (50–60 mM), at least 20 % of the pyruvate utilized was metabolized to acetoin + diacetyl.

Presence of capric acid. The metabolism of cells treated with capric acid was

similar to that of cells treated with oleic acid in that there was an increased formation of acetoin + diacetyl at pH 7·0 (Table 6). However, this increase was considerably less than that which occurred in the presence of oleic acid.
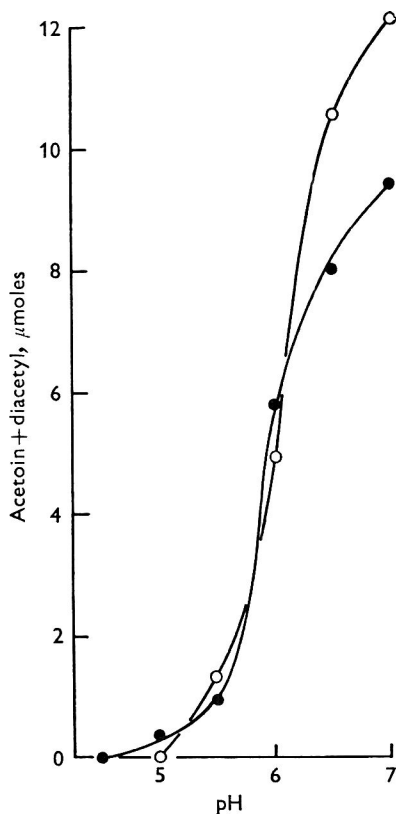
Fig. 5. The effect of pH on the formation of acetoin + diacetyl by *Streptococcus cremoris* strain C13 (○) acetone-dried, (●) in the presence of 5 mM oleic acid. Details of the reaction are given in Fig. 1.

Table 4. *Effect of arsenite on the metabolism of pyruvate in* Streptococcus cremoris *strain C 13*

pH	O$_2$ taken up, μmoles		CO$_2$ evolved, μmoles		Acetoin + diacetyl formed, μmoles		Acetate formed, μmoles	
	− As	+ As	− As	+ As	− As	+ As	+ As	+ As
6·5	3·3	0·9	5·2	2·8	0·04	0·59	5·3	1·0
5·0	8·1	0·0	25·0	3·7	3·5	2·3	18·0	0·0
4·5	6·5	0·0	32·0	3·9	8·3	2·5	17·1	0·0

Reactions were carried out as described in Table 1. The concentration of arsenite used was 1 mM.

Formation of lactate

Lactate was not formed in any appreciable amount from pyruvate under the conditions used in the manometric assays. Moreover, when suspensions of whole cells of *Str. cremoris* strain C 13 were examined spectrophotometrically for lactate dehydro-

genase activity no activity was observed (Table 7), presumably because of the impermeability of the cell to the large reduced NAD molecule. When capric acid was added to the reaction mixture, marked dehydrogenase activity became immediately apparent. Essentially the same effect was observed with the oleic acid-

Table 5. *Effect of substrate concentration on the metabolism of pyruvate in* Streptococcus cremoris *strain C* 13

Concentration of pyruvate, mM	Pyruvate consumed, μmoles	O_2 taken up, μmoles	CO_2 evolved, μmoles	Acetoin + diacetyl formed, μmoles	Acetate formed, μmoles
10	18·2	8·2	16·9	0·1	17·4
20	25·1	9·6	21·5	0·5	22·0
30	31·0	12·8	34·6	1·6	26·4
40	38·2	12·9	40·1	2·1	26·8
50	49·8	12·9	46·6	4·2	27·4
60	47·6	12·9	49·5	5·5	28·5

Reactions were carried out as described in Table 1, at pH 5·5 and at the pyruvate concentrations shown.

Table 6. *Effect of capric acid on the metabolism of pyruvate by* Streptococcus cremoris *strain C* 13

Concentration of capric acid, mM	O_2 taken up, μmoles	CO_2 evolved, μmoles	Acetoin + diacetyl formed, μmoles	Acetate formed, μmoles
0	4·6	7·4	0·0	4·5
5	5·2 (0·4)	9·6 (13·3)	4·8 (9·8)	1·3 (1·0)

Reactions were carried out as described in Table 1 at pH 7·0. In parentheses are the values obtained in presence of 5 mM oleic acid.

Table 7. *Lactate dehydrogenase activity of suspensions of whole cells of Group N streptococci in solutions of fatty acids*

Species	Strain	Relative activity				
		a	b	c	d	e
Streptococcus cremoris	C13	−	−	+ + +	−	+ + + +
Str. cremoris	C13*	−	−	+ + +	−	+ + + +
Streptococcus lactis	C10	−	−	+ + +	+	+ + + +
Streptococcus diacetilactis	DRC1	+	−	+ +	+	+ + +

* Variant resistant to oleic acid (Anders & Jago, 1964b). a, No fatty acid; b, 5 mM oleic acid; c, 5 mM capric acid; d, cells incubated in 5 mM oleic acid for 1 h before the assay; e, cells incubated in 5 mM capric acid for 1 h before the assay.

Experimental details for the assay of lactate dehydrogenase activity are given under Methods. Approximately 1 mg (dry wt) of cells was used in each assay.

resistant *Str. cremoris* strain C13, *Str. lactis* strain C10 and *Str. diacetilactis* strain DRC1. However, strain DRC1 did not exhibit as great a response to the addition of capric acid as did the other 3 strains (Table 7). The addition of oleic acid to the reaction mixture did not produce this effect.

When the cell suspensions were incubated with fatty acid for 1 h before the assay

of lactate dehydrogenase activity, the effect of capric acid was slightly more pronounced. Whereas oleic acid still had no effect on either of the *Str. cremoris* strains, cell suspensions of *Str. lactis* strain C 10 and *Str. diacetilactis* strain DRC 1 did exhibit dehydrogenase activity under these conditions (Table 7).

When cells of these organisms were dried in acetone, before the assay of lactate dehydrogenase activity, marked activity was demonstrated in all the organisms. As shown in Table 8, the addition of oleic acid to the assay system inhibited this activity, whereas the addition of capric acid caused a marked stimulation.

Table 8. *Lactate dehydrogenase activity of suspensions of acetone-dried cells of Group N streptococci in solutions of fatty acids*

| | | Relative activity | | |
| | | No fatty acid | 5 mM oleic acid | 5 mM capric acid |
Species	Strain			
Streptococcus cremoris	C13	+ + +	+	+ + + +
Str. cremoris	C13*	+ + +	+	+ + + +
Streptococcus lactis	C10	+ + +	+	+ + + +
Streptococcus diacetilactis	DRC1	+ + +	+	+ + + +

* Variant of C 13 resistant to oleic acid (Anders & Jago, 1964 b).

Experimental details for the assay of lactate dehydrogenase activity are given under Methods; 1 mg (dry wt) of acetone-dried cells was used in each assay.

DISCUSSION

Metabolism of pyruvate. Pyruvate was metabolized optimally at pH 4·5 by suspensions of whole cells of *Str. cremoris* strain C 13. This pH value probably does not represent the pH optimum of the enzymes utilizing pyruvate, but rather the pH at which exogenous pyruvate passes most easily into the cells.

In the absence of an active transport mechanism, pyruvate would pass more easily into the cell at pH 4·5 than at pH 7·0 because, at the lower pH, more pyruvate ($K = 3·2 \times 10^{-3}$) would exist in the uncharged non-dissociated form. Thus, when the cell membrane was made more permeable by treating the cell with acetone the utilization of pyruvate was increased at pH 7·0.

Acetoin + diacetyl, volatile acid and carbon dioxide were the only products detected when suspensions of whole cells of *Str. cremoris* strain C 13 were incubated with pyruvate in a conventional Warburg apparatus. Volatile acid production, which was closely paralleled by the uptake of oxygen, was assumed to be predominantly acetate formed via a pyruvate dehydrogenase system closely coupled to terminal oxidases.

In the present investigation no attempt was made to distinguish between acetoin and diacetyl formation. While pyruvate is the precursor of both of these compounds in Group N streptococci, diacetyl cannot be formed in the absence of acetyl-CoA (Speckman & Collins, 1968). Thus inhibition of the pyruvate dehydrogenase system by oleic acid, as shown by the inhibition of oxygen uptake and acetate formation, would preclude the formation of diacetyl in the presence of oleic acid. The values for acetoin + diacetyl produced in the presence of oleic acid would be, presumably, those for acetoin only.

The formation of acetoin and diacetyl from pyruvate proceeds with the liberation of 2 moles of carbon dioxide for each mole of acetoin or diacetyl formed. In this investigation a 2:1 ratio for these products was not always obtained. However, when studies were carried out with acetone-dried cells and carbon dioxide and acetoin + diacetyl were the only products formed, a ratio of 2:1 was obtained over a wide pH range.

In many experiments it was not possible to account for all of the pyruvate utilized. Retention of pyruvate (and products) within the cells may, in part, explain this. The greatest discrepancy between pyruvate utilized and that accounted for as products was found in incubation mixtures where acetoin + diacetyl were the only products formed. Gunsalus (1958) has reported that the pathway forming acetoin in lactic acid bacteria required a substrate concentration which was higher than that required by other pathways utilizing pyruvate, from which it may be inferred that one or more of the enzymes involved had a high Michaelis constant. If this is so, and the results of the present investigation with lactic acid streptococci support this view, the intracellular concentration of pyruvate would be higher when the pathway forming acetoin + diacetyl was the only pathway utilizing pyruvate.

Of interest was the fact that lactate did not appear as a product of the metabolism of pyruvate under the conditions used in this investigation. It made no difference whether the reaction mixtures were incubated aerobically or anaerobically. The formation of lactate from pyruvate is dependent on a supply of reduced coenzyme. In the absence of glucose, and when pyruvate is the only substrate present, the only source of reduced NAD would be that arising from the oxidative decarboxylation of pyruvate to acetate. Thus, under aerobic conditions, reduced NAD from this source appeared to be preferentially reoxidized in these bacteria by molecular oxygen. As no acetate was formed from pyruvate anaerobically, no reduced NAD would be formed under these conditions. These results indicated that the Group N streptococci do not couple reduction of pyruvate with reoxidation of reduced NAD formed via the pyruvate dehydrogenase system and therefore lack the dismutative activity found in *Lactobacillus delbrueckii* (Hager & Lipmann, 1955).

Effect of oleic acid on the metabolism of pyruvate. Oleic acid added to suspensions of whole cells was shown markedly to alter the metabolism of pyruvate by Group N streptococci. It is suggested that the altered pattern of metabolism is the result of a 2-fold effect by the fatty acid.

First, treatment of the cell with oleic acid appears to alter the permeability of the cell membrane so that the cell can no longer regulate the intracellular pH independently of the external pH. This effect was postulated by Coles & Lichstein (1963) to explain partly the inhibition of the malic enzyme in *Lactobacillus arabinosus* by oleic acid.

Secondly, oleic acid appears to inhibit specifically an enzyme involved in the formation of acetate. This pathway required a lower concentration of substrate for maximum activity than did the pathway forming acetoin and diacetyl (see Table 5). Therefore at pH 7·0, in absence of oleic acid, the intracellular concentration of pyruvate is maintained at a low level because the rate at which acetate is formed is limited by the low rate at which pyruvate enters the cell. Under these conditions acetoin + diacetyl is not formed. In presence of oleic acid the intracellular concentra-

tion of pyruvate increases because of the increased permeability of the cell to pyruvate and because the pathway forming acetate is inhibited. Under these latter conditions the concentration of pyruvate in the cell reaches a level at which the enzymes forming acetoin + diacetyl become active. This explanation of the effect of oleic acid is supported by the changes produced in the metabolism of pyruvate by other treatments applied to the bacterial cell. Treatment with arsenite inhibited the formation of acetate and the uptake of oxygen and stimulated the rate of formation of acetoin + diacetyl. Increasing the concentration of pyruvate in the reaction mixture led to an increased formation of acetoin + diacetyl only when the concentration of pyruvate was high enough to saturate the acetate pathway. Treatment with capric acid did not inhibit the formation of acetate but it did increase the amount of acetoin + diacetyl formed at pH 7·0, presumably by damaging the cell membrane so that it became a less efficient barrier to the pyruvate anion.

As the effect of oleic acid on the metabolism of pyruvate by *Str. cremoris* strain C13 required a higher concentration of oleic acid than was required for inhibition of growth, it seems doubtful whether the growth inhibition was related to the effect on pyruvate metabolism. Other organisms which were resistant to inhibition by relatively high concentrations of oleic acid also showed an altered metabolism of pyruvate in presence of oleic acid.

It is evident that the accumulation of fatty acids, particularly oleic, in cultured dairy products could influence the metabolism of the organisms present and thereby the nature of the end-products formed. For example, inhibition by oleic acid of the pyruvate dehydrogenase system in Group N streptococci would inhibit the formation of such flavour compounds as diacetyl, acetaldehyde and ethanol.

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The inactivation of lactoperoxidase by group N streptococci

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The inactivation of lactoperoxidase (LP) by cultures of Group N streptococci was originally reported by Kiermeier & Kayser (1960). However, Pickering, Oram & Reiter (1962) found that incubation of *Streptococcus cremoris* 803 with LP did not completely inactivate the enzyme, but only modified its activity with certain substrates. This observation was subsequently confirmed by Kiermeier & Lechner (1966, 1967). In the present investigation, several strains of Group N streptococci were found to modify LP in such a way as to cause total or partial loss of enzymic activity with guaiacol. This loss of LP activity was found to be a consequence of the production of hydrogen peroxide by these organisms.

When LP, prepared by the method of Hogg & Jago (1970), was incubated with a suspension of *Streptococcus lactis* C 10, the activity of the enzyme, as measured by the rate of oxidation of guaiacol (Maehly & Chance, 1954), rapidly decreased. The rate of inactivation increased with cell concentration, as is shown in Fig. 1.

The LP-inactivating factor was found to be present in the supernatant liquid obtained by centrifuging a suspension of *Str. lactis* C 10 cells in 0·05 M-sodium phosphate solution of pH 7·0, after incubation under aerobic conditions. The ability of the supernatant to inactivate LP was destroyed by the addition of catalase. The supernatant obtained after incubating the cell suspension anaerobically was not active against LP. The dependence of oxygen for its formation, and its destruction by catalase, strongly suggested that the substance which inactivated LP was hydrogen peroxide.

Estimation of the hydrogen peroxide concentrations in supernatants of strepto-coccal cultures revealed a correlation between hydrogen peroxide concentrations and the rates at which the supernatants inactivated LP. As is shown in Table 1, most strains of Group N streptococci inactivated LP to approximately the same extent but *Str. lactis* C 10 showed considerably greater activity which was found to be cor-related with a relatively high concentration of hydrogen peroxide in the incubation medium.

Significant inactivation of LP by hydrogen peroxide at concentrations of 0·176 mM was reported by Chance (1950), and kinetic studies of various LP-catalysed reactions also indicated marked inhibition of LP activity at hydrogen peroxide concentrations of 0·1 mM (Hogg & Jago, 1970). In the present studies, the hydrogen peroxide con-centrations in the supernatants associated with inactivation of LP ranged from 0·024 to 0·111 mM, values which according to the results quoted above would be expected to bring about significant inactivation of LP.

The formation of hydrogen peroxide by Group N streptococci can occur through the action of NADH oxidase (Anders, Hogg & Jago, 1970) which catalyses the reaction

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2.$$
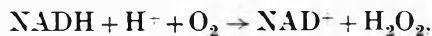
while the utilization of hydrogen peroxide can occur through the action of an NADH peroxidase which catalyses the reaction,

$$NADH + H^+ + H_2O_2 \rightarrow NAD^+ + 2H_2O.$$
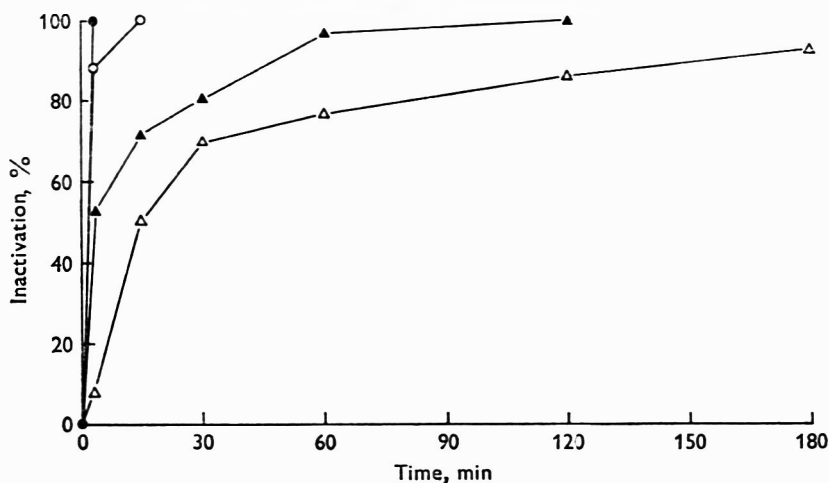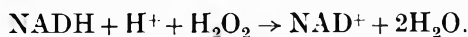
Fig. 1. The effect of cell concentration on the activation of LP by *Streptococcus lactis* C10. Cells of *Str. lactis* C10 were incubated with 0·14 μM-LP in 0·05 M-sodium phosphate, pH 7·0, at 30 C. Cell concentrations, measured by absorbance at 500 nm, were as follows: ●, 0·053: ○, 0·027: ▲, 0·013: △, 0·007.

Table 1. *Accumulation of hydrogen peroxide by Group N streptococci*

Organism	Inactivation of LP, %		Concentration of H_2O_2 in supernatant, μM
	Cells	Supernatant	
Streptococcus lactis C10	100	89	111
Streptococcus cremoris C13	56	50	24
Str. cremoris 972	67	67	27
Str. cremoris ML1	64	64	25

Cell suspensions and supernatants were incubated for 15 min at 30 °C with 0·33 μM-LP in 0·05 M-sodium phosphate solution of pH 7·0. Hydrogen peroxide in cell supernatants was estimated colorimetrically by the oxidation of guaiacol in the presence of horseradish peroxidase. The reaction mixture contained 1·9 ml of H_2O_2 solution, 1·0 ml of 0·020 M guaiacol and 0·1 ml of horseradish peroxidase (L. Light & Co.) at a sufficient concentration to cause immediate utilization of all the H_2O_2 in the oxidation of guaiacol. The absorbance at 470 nm, which was measured immediately after completion of the reaction, was found to be directly proportional to H_2O_2 concentrations over the range 0–0·15 mM.

These 2 reactions would compete for available NADH, and the relative extent to which they occurred would determine the amount of hydrogen peroxide that accumulated (Stadhouders & Veringa, 1962). It has been found that the NADH oxidase from *Str. lactis* C10 has a greater affinity for oxygen and NADH than have the correspond-

ing enzymes in other strains, and that the ratio of NADH oxidase activity to NADH peroxidase activity under the conditions used was also greater in this strain (Anders *et al.* 1970), thereby favouring higher levels of hydrogen peroxide accumulation.

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461

Nutritional deficiencies of co-precipitate for the growth of a strain of *Lactobacillus acidophilus*

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SUMMARY. The nutritional deficiencies of co-precipitate as a growth medium for a strain of *Lactobacillus acidophilus* have been studied. Acid production by this organism was insignificant in a medium composed of 4 % low-calcium co-precipitate and 5 % lactose.

Additions of the amino acids phenylalanine, tyrosine, valine, cysteine and threonine as well as of nicotinic acid, manganese, magnesium and citric acid all increased acid production.

When only half the milk solids of skim-milk powder was replaced by the co-precipitate, fortification with manganese only was required.

Following the development of a continuous process for the manufacture of a range of co-precipitates (casein plus most of the whey proteins) of varying calcium contents, the manufacture of these products has grown rapidly in Australia. They are made by the precipitation of the proteins from milk by the use of calcium chloride, acid and heat (Buchanan, Snow & Hayes, 1965).

Co-precipitates are at present finding an increasingly wide use in the manufacture of various foods. There has been interest in Japan in the use of co-precipitate as a substitute for some of the skim-milk powder used in the manufacture of fermented milks. The organism used in the fermentation is usually *Lactobacillus acidophilus*. Acid production by this organism in co-precipitate solutions was significantly slower than in reconstituted skim-milk powder even where a co-precipitate was used to replace only a part of the milk solids. A study has therefore been made of the nutritional deficiencies of co-precipitate as a growth medium for this organism.

EXPERIMENTAL

A culture of *L. acidophilus* strain NZ was grown in reconstituted skim-milk for 24 h at 37 °C. A 3 % inoculum from this culture was added to the test medium.

A complete medium containing a low-calcium co-precipitate, lactose and the substances listed in Table 1 was prepared. Of this medium 10-ml portions were inoculated with *L. acidophilus* strain NZ. Each of the substances included in Table 1 was omitted in turn and the activities of the *L. acidophilus* in these media and in the complete medium were compared. Activities were measured by titrating the acidity to the end-point at pH 8·3 after incubation for 16 h at 37 °C. A blank titration was performed for each medium.

The minimum levels of the various components which were found in the above tests to be stimulatory to acid production were determined by varying their concentration in turn while the concentrations of all the other components were kept constant. The substances and levels tested are listed in Table 2. Possible effects of minerals on the buffering capacity and thus on the acid development were checked by obtaining a titration curve for each medium.

Table 1. *Basal medium*

	mg/ml		μg/ml
L-Cystoine-HCl	0·2	Riboflavin	2·0
L-Tryptophan	0·4	Folic acid	0·2
L-Tyrosine	0·08	Vitamin B12	0·002
L-Phenylalanine	0·08	Nicotinic acid	2·0
L-Proline	0·04	Pantothenic acid	2·0
L-Histidine	0·04	Thiamin	2·0
L-Methionine	0·2	Biotin	0·01
L-Lysine	0·2	Pyridoxal	2·0
L-Alanine	0·05	Para-aminobenzoic acid	2·0
L-Arginine	0·05	Lipoic acid	2·0
L-Aspartic acid	0·05	Adenine	5·0
L-Glutamic acid	0·05	Guanine	5·0
L-Glycine	0·05	Uracil	5·0
L-Isoloucine	0·03	Xanthine	5·0
L-Leucine	0·05	Citric acid	500·0
L-Serine	0·05		
L-Threonine	0·05		
L-Valine	0·05		

In addition the medium contained 4% low-calcium co-precipitate, 5% lactose, 0.33% K_2HPO_4 and the following minerals (in mg/ml): $MgSO_4$, $7H_2O$, 0·23; $ZnSO_4$, $7H_2O$, 0·005; $CuSO_4$, $5H_2O$, 0·0025; $FeCl_3$, $6H_2O$, 0·005; $MnSO_4$, $5H_2O$, 0·0027; VSO_4, 0·0025; $CaCl_2$, 0·05; $CoCl_2$, $6H_2O$, 0·0025; Na_2MoO_4, 0·0025.

Table 2. *Range of concentrations tested*

Stimulatory substance	Range of concentrations tested, %	
Amino acids: threonine, valine, tyrosine, phenylalanine	0·00125–0·025 Serial dilutions: 2-fold	
Cysteine-HCl	0·002–0·08	2-fold
Vitamins:		
Nicotinic acid	0·00002–0·002	10-fold
Minerals:		
$MgSO_4$, $7H_2O$	0·0023–0·018	2-fold
$MnSO_4$, $5H_2O$	0·00136–0·011	2-fold
Organic compounds:		
Citric acid	0·005–0·5	10-fold

The effect of heat treatment of co-precipitate on its nutritional properties for this strain of *L. acidophilus* was also studied. For this purpose some of the co-precipitate solutions were autoclaved momentarily to 121 °C and others were autoclaved for 10 min at 121 °C.

Comparison of casein and co-precipitate

The nutritional properties of casein for *L. acidophilus* strain NZ were compared with those of co-precipitate by substituting casein for the co-precipitate in the basal

medium and determining the growth of this organism with and without the factors found to be stimulatory when co-precipitate was used. As a control, co-precipitate with and without these factors was used.

Co-precipitate as a substitute for milk solids

Milk solids other than lactose in reconstituted skim-milk were replaced with low-calcium co-precipitate to the extent of $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{3}{4}$. The mixtures were fortified with the stimulatory factors found necessary in the previous experiments. Each of these factors was omitted in turn to determine whether the milk powder would provide a sufficient level of any of these factors when the co-precipitate and milk powder were combined. The activities of the culture tested in all these mixtures were compared with that of the culture grown in pure reconstituted skim-milk. Finally, each of the factors found to be stimulatory was tested for its effect when added as the sole supplement.

RESULTS

Acid production by the strain of *L. acidophilus*, tested in a co-precipitate medium fortified with lactose alone, was almost imperceptible. Table 3 shows that for maximum acid production in co-precipitate media this strain required phenylalanine, tyrosine, valine, cysteine, threonine, nicotinic acid, manganese, magnesium and citric acid in addition to lactose.

Table 3. *Composition of medium supporting acid production to a level approximately similar to that produced in skim-milk*

	%
Co-precipitate (low-calcium)	4
Lactose	5
K_2HPO_4	0·33
L-Phenylalanine	0·0025
L-Tyrosine	0·0025
L-Valine	0·0025
L-Threonine	0·0025
L-Cysteine-HCl	0·025
Nicotinic acid	0·00002
$MgSO_4$, $7H_2O$	0·018
$MnSO_4$, $5H_2O$	0·00136
Citric acid	0·05

Cysteine-HCl was found to be stimulatory but the amount required was influenced by the degree of heat treatment to which the co-precipitate solution had been subjected. In the less severely heat-treated solution the effect of increasing cysteine levels from 0·005 to 0·025 % was more marked than in the solution sterilized at 121 °C for 10 min, where the increase caused no significant increase in acid production. Sodium sulphite (M/2000 to M/500) had no effect.

Other data, not shown in Table 3, indicated that the concentration of nicotinic acid had a bearing on the minimum levels of the various amino acids necessary for stimulation of acid production. When the nicotinic acid was increased from 0·00002 to 0·0002 % the concentrations of phenylalanine, tyrosine, valine and threonine had to be doubled (to 0·005 %) to obtain similar stimulatory effects.

When the casein medium was fortified with the same substances as the co-precipitate medium, a slightly greater stimulatory effect on acid production was observed. Without the fortification the rate of acid production in the 2 media was almost identical.

The tests also showed that when L-serine (0·005 %) and riboflavin (0·00002 %) were added to the medium they were inhibitory to acid production by this strain, although L-serine was not inhibitory when all the amino acids and vitamins listed in Table 1 were present. The effect of the added minerals appeared not to be due to the alteration in the buffering capacity of the medium.

When co-precipitate replaced only one-third of the milk solids (other than lactose), no fortification was found necessary for adequate fermentation. When half of the milk solids was replaced the rate of acid production was reduced and fortification only with 0·034 mg of $MnSO_4. 5H_2O$/g of co-precipitate was required.

When a greater proportion of the milk solids was replaced with co-precipitate, then further stimulatory substances were required.

CONCLUSIONS

It can be concluded from these studies that of the amino acids, additions of only phenylalanine, tyrosine, valine, cysteine and threonine were required by the strain of *L. acidophilus* NZ in a co-precipitate medium. The lack of stimulatory effect with sodium sulphite suggests that cysteine acts as a growth factor rather than as a reducing agent.

It is difficult to account for the inhibitory effect of nicotinic acid at a higher concentration unless one assumes some interference, as a co-enzyme, in the enzymic pathways involved in amino acid metabolism.

The protein of skim-milk powder can be replaced with co-precipitate provided the medium is fortified with other substances. When only one-third of the milk solids other than lactose was replaced there appeared to be a sufficient concentration of stimulatory factors to permit a normal rate of acid production. The extent of fortification required will thus be influenced by the degree of replacement. The necessity of adding lactose to the co-precipitate skim-milk powder mixture would therefore depend on the desired period of incubation and the acidity required in the final product.

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465

ANNOUNCEMENT

Electronic counting of somatic cells in milk

A recommended procedure for milk sample preparation and cell counting with a Coulter counter

To assist in the quality control of milk supplies and in monitoring the extent of mastitis in dairy herds, a test for the somatic cell content of milk is of considerable value. During the past few years electronic methods of counting somatic cells in milk have received a great deal of attention, but there is an urgent need for the standardization of procedures for milk sample preparation and the performance of the test.

Accordingly, on 10 September 1969, a group of workers in the United Kingdom and an observer from the Republic of Ireland met in Belfast, Northern Ireland, to consider the various techniques available. It was concluded that a technique based on the chemical method of preparing milk samples described by Tolle, Zeidler & Heeschen (1966) and Zeidler, Tolle & Heeschen (1968) was the most suitable at the present time, and a modification of their procedure has been agreed. In reaching this conclusion, the findings in the accompanying paper (Pearson, Wright & Greer, 1970) were taken into account. A leaflet describing the full procedure for determining the somatic cell content of milk has now been prepared and is available on request from any of the following laboratories which, with others, took part in the collaborative study.

Government of Northern Ireland, Ministry of Agriculture, Veterinary Research Laboratories, Stormont, Belfast, Northern Ireland.

National Institute for Research in Dairying, Shinfield, Reading, Berkshire.

Milk Marketing Board of England and Wales, Mastitis Research Unit, School Lane, Bamber Bridge, Preston, Lancashire.

March 1970

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A study of methods for estimating the cell content of bulk milk

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Summary. Four methods of estimating the cell content of bulk milk were studied, correlated statistically and their degree of reproducibility estimated. The relative merits of the 4 methods are discussed, and the need for defining the object of testing before selecting a test is stressed. If designed for use in a programme for mastitis control, or the control of milk quality based on cell content, each country must choose a screening test or a precise cell-counting technique, depending on the availability of laboratory resources, extension advisory services and other economic factors.

Screening tests studied were the California Mastitis Test (CMT) and the Brabant Mastitis Test (BMR), the former showing a correlation coefficient of 0·817 against the Electronic Cell Count (ECC) method in 320 samples. The more objective BMR correlated well with the ECC method, showing a correlation coefficient of 0·987 for 406 samples. Direct microscopic counts (DMC) were correlated with a centrifugal method and with a chemical method of preparing samples for electronic cell counting. For 430 samples in each case the correlation coefficients obtained were 0·930 and 0·966 respectively. The 2 ECC techniques were compared using 430 samples, and a very close relationship was found ($r = 0·988$).

For regular examination of large numbers of milk samples using the electronic cell counter we consider the chemical method of sample preparation more suitable than the centrifugal method. Differences in the reproducibility and accuracy of both tests are negligible, but the former procedure is quicker, more economical in terms of cost, and more adaptable to a national testing programme.

The Ministry of Agriculture in Northern Ireland has been using a form of the California Mastitis Test (CMT) for detecting high cell content milk in the national herd since 1965. The test has strict limitations in any notification scheme however, primarily because of the wide overlap of cell ranges and on account of its subjectiveness. The results of these tests have been used simply for notification of the farmer, who is encouraged to take action on control measures through his private veterinary surgeon. There is no national plan for controlling mastitis, though government laboratory and milk advisory services are available to assist veterinarians, wherever these services are relevant.

In 1966 it was obvious that a much more accurate test for assessing the cell content of bulk milk was required, and a study of 4 methods was undertaken. Two objective

methods were included—a modified Brabant Mastitis Test (BMR) and electronic counting techniques (ECC)—together with 2 subjective methods—the California Mastitis Test (CMT) which is already in use and direct microscopic counting (DMC). The techniques and methods used were as described in this paper, and the results obtained by these different methods were correlated.

A great volume of literature has accumulated since the early 1960s on direct and indirect cell counts in milk and on other tests used for the detection of abnormal milk. Cullen (1966) and Whittlestone & de Langen (1965) have reviewed papers up to 1966 and, as many of the tests used relate to quarter sample milk, mainly those relating to the testing of bulk milk will be referred to here.

It is 60 years since Prescott & Breed (1910) described a method for the counting of cells directly by the microscope. The test (or modifications of it) are still used today, and despite the development of numerous direct and indirect tests since that time, only recently has it been realized that more objective techniques must be based on the original Prescott–Breed method. Many variations of the microscopic technique (DMC) have emerged and some of the more important contributions have come from Levowitz & Weber (1956) and Paape, Hafs & Snyder (1963), on staining; from Newbould & Phipps (1967) on technique, and from Schneider & Jasper (1965, 1966a, b) on the importance of working factors. The National Mastitis Council Report (1969) stressed the need for all workers in this field to relate new cell counting techniques to a standard microscopic counting procedure using eyepiece reticles. Smith (p. 77) outlined such a method in detail in that report, while Schultze (1968) described reticle design in an earlier paper.

Following the development of the CMT by Schalm & Noorlander (1957) as an indirect method for estimating the cell content of milk, Schneider & Jasper (1964) described a method of standardization, while Blackburn (1965) suggested modifications to the composition of the reagent. Many workers, including Pearson (1965), Schneider, Jasper & Eide (1966) and Daniel, Smith & Barnum (1966), have discussed its use and meaning in the testing of bulk milk, but basic research studies are still being carried out to determine how the test actually works (cf. Kernohan, 1968; Nageswararao & Derbyshire, 1969).

To overcome the subjectiveness of the CMT, Jaartsveld (1961) described a means of using the test objectively in the laboratory and this has been studied in parallel with other tests by a number of workers in Europe, Australasia and the U.S.A. This so-called BMR was selected by us for comparative work, as other procedures such as the Wisconsin Mastitis Test (WMT) were not so fully described or developed when we commenced our study.

To combine objectivity with precise estimation of cell numbers, we chose electronic cell counting methods as our fourth procedure for study, and in this paper 2 ECC procedures are described and compared. The method of Cullen (1967) was used as a basis for our centrifugal method, while the chemical method used was based on the work of Tolle, Zeidler & Heeschen (1966) and Zeidler, Tolle & Heeschen (1968). A preliminary note on our comparative work has already been published (Wright & Pearson, 1969). Other useful papers to date on ECC methods and their application come from Read, Reyes, Bradshaw & Peeler (1967); Phipps (1968); Phipps & Newbould (1966); Klein & Thomas (1968); Kleinschroth, Richter & Schumann (1968);

Dijkman, Schipper & Walstra (1966); Dijkman (1968) and Dijkman, Schipper, Booy & Posthumus (1969). It is necessary to study all these papers thoroughly in order to appreciate the many variable factors that may contribute to conflicting results obtained at different research institutes.

This difficulty, in fact, applies to all the tests, and the main issues relating to difficulties in standardizing them internationally, or even nationally, are discussed in the present paper.

<div style="text-align:center">MATERIALS AND METHODS</div>

<div style="text-align:center">*Milk samples*</div>

All milk samples were taken direct from bulked herd milk, and tested or preserved for testing within 24 h. Emphasis was placed on the need for adequate mixing or agitation of the bulk milk before sampling. Four hundred and thirty-six herds were included in the trial, containing from 9 to 152 cows in milk at the time of testing.

<div style="text-align:center">*Test procedures*</div>

The California Mastitis Test (CMT). This was carried out in the laboratory at room temperature, using a blackened tile measuring 6 in. × 6 in. and divided into 16 testing areas each $1\frac{1}{2}$ in. square. Equal quantities of agitated milk and of the CMT reagent described by Blackburn (1965) were stirred together and the degree of viscosity estimated visually within 7 s. Reactions were graded nil, 1, 2 or 3 and following a study of over 2000 correlated DMC tests were associated with cell-count ranges in the following way:

Negative reaction ($-$), no viscosity ($< 2 \times 10^5$).

Grade 1 reaction, slight viscosity ($2 \times 10^5 - 6 \times 10^5$).

Grade 2 reaction, marked viscosity on stirring within 7 s but absence of solid gelling at rest ($5 \times 10^5 - 2\cdot7 \times 10^6$).

Grade 3 reaction, marked viscosity within 3 s and remaining as a solid gel sticking to the glass rod ($1\cdot7 \times 10^6 - 8 \times 10^6$).

A CMT 3 reaction was seldom observed by us with bulk milk, but it is of course frequently found in samples taken from quarters with subclinical mastitis.

The Brabant Mastitis Reaction (BMR). This test was carried out at room temperature using as a basis the apparatus and technique described by Jaartsveld (1961). Several steps of the original test were considered unnecessary and were omitted, and modifications in the photographing and timing of reactions were introduced. The main changes from Jaartsfeld's original procedure were as follows: (*a*) the Brabant reagent was delivered into the milk tubes by means of an automatic pipette; (*b*) a clock, with a dial marked in seconds, was photographed with each test; (c) a single lens reflex camera was used to photograph the flow-through time; (*d*) photographs were taken every $2\frac{1}{2}$ s up to 20 s, and then every 5 s up to 40 s.

Mean cell count values up to 15 s were as shown in Table 1.

The Direct Microscopic Count (DMC). Counts were obtained using standard $2 \times \frac{1}{2}$ cm smears prepared from 0·01 ml portions of milk, and staining with a defatting solution of methylene blue. Using a working factor of 20000, the cells in 20 fields were counted and the result multiplied by 2×10^4.

Electronic Cell Counting (ECC). The particle counters used for this work were

purchased from Coulter Electronics Ltd, Dunstable, Beds., Models B and Fn being used.

(a) *Centrifugation Method* (CG). The technique using centrifugation for fat removal was based on the method developed by Cullen (1967). The main modifications adopted by us were the centrifugation of samples for 30 min at 1000 g whereas Cullen used 280 g for 1 h; we found it preferable to count particles of sizes down to 4·7 μm diam. instead of 6·3 μm.

Table 1

Time, s	Number of samples (totalling 393)	Cell count (ECC) $\times 10^3$
0–2½	70	374
2½–5	180	593
5–7½	69	776
7½–10	34	920
10–12½	19	1161
12½–15	21	1183

(b) *Chemical Method* (CM). The method employed basically was that described by Tolle *et al.* (1966). Formalin preservative 1:500 final concentration was allowed to act upon the milk sample for 24 h. A 1:100 dilution of the treated sample was made in a defatting solution containing 84·5% of 0·9% sodium chloride solution, 12·5% methylated spirit, 2·0% Triton X-100 and 1·0% of a 40% solution of formaldehyde. The mixture was then heated at 80 °C for 10 min in a water bath. After cooling the sample to room temperature, counting was carried out at a size threshold set to count a minimum particle size of 4·7 μm. Fuller details of the test together with the method of sample preparation are available from this laboratory.

RESULTS AND DISCUSSION

Table 2 summarizes the results obtained from 1290 field samples. The samples were split into 3 groups of 430 samples and duplicate counts were made on each sample by each method. No significant difference was found between the mean cell counts obtained by any of the methods.

Reproducibility and accuracy of methods

The degree of reproducibility established by the ECC and DMC methods is demonstrated by calculating the coefficients of variation, the reproducibility of counts on replicated milk samples within various cell content ranges being shown in Table 3. In the case of the BMR, 372 samples were grouped in cell-count ranges estimated by the ECC (CG) method, and the results are shown in Table 4.

The errors in sampling a random distribution of cells in milk follows a Poisson distribution. The standard error of a microscope count varies directly with any multiplication factor involved in the test, and the coefficient of variation increases as the count decreases (Schneider & Jasper, 1966b). Both ECC methods have a constant working factor of 200. When using the DMC it has been calculated that a working factor of less than 5000 is needed where a good estimate is required, and expected coefficients of variation range from 24 to 2% depending upon the number of cells counted

(Schultze, 1968). Using an improved but tedious method involving stratified within-smear sampling, the mean between smear coefficients of variation may be reduced to less than 10 % where the cell count exceeds 350000 cells/ml (Newbould & Phipps, 1967). DMC results from replicated samples in Table 3 show coefficients of variation from 12·9 to 7·6 %, varying inversely with the cell content of the sample.

Table 2. *Results obtained from comparison of Electronic Cell Count (ECC) Centrifugation Method (CG), ECC Chemical Method (CM) and Direct Microscopic Count (DMC) techniques, showing methods compared, number of observations, cell-count ranges, mean cell count, standard error and coefficients of variation*

Methods compared	No. of observations	Cell-count ranges, cells/ml	Arithmetic mean	S.E.M.	Coeff. var., %
ECC (CG)					
v. DMC	430	162000–2334000	704 409	15930	46·9
	430	120000–2400000	689 153	17080	51·4
ECC (CM)					
v. DMC	430	146000–2152000	700795	14690	43·48
	430	160000–2440000	702019	15580	46
ECC (CG)					
v. ECC (CM)	430	169000–2245000	682207	16760	52
	430	124000–2412000	675718	16970	50·89

Table 3. *Results obtained for replicated milk samples in different cell-content ranges by 3 methods, showing means and coefficients of variation of the mean*

Cell content/ml × 10^3

ECC (CG)		ECC (CM)		DMC	
Mean	Coeff. var., %	Mean	Coeff. var., %	Mean	Coeff. var., %
427	7·1	415	2·5	435	12·9
865	5·2	737	2·0	711	10·4
1,093	4·0	986	1·6	1,075	8·4
1,405	4·5	1,242	0·9	1,247	7·6

(4 Samples each test, 10 replicates at each cell count level.)

The results demonstrate that the ECC methods provide accurate and highly reproducible values besides being rapid in use and suitable for bulk milk samples. The within-sample coefficients of variation calculated from values for replicated samples in various cell-count ranges are also shown. The electronic methods gave somatic cell counts that reproduced closely in replicate samples, the chemical method proving the more precise with an average coefficient of variation of 1·755 %.

The CMT used in earlier work done by us proved to be highly reproducible with individual operators but lacks precision in the low and high cell-count ranges, and the cell content cannot be expressed satisfactorily. To overcome the subjectivity of this test the BMR has been employed but has the disadvantage that it is influenced by age of milk sample, temperature, technique, and pH. Table 4 shows the means, standard errors and coefficients of variation of various cell-count ranges obtained by the BMR on 372 bulk milk samples, and the cell counts obtained for the same milk samples using the ECC centrifugal technique.

Correlation of tests

The tests were correlated in 5 different ways and Table 5 summarizes the statistical relationship between them; the number of samples is shown as (N), the correlation coefficient as (r) and the standard error as (S.E.). The regression line formula is also shown in each case and was estimated from the data recorded in Tables 2 and 4, and in Fig. 5.

Table 4. *Comparison of the Electronic Cell Counting (ECC) (Centrifugation Method) (CG) and the Brabant Mastitis Reaction on 372 bulked milk samples*

	ECC (CG), cells/ml × 10³				BMR, s		
No. of samples	Cell count median	Actual mean	S.E.M.	Coeff. var., %	Mean	S.E.M.	Coeff. var., %
187	500	441	8·4	26·0	4·2	0·1	33·2
121	750	742	6·2	9·2	6·5	0·2	28·7
57	1,000	1,001	12·4	9·3	9·0	0·35	29·3
7	1,250	1,464	67·3	12·0	11·8	0·5	10·3

Table 5. *Summary of correlation data*

Tests compared			N	Regression formula	r	S.E. of r
y		x				
DMC	$v.$	ECC (CG)	430	$y = 0.9978x - 13.7276$ ± 0.0190*	0·9303	0·0065
DMC	$v.$	ECC (CM)	430	$y = 1.0246x - 16.0085$ ± 0.0132*	0·9664	0·0031
ECC (CM)	$v.$	ECC (CG)	430	$y = 1.0006x - 6.8679$ ± 0.0076*	0·9878	0·0011
ECC (CG)	$v.$	BRM†	406	$y = 64.0144x - 266.1035$	0·9870	0·0010
Log ECC (CG)	$v.$	CMT	320	$y = 0.1490x + 5.1927$	0·8170	0·0186

y and x are in thousands. * S.E. of regression coefficient. † Up to 12·5 s only.

Correlation of ECC and DMC counts

Fig. 1 illustrates the line of regression together with the correlation coefficient and statistical data. No corrections for the coincident passage of cells through the counting aperture were considered necessary, in view of the fact that only about 13 % of the cell counts exceeded 1 million cells/ml.

A correlation coefficient of 0·9303 is highly satisfactory, particularly in view of the number of observations and cell-count ranges, and it seems probable that the agreement between the cell counts at all levels results principally from the counting of particles at a 4·7 μm threshold. Cullen (1967) chose to count at 6·3 μm. Phipps & Newbould (1966) used a 7·5 μm level and adjusted the counts by multiplying by a correlation factor. These authors used Model A counters, and most of their work was carried out on quarter milk samples, many of which had very high cell counts.

Read *et al.* (1967) counted at 7·2 μm using a Model B counter, obtaining a correlation coefficient of 0·997 with the direct microscopic count; a regression coefficient of 1·0 was found with cell counts > 300000 cells/ml, including counts of up to 60

million cells/ml. An almost identical correlation was found in their later work (1969), but a number of workers may be critical of the method they used to calibrate the electronic counter. Mitchell, Newbould & Platonow (1967) pointed out differences obtained in studies between bulk tank milk and fresh composite milk from individual cows comprising milk from all 4 quarters. They consider that their electronic methods of counting cells in bulk milk are unreliable, as they are high in comparison to the microscopic count. In their work, however, the average bulk cell counts were relatively low, none being greater than 600 000 cells/ml.
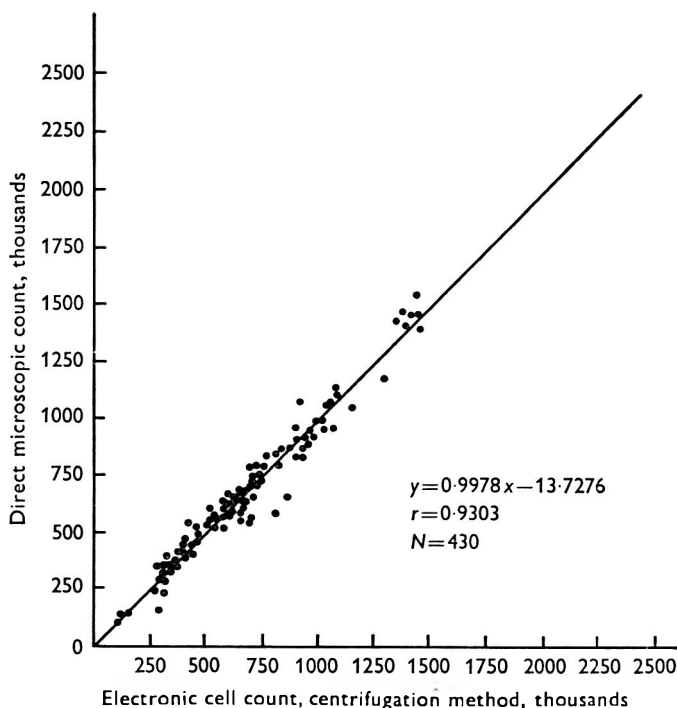
Fig. 1. Regression line of Direct Microscopic Count on Electronic Cell Count, Centrifugation Method. For the sake of clarity only 100 points are shown representing an accurate proportion of the total number of observations at each cell-count level.

In our present work the 430 routine bulk tank samples were studied, in contrast to the 81 samples specifically selected in our preliminary paper (Wright & Pearson, 1969), but despite this lack of selection a high correlation coefficient was obtained. It would be difficult to improve upon this within the wide range of cell counts of 162 000–2 400 000 cells/ml estimated by all methods, and indeed any improvement would be irrelevant in the application of the test to field conditions.

Correlation of ECC (CM) and DMC counts

Fig. 2 shows the correlation graph together with the regression line formula and correlation coefficient for the 430 samples used. As with the centrifugation method the correlation coefficient of 0·9664 is very satisfactory, particularly as the 430 samples were taken from routine milk supplies and covered a wide range of cells/ml.

It is interesting that Tolle *et al.* (1966) obtained a correlation coefficient of 0·98 on 100 selected samples, counting at 5 μm where we adopted 4·7 μm. Some electronic counter models may require a different threshold to produce similar results to others. Tolle *et al.* used mainly a Model A in their experiments. In a comparative experiment (Dr L. W. Phipps, personal communication) the size distributions obtained with the A model were broader and skewed compared with the Fn model as used in our experiments. It must be appreciated that it is virtually the cell nuclei that are being counted following treatment by the chemical method, as against whole cells by the centrifugation technique.
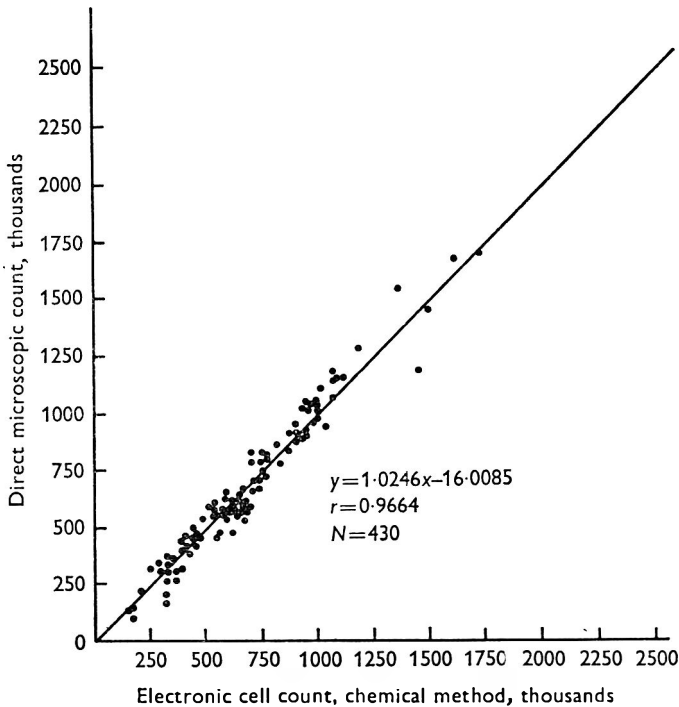
Fig. 2. Regression line of Direct Microscopic Count on Electronic Cell Count, Chemical Method. For the sake of clarity only 100 points are shown representing an accurate proportion of the total number of observations at each cell-count level.

Dijkman *et al.* (1969) found a good relationship between the chemical ECC method and microscopic count, although they observed, like ourselves and others (Cullen, 1967; Dijkman *et al.* 1966; Phipps, 1968), that with low counts the ECC figures are higher than they should be. The small error may not matter much in practice, and is due to the fact that electronic counts include relatively more particles that are not cells.

Correlation of ECC (CG) and ECC (CM) methods

The 2 electronic methods correlated well with each other and their relationship is shown diagrammatically in Fig. 3. A correlation coefficient of 0·9878 was obtained on the 430 samples. Phipps (1968) also found a satisfactory correlation between the 2 tests, despite the fact that he used the detergent Witopal Co, counted at 5 μm, in

contrast to our 4·7 μm, and observed 2 exceptionally high counts possibly because of inadequate fat dispersal.

In general, cell counts using the centrifugal technique tend to be fractionally higher than those obtained by the chemical method, possibly because there is more risk of counting debris. At low threshold levels red blood cells, if present, would also be counted (Phipps & Newbould, 1966). Any loss of particles using the chemical method in high cell-count ranges, however, is probably unimportant, as the most critical range in our circumstances at present is between 0·5 and 1·5 × 10^6 cells/ml, and here we have a good relationship.
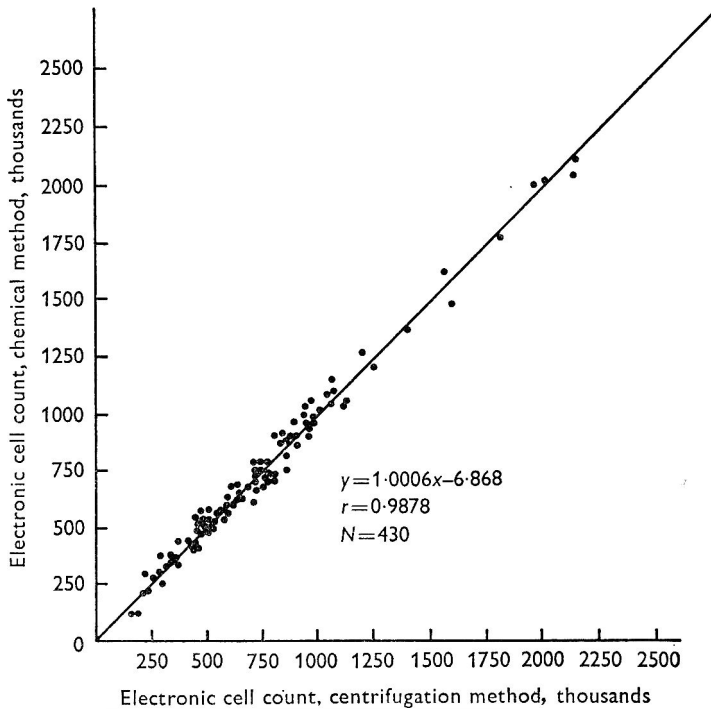
Fig. 3. Regression line of Electronic Cell Count, Chemical Method on Electronic Cell Count, Centrifugation Method. For the sake of clarity only 100 points are shown representing an accurate proportion of the total number of observations at each cell-count level.

Our preference between the tests is thus in favour of the chemical method of sample preparation, as the method is highly reproducible and accurate, and is much more suitable than the centrifugal technique for the examination of large numbers of milk samples.

Correlation of ECC (CG) and BMR

Previous work by Wright & Pearson (1969) is reproduced in Fig. 4 for convenient reference. Four hundred and six samples were tested and a very high correlation between the tests was obtained ($r = 0.987$), although the linear relationship ceased after 12·5 s.

The advantages of photographing reactions at $2\frac{1}{2}$-s intervals are clearly shown, particularly up to 15 s; and the test used in this way might be acceptable as a

screening test to eliminate all samples in a low cell-count range. For example, if 85 % of the national sample was below 1 000 000 cells/ml, as in our present survey, it might be possible to apply the Brabant testing system monthly to all supplies and carry out critical ECC tests on the top 15 or 20 % of Brabant reactors, i.e. all those showing reaction times of over 10–12½ s. Adjustments to the method of combining the 2 tests in other countries would have to be made, depending on (a) the national cell-count mean and (b) the reason for testing, i.e. for awareness notification or for financial penalty based on precise counts. A broader selection of Brabant positives might be required for the latter.
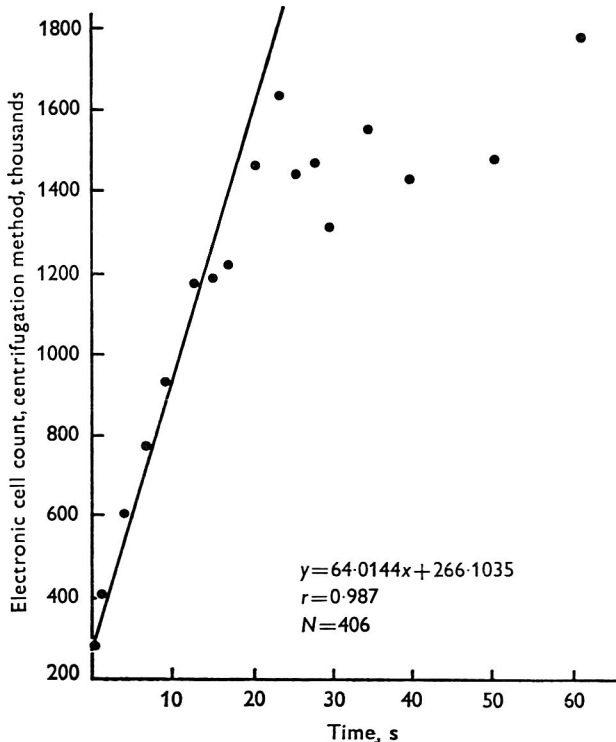
Fig. 4. Regression line of total cell count on the Brabant Mastitis Test reaction time, up to 12·5 s showing 17 points representing the total samples.

It is difficult to understand why different workers obtain different mean cell-count values at approximately the same flow-through times. Ageing of samples is important (Dijkman *et al.* 1969) but it is more likely that sampling procedure and the technique of carrying out the test (van der Schaaf, Jaartsveld & Kramer-Zeeuw, 1964) are more important. There is no obvious reason why such an objective test should not be reproducible, and O'Reilly & Dodd (1969) have found this to be so up to 800000 cells/ ml at 20 °C. They emphasize the importance of performing the test at a standard temperature (20 °C), and refer to a breakdown in the relationship between the BMR and higher cell counts, particularly when the BMR test is carried out at 37 °C.

Each laboratory must therefore study very closely its techniques and apparatus used, before concluding that a true relationship between tests has been found or that

the results are comparable with those of other laboratories. For this reason we emphasize again that the photographing of reactions at short intervals up to at least 15 s is essential.

The greatest difficulty in applying the Brabant test in any national plan, however, is that fresh milk must be used. Like many other workers, we have found that the value of the test diminishes considerably if the bulk samples are more than 24-h old.

Correlation of ECC (CG) and the CMT

Few workers have looked at this relationship, possibly because electronic counting is a precise and objective test, whereas the CMT is subjective, and each reaction covers a wide cell-count range.
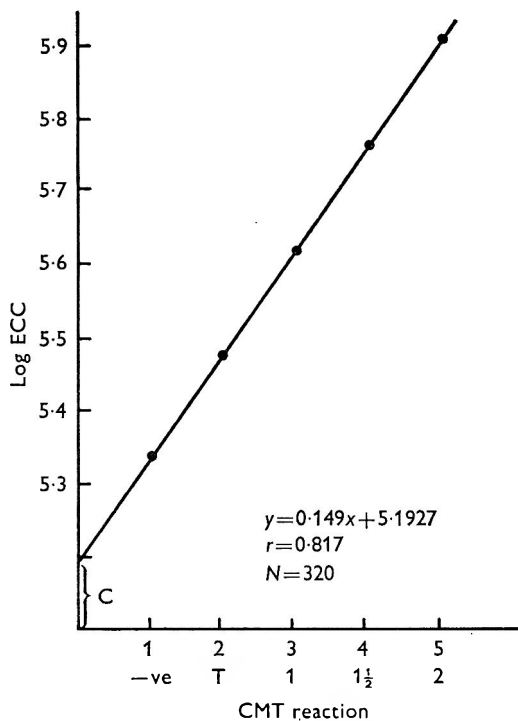
Fig. 5. Regression line of log Electronic Cell Count (ECC) on California Mastitis Test (CMT) reaction at 7 s. Points on graph represent the mean of the log cell counts for each CMT score.

No. of samples	CMT score	Mean log ECC	Mean ECC, thousands	s.e. of means in logs
16	1	5·3459	222	± 0·781
45	2	5·5117	324	± 0·452
85	3	5·6191	416	± 0·695
103	4	5·7501	562	± 1·029
71	5	5·9717	937	± 2·650

Nevertheless, the CMT is of value as a simple screening test for cells, and can be used at creamery laboratories on well-mixed churn milk, by staff with very little training. Fig. 5 shows the relationship between ratings for 320 samples by the CMT

method compared with those obtained by the centrifugal ECC method. To obtain a better fit logarithmic ECC values are used, and the CMT results are coded 1, 2, 3, 4 and 5 to correspond with our more critical interpretation of reactions for this exercise, i.e. Negative, Trace, 1, $1\frac{1}{2}$ and 2. We did not obtain a CMT reaction of 3 at any time during this experiment. A high correlation is to be expected between the CMT and cell counts, particularly when the CMT is carried out by experienced workers, and also because each CMT score covers such a wide range of cells; in fact a correlation coefficient of 0·817 was obtained.

Schneider & Jasper (1964) studied 2 basic milk samples, one strongly CMT-positive and the other negative, and attempted to identify a precise range of cells with each CMT reaction. This work was very helpful, but the human error associated with interpretation was found to be a limiting factor. Kernohan (1968) used lymph cells to determine a relationship with CMT scores, but found her actual cell counts to be low in comparison with other workers who assessed their results using milk.

Numerous authors have studied various forms of the CMT. Most have used quarter sample milk in which cell counts may be extremely high, and a CMT reaction of 3 is commonly associated with the mean cell counts of 4–7 million cells/ml. This appears to us to be much too high and would be associated more with clinical disease by our interpretation. Some of the most useful work on bulk samples, however, is described in papers by Ewbank (1962), Postle & Blobel (1965), Daniel *et al.* (1966), Kroger & Jasper (1967) and Janzen (1969), while Barnum & Newbould (1961), Nyhan (1965) and Schneider *et al.* (1966) are among those who have looked for a relationship between the CMT reaction in bulk milk and the incidence of mastitis in cows and quarters in the herd.

Conclusions

Most workers agree that the estimation of somatic cells in the bulk milk from a herd provides a good guide to the degree of mastitis in that herd. It is obvious that, whatever test is used, it must be carried out on the herd milk fairly frequently, in order to minimize some of the variable factors which might contribute to false positive results, e.g. a high proportion of late lactation cows at the time of test.

In very small herds, relatively simple tests may be adequate and possibly this is why in Switzerland the Whiteside test has maintained its popularity for so long (Bieri, 1966), although Temple (1963) refers to its value in a State mastitis control programme in New York. Mastitis awareness and control programmes using the CMT are referred to by Jasper (1967) and Pearson (1965), while DMC methods are applied in Denmark. It is difficult, however, to remove the subjectivity of these tests, hence the search by ourselves and many others for a more objective routine for assessing the cell content of bulk milk in the national herd.

Much depends on the purpose for which the testing is carried out, and what in fact the milk producer and the veterinarian are supposed to do when the results are made available to them. Giesecke & van der Heever (1967) discuss this point, and state that there is little point in having an efficient testing system in the absence of an adequate follow up and advisory programme.

The results of the present work, however, are on the tests themselves, and it is clear that, at the present time, ECC methods provide the most accurate and suitable

means of monitoring somatic cells in bulk milk. The chemical method is to be preferred to the centrifugal method as it can be more readily and economically designed to cope with large numbers of samples. The BMR test, however, might be acceptable in some countries as a screening test, perhaps coupled with ECC procedures for the more precise counting of cells in higher cell-count ranges only. This, in theory, is an economically sound idea, but in practice it might be difficult to obtain fresh milk samples consistently for the Brabant test.

It is necessary all the same to keep an open mind about the possible development of newer testing procedures, and fluorescent microscopy (Madsen, 1968) or Feulgen–DNA tests (Paape, Hafs & Tucker, 1964; Hauke, 1967; and de Langen, 1967) or some other biochemical test may ultimately become more accurate, economical and acceptable. In view of this, we have considered it essential to standardize the chemical ECC method described in this paper, and this has now been agreed between 3 laboratories within the United Kingdom (Pearson, Wright, Greer, Phipps & Booth, 1970). The benefits of having an agreed national standard test of this kind are considerable.

The significance of the bulk milk cell count in relation to cow-and-quarter-disease incidence in the herd is of considerable importance and has been discussed by a number of authors (Schneider *et al.* 1966; Gray & Schalm, 1960; Nyhan, 1965; Postle, 1967 and Kleinschroth *et al.* 1968). Although it is clear that the numbers of cows and quarters affected cannot be assessed from the bulk count score, owing to physiological factors, yield, distribution of diseased quarters, etc., a broad picture of the herd incidence can be obtained from a series of tests. The most important source of cells is the number of subclinical quarters in the herd (Barnum & Newbould, 1961; Philpot, 1969; Pearson, Greer & Spence, 1970). There appears to be little point in developing tests for determining the cell content of bulked milk from herds, unless the results are incorporated in a notification programme to the producer and an extension advisory service on mastitis control. The milk producer must be guided on the interpretation of cell-count results as a natural sequel in a programme of monitoring and controlling udder disease.

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The associative effect of level of energy and protein intake in the dairy cow

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SUMMARY. Eight lactating cows were used in a Latin square experiment, to study the associative effects of level of energy and protein intake on milk yield and composition. Four diets were used, supplying 80 and 120% of estimated energy requirements and 80 and 120% of estimated protein requirements. The level of energy intake significantly affected milk yield, milk energy output, percentage butterfat, ash and non-protein nitrogen. The level of protein intake only significantly affected milk energy output and the non-protein nitrogen content of the milk. Although only the interaction of the effects of energy and protein intake on the milk content of solids-not-fat (SNF) and ash was significant, it was evident that the effect of each of these factors on milk yield or composition was related to the level of the other in the diet.

Input–output relationships within each protein level were used to compute the response in milk energy output and bodyweight change to a change in energy intake. These showed a greater partitioning of additional energy toward milk energy output with the high than with the low protein level. Multiple regression analysis within each level of protein intake was used to partition energy intake between that used for maintenance, milk energy output and liveweight change. The results showed efficiencies of utilization of metabolizable energy for milk output of 63 and 50% on the high- and low-protein diets, respectively.

Nitrogen balance data are presented.

Burt (1957) and Rook (1961) have reviewed the effects of level of energy and protein intake of the dairy cow on milk yield and composition. They concluded that an increase in energy intake increases yield, SNF content and, to a lesser extent, lactose content, and decreases the fat content. The level of protein intake has little effect on milk yield, or on composition, except for the non-protein nitrogen (NPN) content, unless low levels of dietary protein are given. Few data are available on the associative effects of energy and protein intake on milk yield and composition. Rook (1953) was unable to show any significant energy × protein interaction when both energy and protein were given at and above normal standards (Woodman, 1948). However, the results of Reid & Holmes (1956) and Logan, Miles & Haskell (1959) indicate that the response from additional energy is reduced when lower protein diets are given. The object of the present investigation was to provide information on the associative effects of energy and protein intake on milk yield, milk composition and bodyweight change, when given above and below normal standards.

EXPERIMENTAL

Eight dairy Shorthorn cows in their 6th–10th week of lactation were used. All cows were in the second or later lactations. The cows were housed in individual stalls and milked twice daily. During the preliminary period they were given sufficient medium quality hay for maintenance (Blaxter, 1959) and concentrates at the rate of 0·4 kg/kg of milk produced. The cows were weighed on 3 consecutive days at the end of the preliminary period and the mean used to calculate their metabolic bodyweight ($W^{0·73}$). This metabolic bodyweight was used throughout the remainder of the experiment.

The experimental layout was a Latin square design balanced for the estimation of first residual effects. At the end of a 3-week preliminary period the animals were grouped into 2 blocks according to milk yield and composition and allocated at random within each block to the 4 treatments. The test diets provided: (1) 80% of energy requirements and 120% of protein requirements; (2) 120% of both energy and protein requirements; (3) 120% of energy requirements and 80% of protein requirements and (4) 80% of both energy and protein requirements. The values taken to represent the energy and protein requirements were those given by Blaxter (1959). Each animal was subjected to a sequence of treatments as indicated by Williams (1949). There were 4 comparison periods, the first being of 6 and the remainder of 4-weeks duration. Nutrient requirements at each changeover point were calculated by the method of equalized feeding (Lucas, 1943) with a yield persistency factor being calculated within each block using data only from those cows on the higher energy treatments.

The milk was sampled at each milking during the last 6 days of each period and the samples bulked according to yield to provide two 3-day composite samples for each animal. The cows were weighed on each of the last 3 days of each period.

Throughout the experimental periods all the cows were given 5·5 kg of hay, of which the starch equivalent and digestible crude protein content had been determined previously from sheep digestibility data. The hay contained 10·9% crude protein and 34·3% crude fibre in the dry matter and had a starch equivalent content of 39. Due to the variations in bodyweight and milk yield between animals and in the standard amount of hay being given, the supplementary energy and protein intakes required on each treatment could not be provided by adjusting the intake of a standard concentrate within each treatment. Thus, within each treatment the composition of the concentrate mixture was altered to meet the requirements of each individual animal. The compositions of the concentrate mixtures are presented in Table 1 as treatment means for each constituent with their standard errors to indicate the extent of the within treatment adjustment. In order to produce a diet sufficiently low in protein on treatment 3 it was necessary to replace the flaked maize by maize starch, in addition to eliminating soybean and groundnut meal. It was considered that the difference in starch form would not affect the results obtained.

The results were analysed according to the method of Patterson & Lucas (1962) and treatment means compared by the multiple range test of Duncan (1955). A multiple regression technique was used to relate the milk yield and weight change within each protein level to the level of energy intake and also to estimate the

proportions of digestible organic matter intake (DOMI) utilized for maintenance and production.

Nitrogen balances. Seven-day digestibility and N-balance trials were carried out using 4 cows (1/treatment) on the thirty-second–thirty-ninth day of period 1 and on the eighteenth–twenty-fifth day of each of periods 2–4. A technique was adopted that allowed separate collection of faeces and urine without the use of a harness, and without removing the animal from its normal stall. The urine was channelled into a container by means of 500 gauge plastic tube. An adhesive was used to attach the tube to the cow. The faeces were collected on plastic sheeting behind each stall.

Table 1. *Mean composition of the concentrate mixtures,* %

	Treatments			
	1	2	3	4
Flaked barley	44·7 ± 0·4	62·7 ± 0·4	82·4 ± 2·1	63·3 ± 0·3
Flaked maize	10·9 ± 0·2	20·9 ± 0·1	—	21·1 ± 0·1
Soybean meal	27·9 ± 0·2	7·1 ± 0·4	—	6·3 ± 0·2
Groundnut meal	10·4 ± 0·7	4·0 ± 0·3	—	3·9 ± 0·3
Maize starch	—	—	12·1 ± 2·3	—
Minerals*	5·6 ± 0·0	5·3 ± 0·1	5·4 ± 0·1	5·3 ± 0·0

* Declared composition: P, 5·7%; Ca, 21·4%; NaCl, 33·3%; Fe, 0·3%; Mn, 800 ppm.; Cu, 300 ppm.; Co, 100 ppm.; I, 200 ppm.

The milking and feeding procedure adopted during balances was similar to that used throughout the remainder of the experiment. Two days before and during the balance period a sample equivalent to 5 % of the animal's daily hay and concentrate intake was retained. At the end of the balance period the daily samples for each cow were bulked and subsampled for chemical analysis. The faeces and urine were collected each morning after milking. Ten per cent of the daily faeces output and 5 % of the daily urine output were retained. The faeces samples were stored separately at 0 °C using approximately 0·5 ml toluene as a bacteriostat. The urine samples were acidified with glacial acetic acid and bulked for each animal. At the end of each balance the daily faeces samples for each cow were bulked, thoroughly mixed and sampled for chemical analysis. Nitrogen determinations were made on the fresh material by the macro-Kjeldahl method and dry-matter content estimated by drying at 100 °C for 24 h. The bulked 7-day urine samples were also thoroughly mixed and 10-ml samples analysed for nitrogen content.

Milk analysis. Each 3-day composite milk sample was analysed for butterfat by the Gerber method, for total solids by the gravimetric method, for protein by the macro-Kjeldahl method ($N \times 6.38$), for lactose by Rowland's modification of the method of Hinton & Macara as given by Ling (1963), and for ash and non-protein nitrogen by the method of Ling (1963). The mean composition obtained from the 2 composite samples was used for treatment comparisons. Milk energy was calculated from the total output of milk constituents and the energy content of these constituents as given by Kleiber (1961).

RESULTS

The intakes of energy and digestible crude protein, expressed as a percentage of the animals' requirements (Blaxter, 1959) at the beginning of each 4-week comparison period, are presented in Table 2. Since digestibility and N balance trials were being carried out in each period, nutrient intakes were not altered within each period.

Table 2. *Mean intakes of energy and digestible crude protein expressed as a percentage of requirements* (Blaxter, 1959) *at the beginning of each period*

	Treatments			
	1	2	3	4
s.e. intake	82·1	119·7	118·0	80·0
DCP	122·2	118·8	79·6	76·6

Table 3. *Milk yield, milk energy output and bodyweight changes*

Adjusted for residual effects

	Treatments					Statistical comparisons		
	1	2	3	4	S.E.M.	Energy	Protein	Treatments
Milk yield, kg/day	10·82	13·18	11·32	10·41	±0·58	HE > LE**	NS	4 < 2**; 1 < 2,* 3 < 2*
Milk energy, kcal/day	7387	8886	7434	7120	±360	HE > LE*	HP > LP*	4 < 2**; 1 < 2,* 3 < 2*
Bodyweight change, kg/day†	−0·63	+0·47	+0·31	−0·70	±0·14	HE > LE***	NS	1 < 2,** 1 < 3,** 4 < 2,** 4 < 3**

NS, non-significant.
* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
† Residual effects significant ($P < 0.01$).

The mean milk yield, milk energy output and bodyweight changes adjusted for residual effects are presented in Table 3. The level of energy intake had a significant effect both on milk yield ($P < 0.01$) and on milk energy output ($P < 0.05$), with the higher energy intake producing on average 1·63 kg more milk and 906 kcal more energy than the lower energy intake. The effect of protein intake was similar, with the higher protein diet producing 1·12 kg more milk and 860 kcal more energy than the low protein diet, although only the effect on milk energy output reached significance ($P < 0.05$). Bodyweight change was significantly affected by level of energy ($P < 0.001$) with the animals on the higher energy intake gaining on average 0·39 kg/day while those on the lower intake lost 0·67 kg/day. Protein intake had no significant effect on bodyweight change.

The treatment means, adjusted for residual effects, for the composition of the milk are presented in Table 4. The contents of total solids, protein and lactose were not significantly affected by any of the treatments. However, the butterfat content was significantly affected by the level of energy intake ($P < 0.01$), with that on treatment 3 being significantly lower than on treatments 4 and 1 ($P < 0.01$). The SNF content on treatment 2 was significantly greater than on treatments 1 ($P < 0.01$) and 3 ($P < 0.05$). However, due to the significant energy × protein interaction ($P < 0.05$)

there was no significant overall energy or protein effect on SNF. The ash content of the milk was significantly affected by level of dietary energy ($P < 0.01$). The NPN content of the milk was significantly affected both by the level of dietary energy ($P < 0.01$) and of protein ($P < 0.001$).

The adjusted treatment means for the total yields of the main milk constituents are presented in Table 5. The total output of butterfat was significantly affected by the level of protein intake ($P < 0.05$) but not by the level of energy intake. Both the

Table 4. *Composition of the milk*

Means adjusted for residual effects

| | Treatments | | | | | Statistical comparisons | | |
	1	2	3	4	s.e.m.	Energy	Protein	Individual treatments
solids, %	11·79	11·87	11·50	11·95	±0·151	NS	NS	NS
⸱rfat, %	3·66	3·39	3·28	3·62	±0·101	HE<LE**	NS	3<4,* 3<1*
%	8·17	8·50	8·24	8·36	±0·004	NS	NS	1<2,**; 3<2*
⸱in, %	3·27	3·38	3·32	3·32	±0·045	NS	NS	NS
⸱se, %	4·07	4·32	4·19	4·20	±0·084	NS	NS	NS
%	0·76	0·80	0·79	0·78	±0·008	HE>LE**	NS	1<2,**= 1<3*
, mg/100 g	42·9	34·2	27·3	31·1	±2·13	HE<LE**	HP>LP***	3<1,** 4<1,**; 3<2,* 2<1*

NS, non-significant.
* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.
The energy × protein interaction of SNF and ash was significant at the 5 % level.

Table 5. *Mean output of butterfat, solids-not-fat, protein and lactose*

Means adjusted for residual effects

| | Treatments | | | | | Statistical comparisons | | |
	1	2	3	4	s.e.m.	Energy	Protein	Individual treatments
⸱rfat, kg/day	0·39	0·44	0·37	0·37	±0·018	NS	HP>LP*	3<2,* 4<2*
kg/day	0·89	1·12	0·94	0·87	±0·048	HE>LE**	HP>LP*	4<2,** 1<2,**; 3<2*
⸱in, kg/day	0·35	0·44	0·37	0·34	±0·019	HE>LE**	HP>LP*	4<2,** 1<2,**; 3<2*
⸱se, kg/day	0·46	0·57	0·49	0·48	±0·025	HE>LE**	NS	1<2,* 4<2,* 3<2*

NS, non-significant.
* $P < 0.05$, **$P < 0.01$.
The energy × protein interaction on lactose was significant at the 5 % level.

Table 6. *Nitrogen balance data*

Means adjusted for residual effects

| | Treatments | | | | | Statistical comparisons | | |
	1	2	3	4	s.e.m.	Energy	Protein	Individual treatments
⸱ake, g/day	230·5	252·8	174·5	176·8	±13·2			
⸱rent digesti-⸱ity of N, %	73·9	67·5	58·6	67·9	±1·4	HE<LE**	HP>LP**	3<4,** 3<2,** 3<1,**; 2<1,* 4<1*
⸱ined N, g/day	−1·4	+23·3	+13·8	−0·4	±3·4	HE>E***	NS	1<2,** 4<2,**; 1<3,* 4<3*
⸱ined N+milk g/day	59·1	86·9	65·3	50·0	±8·6	HE>LE*	NS	4<2*

NS, non-significant.
* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

higher protein and higher energy diets resulted in an approximately similar percentage increase in the total output of milk protein. However, the non-significant effect of protein intake on lactose (6%) was much smaller than that due to energy intake (12%; $P < 0.01$). This resulted in the output of SNF being affected more by the level of energy ($P < 0.01$) than of protein intake ($P < 0.05$). There was a significant energy × protein interaction ($P < 0.05$) on the output of lactose.

The organic-matter digestibilities of the total diets—71·8, 74·5, 73·7 and 72·3 on treatments 1–4, respectively—were significantly affected by the level of energy ($P < 0.05$) but not by protein intake. The treatment means for the N balance data are presented in Table 6. The apparent digestibility of N was significantly affected both by the level of energy and of protein intake ($P < 0.01$). The digestibility on treatment 1 was significantly higher than on treatments 3 ($P < 0.01$) and 2 and 4 ($P < 0.05$) and significantly lower on treatment 3 than on each of the other three treatments ($P < 0.01$). The level of N retained and N retained plus milk N were significantly affected by level of energy ($P < 0.001$ and $P < 0.05$, respectively), but neither was significantly affected by level of protein intake.

DISCUSSION

Effect of protein. The overall effect of level of dietary protein on milk yield just failed to reach significance at the 5% level, with the animals receiving 120·5% of requirements producing 1·12 kg/day more milk than those receiving 78·1%. The response in yield to increased protein intake was considerably greater at the high than at the low energy level (1·9 and 0·4 kg/day, respectively). The effect of protein intake on milk energy output followed a similar pattern although in this case the overall effect of protein (860 kcal) was significant. This response was only marginally lower than that of 906 kcal due to a similar percentage change in energy intake. The response to increased protein intake was considerably lower at the low than at the high level of energy intake, being 267 and 1452 kcal, respectively. These results on the overall effects of protein level on milk yield agree with those reviewed by Rook (1961). Rook & Line (1962) obtained a reduction in yield of 0·9 kg/day when protein intake was reduced to 80% of Woodman's (1957) standards. Few results are available on the response to changes in protein intake at widely differing levels of energy intake. However, evidence by Reid & Holmes (1956) and Logan *et al.* (1959) would tend to support the view that the response to changes in protein intake increases with level of energy feeding.

Although the level of protein intake did not significantly affect the percentage of total solids, butterfat, SNF, protein, ash or lactose, there was a significant interacting effect of energy and protein intake on milk SNF and ash. The higher protein intake, at the high level of energy intake, resulted in an increase in the SNF content of 0·26% units while at the low level of energy intake there was a decrease in SNF of 0·19% units. A similar but non-significant trend was obtained with milk protein. Rook (1953) obtained no interacting effect of protein and energy intake on either of these milk constituents when the protein was given with normal and high energy intakes. Logan *et al.* (1959) found similar differences in the response to protein at high and low levels of energy intake to those reported here. However, in a second trial

in which normal and high levels of energy intake were used these authors did not obtain this interaction. Therefore, while it is generally recognized that changes in protein intake other than reductions below approximately 70 % of requirements (Woodman, 1957) have little effect on milk SNF or protein when given with the recommended standard levels of energy intake (Rook, 1961), it would appear that there may be a negative correlation between level of protein intake and milk SNF content when the protein is given in conjunction with levels of energy intake below normal standards.

Effect of energy. The level of energy intake significantly affected both milk yield and milk energy output. Cows receiving 119 % of their energy requirements produced 1·6 kg milk and 906 kcal of milk energy/day more than those receiving 81·1 % of requirements. This significant effect agrees with results reviewed by Burt (1957) and Rook (1961) although the magnitude of the response to increased energy intake obtained in the present investigation was lower than that given by Burt (1957). This may have been due either to the lower response to energy at the lower protein intakes in the experiment reported here or to the fact that the data reviewed by Burt were mainly from experiments in which the total plane of nutrition rather than energy intake alone had been varied. The higher level of energy intake also resulted in mean increases of 0·11, 0·07, 0·12, and 0·03 % units in SNF, protein, lactose and ash content of the milk, respectively. Only the increase in ash content reached significance and the mean effects, particularly that on SNF, were considerably lower than those given by Burt (1957). Although the overall effect of energy intake on SNF was small and non-significant, the higher energy intake did result in a significant increase of 0·33 % units in SNF at the higher level of protein feeding but caused a reduction in SNF at the lower protein intake (− 0·12 % units). Similar but less pronounced trends were also evident with milk protein and lactose. Rook (1953) in a similar trial was unable to show any difference in the response of milk constituents to changes in energy intake at 2 levels of protein intake. However, the lowest protein level used by Rook was designed to meet requirements (Woodman, 1948), and it may be that the energy response is only affected when lower levels of protein such as that used in the present trial are used.

Associative effects of energy and protein. The results obtained in the present investigation would suggest that the milk yield at any level of energy intake, and the response in milk yield and composition to changes in energy intake, depend upon the level of protein intake. To further investigate this effect of protein level on the output of milk and energy utilization, the milk energy output of each individual animal within each level of protein feeding was related to the intake of DOM by a multiple-regression technique. In the relationships both E and E^2 were significant at the 5 % level. The 'best fit' relationships were

$$\text{high protein } Ye = 1·953E - 0·0000403E^2 - 3909 \qquad (1)$$

residual standard deviation (R.S.D.) 2328

$$\text{low protein } Ye = 0·361E + 0·0000297E^2 + 3097 \quad \text{R.S.D. } 2108 \qquad (2)$$

where Ye is the milk energy in kcal/day and E is the energy intake in g DOM/day. The DOMI was determined directly for 8 animals/equation and estimated using mean treatment digestibility coefficients (mean of 4) for the other 8 animals.

These equations are presented graphically in Fig. 1. As is indicated in Fig. 1, the difference in the output of milk energy from isocaloric diets containing the 2 levels of protein was not constant but depended upon the level of energy intake. This difference is given by

$$1 \cdot 592E - 0 \cdot 0000700E^2 - 7006.$$

The response in milk energy output to an incremental change in energy intake at each of the protein levels can be obtained by differentiation of the quadratic relationships given in equations (1) and (2)

$$\text{high protein } \frac{dYe}{dE} = 1 \cdot 953 - 0 \cdot 000,0806E$$

$$\text{low protein } \frac{dYe}{dE} = 0 \cdot 361 + 0 \cdot 000,0594E$$

where dYe/dE is the instantaneous change in milk energy output for an incremental change in energy intake. Within the range of data presented here the response to additional energy was always greater on the high than on the low protein intake.
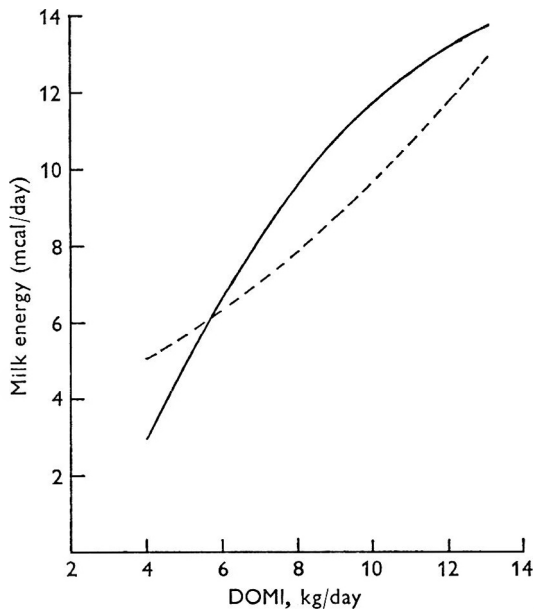
Fig. 1. The relationship between the output of milk energy and the digestible organic matter intake (DOMI) at each of the 2 levels of protein. ———, High protein; -------, low protein.

Blaxter (1966) has shown that the decrease in yield response as the level of energy intake increases is due to a greater proportion of the ingested energy being partitioned towards tissue gain. In general, weight changes in change-over experiments are difficult to interpret due to the short experimental periods in which gut fill changes may be great and the residual effects of treatments. Nevertheless, in order to study the response in liveweight gain to changes in energy intake at each of the 2 protein levels, it was necessary to derive the relationship between liveweight change

(adjusted for residual effects) and energy intake (equations (3) and (4)). In these relationships both E and E^2 were significant at 5 % level

$$\text{high protein } Wc = -0.044E + 0.0000157E^2 - 616 \quad \text{R.S.D. } 534 \tag{3}$$

$$\text{low protein } Wc = 0.742E - 0.0000357E^2 - 3521 \quad \text{R.S.D. } 555 \tag{4}$$

where Wc = the weight change in g/day and E = DOMI in g/day. Differentiation of these relationships gives the response in liveweight to an incremental change in energy intake.

$$\text{High protein } \frac{dWc}{dE} = -0.044 + 0.0000314E.$$

$$\text{Low protein } \frac{dWc}{dE} = 0.742 - 0.0000714E.$$
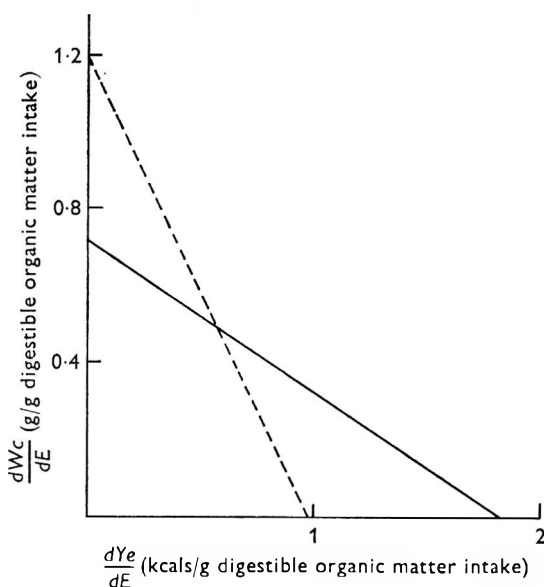
Fig. 2. The relationship between the response in weight change dWc/dE and the response in milk energy output dYe/dE at each of the 2 levels of protein. ———, High protein; ------, low protein.

By relating the milk energy response and bodyweight change response within each protein level the following relationships are obtained

$$\text{high protein } \frac{dYe}{dE} = 1.840 - 2.567 \frac{dWc}{dE}$$

$$\text{low protein } \frac{dYe}{dE} = 0.979 - 0.832 \frac{dWc}{dE}$$

where dWc/dE is the instantaneous change in bodyweight for an incremental change in energy intake.

These 2 relationships are presented graphically in Fig. 2 and show a greater partitioning of incremental additions of energy intake towards milk energy output on

the high than on the low protein diet. If no partitioning towards body gain is assumed (zero tissue balance) the milk energy output/additional g DOMI would be

high protein 1·840 kcal, i.e. approx. 0·49 kcal milk/kcal ME

low protein 0·979 kcal, i.e. approx. 0·26 kcal milk/kcal ME

(assuming 1 g DOM = 3·74 kcal ME (Blaxter & Wainman, 1964)).

Similarly, if there is no response in milk energy output the response in bodyweight/additional g DOM would be

high protein = 0·72 g; low protein = 1·18 g.

The values for the response in milk yield and bodyweight change at the higher level of protein intake agree well with those of Blaxter (1966) who obtained an increase in milk yield of 3·2 kg milk/kg s.e. (compared with 2·83 obtained here assuming milk has an energy content of 715 kcal/kg and 1 kg s.e. = 1·1 kg DOMI) when there was no partitioning of additional energy intake towards liveweight gain. He also obtained a weight gain of 0·82 kg/kg s.e. (compared with 0·79 obtained here) when there was zero increase in milk output. It would appear that the level of protein intake used by Blaxter (1966) may have been only slightly lower than the higher level of protein feeding used here, which may account for the high degree of similarity in the results obtained.

Effect of protein on energy utilization. To further study the partition of energy intake between milk energy output and tissue gain and also the effect of protein level on the efficiency of utilization of energy for milk production, the data within each protein level were analysed by a multiple-regression technique. The energy intake in g DOM/day was partitioned into that used for maintenance, for producing milk, and body-weight change. The equations obtained were

high protein DOMI $= 0·427\,Ye + 1·59\,Wc - 67·7\,W^{0·73} + 10542$ R.S.D. 696   (5)

low protein DOMI $= 0·530\,Ye + 1·791\,Wc + 114·8\,W^{0·73} - 7582$ R.S.D. 1167   (6)

where

$$Ye = \text{yield of milk energy kcal/day,}$$
$$Wc = \text{liveweight change g/day,}$$
$$W^{0·73} = (\text{liveweight in kg})^{0·73}.$$

Using the partial regression coefficients on milk energy output, and assuming both zero tissue balance and no change in maintenance requirements, an increase in energy intake of 0·427 and 0·530 g DOM would be required to produce an increase in milk energy output of 1 kcal on the high and low protein diets, respectively. The standard errors of the regression coefficients were 0·06 and 0·11, respectively. These correspond to efficiencies of conversion of ME to milk energy of 63 % at the higher protein intake and 50 % at the lower protein intake. Both these efficiencies lie within the range of those reported from calorimetric measurements. Van Es (1966) subtracted a standard maintenance allowance and found efficiencies ranging from 55 to 65 %. However, Flatt *et al.* (1966), using higher values for maintenance, obtained efficiencies ranging from 65 to 70 %, which is similar to that given by the Agricultural Research Council (1965). The effect of changes in protein intake on the efficiency with

which ME is utilized for milk production has not received much attention. Mollgaard (1929), cited by Blaxter (1962), has demonstrated that protein over- or under-nutrition decreases the efficiency of utilization of ME. Hashizume, Morimoto, Masubuchi, Abe & Hamada (1965) also obtained a considerable reduction in the utilization of ME for milk production by cows on diets supplying 77 % digestible crude protein standards (National Research Council, 1956) to those receiving 95 %. However, in this trial there was a certain degree of confounding of effects due to a decrease in protein with those due to other factors such as level of fibre intake.

Due to the small amount of variation in the bodyweights of the animals used in derivation of equations (5) and (6), the partial regression coefficients on $W^{0.73}$ are unreliable in estimating maintenance requirements. However, an estimate can be obtained by putting milk energy output and liveweight gain equal to zero and the energy intake obtained relating to the mean metabolic bodyweight within each set of data. By this method the estimates of maintenance were 40·4 and 37·0 g DOMI/kg $W^{0.73}$ on the high- and low protein-diets, respectively. These are equivalent to 151 and 138 kcal/kg $W^{0.73}$ which agree favourably with results reported in the literature from calorimetric trials (Kleiber, Regan & Mead, 1945; Flatt *et al.* 1966).

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Structure of the casein micelle

A proposed model

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Summary. On the basis of complete permeability by high molecular weight reagents of casein micelles in milk and a uniform distribution of the 3 different casein subunits, a model of the micelle structure is proposed. It is composed of an average repeating unit of 1 κ-, 2 α_{s1}- and 2 β-casein subunits assembled in a 3-dimensional network or branched polymer made of 130–130000 monomers, in which the trimers of κ-casein occupy the nodes and the copolymers of α_{s1}- and β-caseins make up the branches. All the associations between subunits are through non-covalent bonds. The chemical composition varies with the number of α_{s1}- and β-casein subunits in the branches. This proposed structure is strongly supported by evidence from electron microscopy and a scale model has been made. It leads to an understanding of the role of κ-casein in micelle formation and opens new perspectives in explaining some properties of the caseins. It offers an interesting example of a new type of quaternary structure of protein subunits.

Casein is synthesized in the secretory cells of the mammary gland in a highly aggregated state making more or less spherical micelles ranging in bovine milk from 300 to 3000 Å in diam. (Nitschmann, 1949). These particles can be conveniently prepared from the milk of lactating mammals and exemplify a relatively simple organized structure produced by a living cell. Their size, stability and structure have long been a challenge to investigators and several models have been proposed (see review by Rose, 1969).

Results already presented (Ribadeau Dumas & Garnier, 1970) show that all the casein subunits in a micelle are accessible to high molecular weight reagents such as carboxypeptidase A, and suggest no preferential positioning of any one of the subunits. This means that inside the casein micelle of milk there are cavities or channels which admit molecules of at least 36000 mol.wt and that the distribution of the 3 casein subunits (α_{s1}, β and κ) is nearly the same at the surface of the micelle as inside it. The existence of such cavities filled with solvent molecules would readily explain the high water content of micelle centrifugate.

One is faced then, with the problem of assembling from 130 to 130000 casein subunits of 3 different kinds to form the globular micelles varying in molecular weight from 3×10^6 to 3×10^9 (Nitschmann, 1949) shown by electron micrographs. As the

casein subunits are relatively small, each made of a single polypeptide chain of 18 700–24 200 mol.wt, the possibility of seeing their spatial arrangement by electron microscopy is doubtful. Furthermore, though electron micrographs of micelle sections published by Shimmin & Hill (1964) confirm an overall homogeneity in the structure they do not reveal any sign of symmetry in the aggregation of the subunits.

This underlines both the difficulties of the task and the limits of confidence that can be given to any proposed model.

In order to understand this structure we first assume that, in spite of the apparent lack of symmetry, the 3 main casein subunits are organized in a basic repeating unit (Garnier & Ribadeau Dumas, 1969). The choice of a suitable repeating unit which can be built into a model of the micelle has been governed by the different requirements listed below. Some of the properties that can be predicted with this model have been compared with those already known and the excellent agreement found gives strong support to the model.

The following points are main requirements for the structure of the repeating unit.

(1) The 3-dimensional structure of the repeating unit should be such that thousands of them may be assembled to form a more or less globular micelle as observed by electron microscopy.

(2) The micelle should have an open sponge-like structure to account for: (a) its high water content of 2–2·5 g/g protein (Ribadeau Dumas & Garnier, 1970); (b) the penetration right through the micelle of dimethylaminonaphthalene-sulphonyl chloride, myoglobin, carboxypeptidase A (Ribadeau Dumas & Garnier, 1970) and also, probably, rennin and β-lactoglobulin, which are known to interact with κ-casein; (c) the accessibility to carboxypeptidase A of the C-terminal ends of all the subunits, to rennin of the bond Phe-Met about 50 amino acid residues from the C-terminal end of κ-casein, and to β-lactoglobulin of at least one SH group of the 2 of κ-casein (Sawyer, Coulter & Jenness, 1963).

(3) A priori, the composition of the repeating unit should, paradoxically, reflect both the average proportions found in milk for the 3 casein subunits, estimated to be 2 α_{s1}, 2 β and 1 κ and the extreme proportions, from 10 to 12 α_{s1} and 1 κ (Zittle, Thompson, Custer & Cerbulis, 1962), down to 1 α_{s1}, 0·3 β and 1 κ (Ribadeau Dumas & Garnier, 1970).

(4) Already known non-covalent associations of the casein subunits are: (a) the functional unit of κ-casein seems to be a trimer of mol.wt about 55 000–60 000 (Swaisgood, Brunner & Lillevik, 1964; Garnier, 1963; 1967), (b) α_{s1}-casein polymerizes in 2 steps: first it forms a tetramer; then several tetramers (up to 6) can make linear polymers (Payens & Schmidt, 1966); (c) β-casein is able to make indefinite linear polymers (Payens & Van Markwijk, 1963); (d) α_{s1}-casein (1 subunit) or β-casein (1 subunit) can form complexes with κ-casein (3 subunits) (Garnier, Yon & Mocquot, 1964; Garnier, 1967)—the complex of α_{s1}- and κ-caseins is the more stable (Mocquot & Garnier, 1965; Garnier, 1967); (e) α_{s1}- and β-caseins can form mixed polymers (Payens, 1968; Garnier, Mocquot, Ribadeau-Dumas & Maubois, 1968).

(5) Its structure should give κ-casein a key role either in building micelles or in the clotting of milk by rennin and other proteases.

(6) Its structure should incorporate divalent ions, phosphate and citrate.

(7) Its structure should lead to a micelle framework consistent with the details in

the electron micrographs recently published (Shimmin & Hill, 1964, 1965; Calapaj, 1968).

Although strict observation of all 7 conditions should lead to a unique model, great uncertainty still arises, especially with regard to point 4, since all the associative properties have been studied in the absence of calcium, i.e. in conditions where micelles do not form. This is due mainly to technical limitations, since with calcium α_{s1}- and β-caseins either precipitate or form with the addition of κ-casein an opaque micellar suspension unsuitable for most standard methods of studying the quaternary structures of proteins.
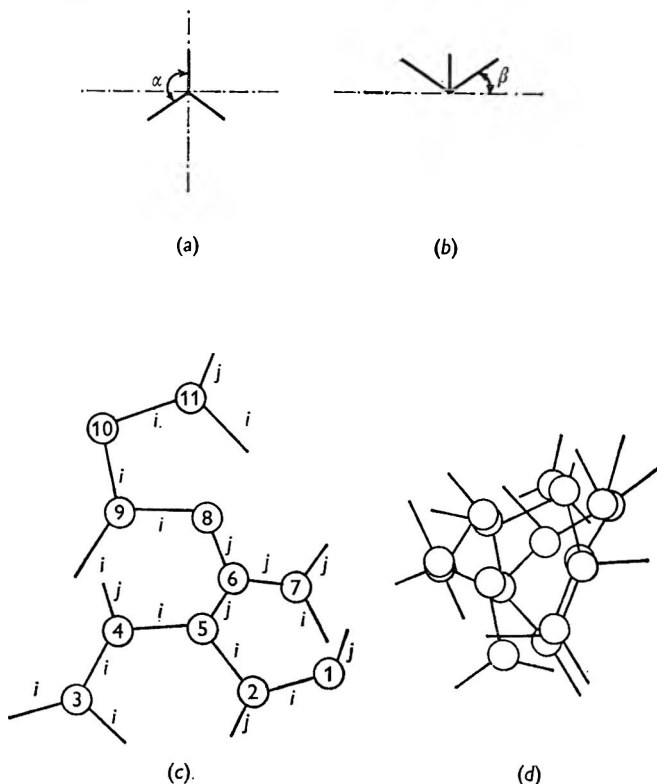
Fig. 1. Scheme of the assembly of trimers of κ-caseins with copolymers of α_{s1}- and β-caseins into the model micelle. (a) and (b) Assembly of one trimer of κ-casein and 3 copolymers of α_{s1}-, β-caseins (dash). (a), seen from the top (projection); (b), seen side face (projection). Angles $\alpha = 120°$, $\beta = 35°$ (see text). (c) Scheme of the micelle model of 161 subunits presented on photographs of Fig. 3; i corresponds to a mixed octamer, 4 α_{s1}- and 4 β-casein subunits, and j to a mixed tetramer, 2 α_{s1}- and 2 β-casein subunits. The numbered circles correspond to trimers of κ-casein forming the nodes of the network of the micelle. (d) Clot of collapsed micelle after transformation of κ-casein into para-κ-casein by rennin. Double circles mean association of 2 trimers of para-κ-casein. Notice the compactness of the new network at the origin of the syneresis.

Structure of the average repeating unit

The simplest 3-dimensional network that one can imagine consists of nodes with 3 branches, one of which is not in the same plane as the other two. The existence of κ-casein in a trimeric form (point 4a) suggests it as the node of the network. Copolymers of α_{s1}- and β-caseins would constitute the branches (point 4b–e) pointing in 3

directions (see Figs 1a, b, 2f and 3d) and joining other trimers of κ-casein. This would make a 3-dimensional network as in Figs 1(c) and 2(f). Where the copolymer of α_{s1}- and β-caseins is an octamer with 4 α_{s1}- and 4 β-casein subunits, the basic repeating unit would consist of 1 κ-, 2 α_{s1}- and 2 β-casein subunits (see Fig. 2f) and would explain the average ratio of the 3 caseins found in milk (point 3). Such a structure should satisfy points 1 and 2 if the casein subunits are so disposed as to be accessible to proteases and the angles α and β (defined as in Fig. 1a, b) are selected so as to give an overall globular shape when a minimum number of subunits is associated (see the 'table tennis balls' model presented below).
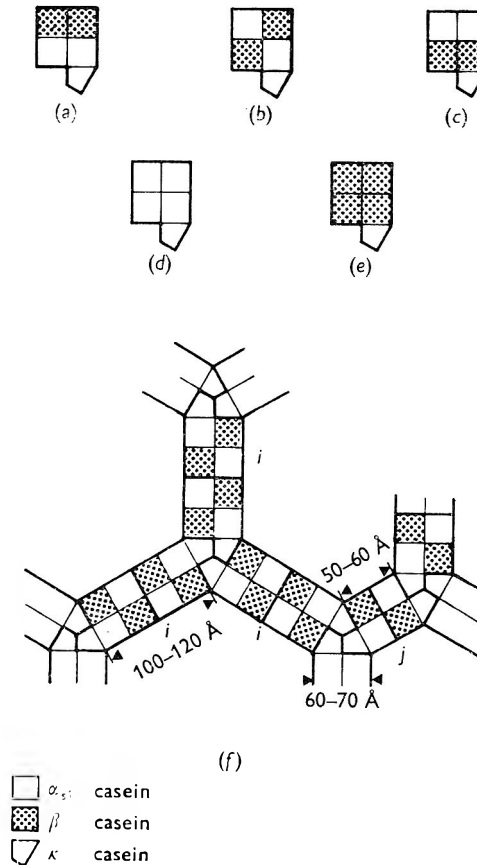
Fig. 2. Detailed structure of the copolymers of κ-, α_{s1}- and β-caseins; (a)–(c) are different types of possible tetramers of α_{s1}- and β-caseins; (d) and (e) correspond to tetramers of only α_{s1}- or β-caseins; (f) gives the approximate dimensions of the network made by the 3 subunits. Strong contour lines underline an elementary repeating unit of 2 α_{s1}, □; 2 β, ▦; 1 κ-caseins, ▽.

This model gives a key role to κ-casein since it orients and limits the polymers of α_{s1}- and β-caseins which otherwise form linear polymers. This is thermodynamically favourable because κ-casein has a higher affinity for α_{s1}- and β-caseins than these 2 subunits have for each other. This satisfies points 5 and 4 b–e. To satisfy point 3 one has to suppose that the copolymers can be made of only α_{s1}-casein subunits and that the length of mixed polymers can vary from 2α_{s1}- and 2 β-caseins (branches j of
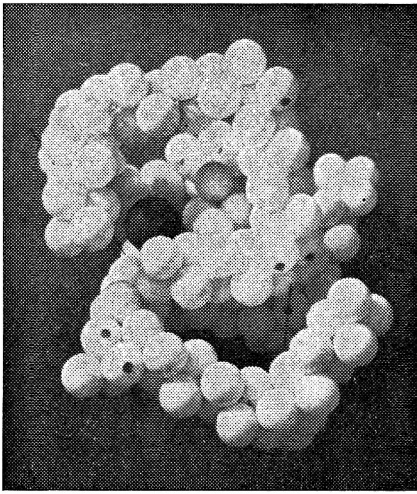
(a)
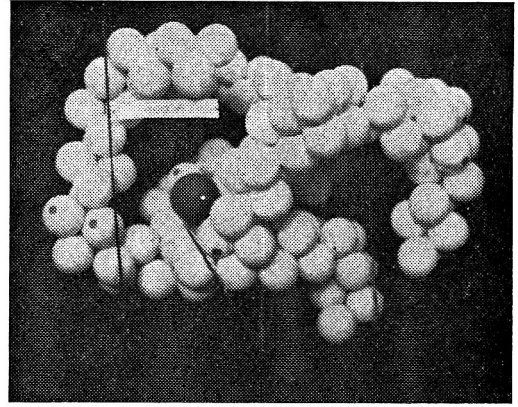
(b)
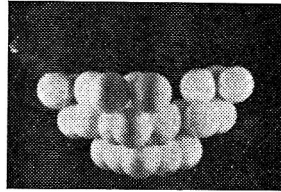
(c)
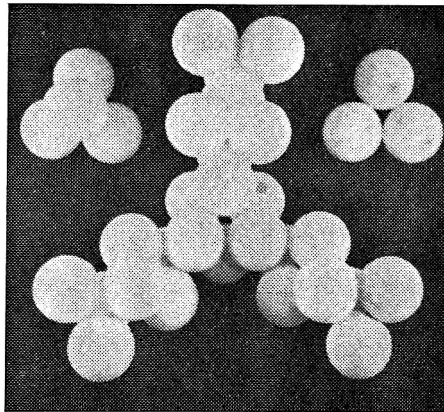
(d)

Fig. 3. Photographs of the scale model of a micelle of 30–35 nm diam. Small black dots on some table-tennis balls of the model represent the caseinomacropeptide region of the κ-casein subunits. The big black ball on (a) and (b) represents at the scale a molecule of rennin hydrolysing a κ-casein subunit or a molecule of carboxypeptidase A splitting the C-terminal end of a subunit. α_{s1}- and β-casein subunits have not been distinguished in this model; (c) side view of a trimer of κ-casein with 3 octamers of the copolymer of α_{s1}- and β-caseins; (d) the same seen from the top including a tetrahedral tetramer of 2 α_{s1}- and 2 β-caseins on the left and a trimer of κ-casein on the right.

Fig. 2*f*) to a higher degree of polymerization (octamers and over) depending on the ratio of κ-casein subunits to α_{s1}- and β-casein subunits. This would allow a flexible chemical composition for the micelle. It is not possible at present to know which of the 3 copolymers of α_{s1}- and β-caseins (*a*), (*b*) or (*c*) (Fig. 2) really exists in the micelles. Evidently this structure tends to play a role similar to α_{s1}- and β-casein subunits.

The trimer of κ-casein can be a closed oligomer with a ternary axis (see Fig. 4*a* where the grey area symbolizes the caseinomacropeptide region and point of rennin
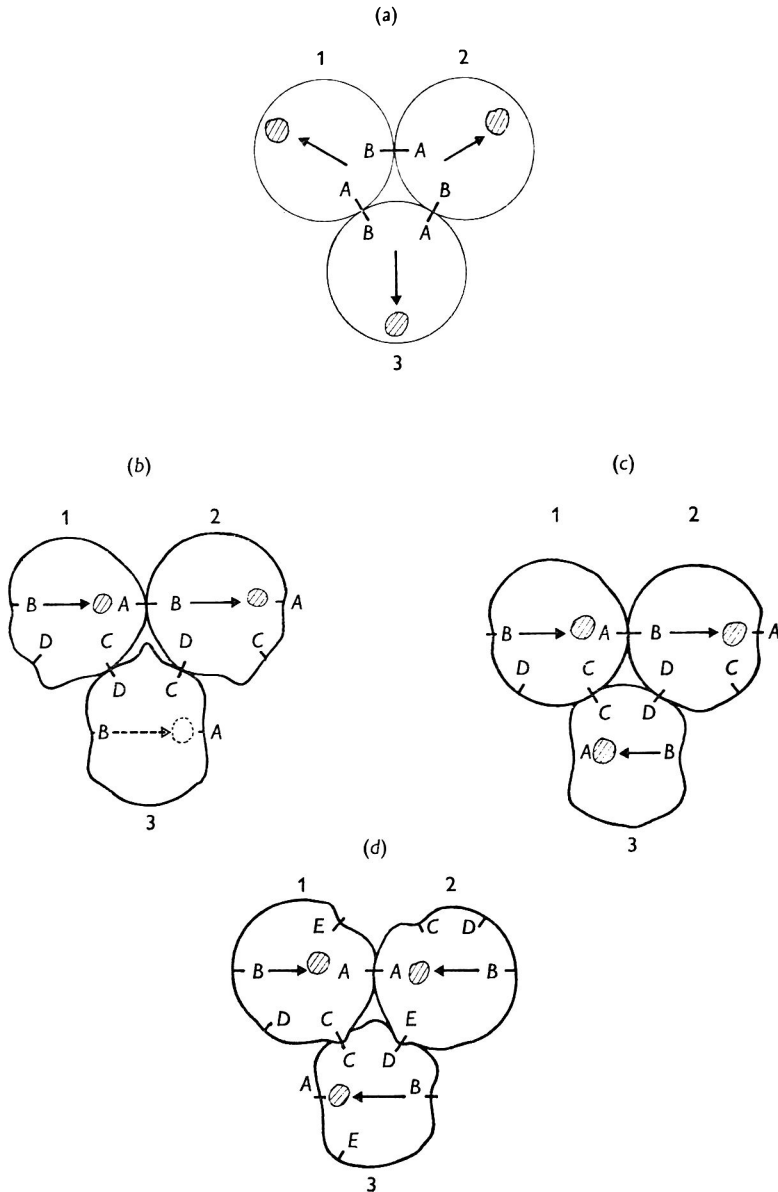
Fig. 4. Different possible structures of the trimer of κ-casein. *A–E* correspond to various areas of association: (*a*) symmetric trimer; (*b–d*) asymmetric trimers; shaded areas symbolize the point of rennin hydrolysis and the caseinomacropeptide region.

hydrolysis). This symmetric trimer offers 3 identical binding sites to the copolymers of α_{s1}- and β-caseins. But this model does not explain why only one proton is released from one of the 3 κ-casein subunits during rennin action (Garnier, 1963; Mercier & Garnier, 1969) from pH 5 up to pH 8, since no chemical heterogeneity can be detected between the 3 subunits. Therefore, one is led to propose an asymmetric closed oligomeric structure as shown in Fig. 4 where 3 possible models (*b*), (*c*) and (*d*) have been drawn. The formation of the *CD*, *CC* and *DD* or *CC* and *DE* bonds would involve a change of the 3 subunits towards a more constrained conformation, for instance, of subunit 3 which will be at the origin of the proton burst after the splitting of the caseinomacropeptide, i.e. it would have in the constrained conformation a masked carboxyl group. This conformational change would prevent further polymerization of the trimer in the direction $B \rightarrow A$ in case (*b*) or (*c*), or the formation of the tetramer in case (*d*) of Fig. 4. This leaves open the possibility of the formation of hexamers or larger polymers in other directions (perpendicular to the plane of Fig. 4 for instance) in solutions containing only κ-casein.

Such an asymmetric trimer offers 3 different binding sites to accommodate either the 3 types (*a*), (*b*) and (*c*) of the copolymers of Fig. 2 or different orientations of the same copolymers of type (*b*) (Fig. 2).

Building up a model of the micelle

A scale model of a micelle on the principles given above would demonstrate whether it can satisfy points 1, 2 and 7. To do this we assumed as a first approximation that the 3 casein subunits can be represented by spheres of the same diameter, in our case a table-tennis ball of 35 mm diam. The molecular weight of the 3 subunits ranges from 18 700 to 24 200 and their average can be taken as 23 000. Then each table-tennis ball represents a molecule of approximately 18 Å radius, corresponding to a magnification of 10^7. We assembled 161 units, including 11 trimers of κ-casein, 12 octamers and 8 tetramers of copolymers of α_{s1}- and β-caseins as described in Fig. 1(*c*). This gives a ratio of 1 κ-casein to 1·9 α_{s1}- and 1·9 β-caseins. Assuming a content of 1 g of protein/2 g water, 161 subunits should give a micelle of average diam. 32 nm*, i.e. 320 mm for our scale model. Since the range in size of micelles found by electron microscopy is 30–300 nm (Nitschmann, 1949), this is one of the smallest. The table-tennis balls were glued together, making all the angles $\alpha = 120°$ and $\beta = 35°$ (Fig. 3*c*, *d*) for the sake of simplicity and assuming a better packing of the copolymers of α_{s1}- and β-caseins on the κ-casein trimer. We also assumed that the copolymers of α_{s1}- and β-caseins have a pyramidal tetrameric configuration (Figs 3*d*) and that the κ-casein trimer has the configuration of Fig. 4(*b*). A photograph of the model is shown in Fig. 3(*a*), (*b*). The trimers of κ-casein numbered 5–7 (Fig. 1*c*) are about in the centre of the model and are joined by tetramers of α_{s1}- and β-caseins forming a nucleus for the micelle. The tetramers appear to be easier to pack than the octamers and hence they prevail at the start.

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* Such a water content gives a calculated density of 1·1 for the micelle, and the number of subunits n for a micelle of radius r (in nm) is

$$n = \frac{4}{3}\pi\frac{1\cdot1}{3} \times \frac{N}{2\cdot3 \times 10^4} \times 10^{-21} \times r^3,$$

N being Avogadro's number, assuming for each subunit an average mol. wt of 23 000.

What is striking in the scale model is that a minimum of 130–160 subunits is needed to form a more or less spherical shape and to saturate most, but not all, of the 'valences' of the κ-casein nodes. This is exactly the minimum size found by electron microscopy and is also the minimum size for observing steric hindrance which would slow down or even interrupt micellar growth with the chosen ratio of subunits. One can see for example that there is not enough space between the α_{s1}-β-casein polymers i and j from κ-casein nodes 9 and 4 to accommodate a trimer of κ-casein and make a closed ring (Fig. 1c). The same applies to nodes 1 and 7. Only the κ-casein nodes 1, 8 and 10 could receive more α_{s1}-β caseins if available. Addition of κ-casein alone could increase only slightly the size (exactly $6 \times 3 = 18$ subunits) of the micelles. The only way to grow the micelles is to add α_{s1}- and β-casein subunits simultaneously and then to decrease the ratio $\kappa/(\alpha_{s1} + \beta)$. This ratio no doubt contributes significantly to an understanding of the size–memory effect observed by Choate, Heckman & Ford (1959) on collections of micelles having different sedimentation coefficients. We do not know the equilibrium conditions or the kinetics of formation for these complex branched polymers with free monomers, but we have shown that if there is such an equilibrium, its establishment requires a rather long time on a molecular scale, i.e. an hour or more (Ribadeau Dumas & Garnier, 1970; Rose, 1968).

An interesting feature of the model is its large interior spaces, allowing easy access of proteases to every subunit (Figs. 3a, b). They are approximately 100–120 Å diam., which is consistent with the size of the dark granules (125 Å) measured by Calapaj (1968) or the clear areas of 100–130 Å diam. visible in the electron micrographs of Shimmin & Hill (1964, 1965).

The dark granules of Calapaj (1968) are therefore deposits in the micellar cavities of the dye used by the author for staining. In the electron micrographs of Shimmin & Hill (1964, 1965) obtained with a different staining process, the black dots could very likely be sections perpendicular to the length of the α_{s1}-β-casein copolymers. Our model predicts a size of 70 Å, and Shimmin & Hill found an average of 80 Å. Bearing in mind the staining process used, we favour this explanation rather than that of Rose & Colvin (1966), who consider the granules to be calcium phosphate aggregates. Miss Michèle Bousquet (personal communication) has shown by electron microscopy the almost complete disappearance of casein micellar structure after treating slices of rabbit udder tissue with pronase before staining. The agreement between these micrographs and our model is then quite good and very well satisfies requirements 1, 2 and 7. Moreover, the enlarged electron micrographs (Plate 1a, b) of casein micelles in the rabbit udder show clearly the kind of network of the model (cf. Plate 1a and Fig. 1c). It is easy in fact to find Y-shaped polymers of proteins in the micelles (Plate 1). The distance between the nodes of these polymers varies from 45 to 100 Å and their width from 40 to 60 Å; again these values agree quite well with those predicted from the model (Fig. 2f). The same type of Y-shaped protein polymers can also be found in electron micrographs published by Shimmin & Hill (1965), Helminen & Ericsson (1968) and Calapaj (1968). These photographs do not rule out the existence of nodes with more than 3 branches, nor do they show that such nodes exist. It can be inferred (Plate 1b) that the angle α is not far from 120°, but no value for the angle β can be proposed from the present electron micrographs.

Finally, it may be seen that our scale model has the dimensions $380 \times 300 \times 350$ mm

corresponding to a micelle of 30–35 nm diam. It is made up of 161 subunits and therefore has the right spacing between protein polymers to admit a water content of 2 g/g protein which is the value found experimentally.

Role of divalent ions

No precise role has yet been given to divalent ions in order to meet, at least partially, point 6 of the general requirement. Calcium may conceivably play a role at the binding sites by making a bridge between 2 carboxyl groups of different subunits; it could also have a conformational role, stabilizing subunits as they form branched copolymers of the proposed type. By linking 2 carboxyl or phosphate groups of the same subunit, calcium ions could select and stabilize one of the possible conformations. A similar role might also be given to calcium phosphate: Bohren & Wenner (1962) have suggested that bivalent phosphate binds 2 carboxyl groups through calcium bridges.

Such a possibility, at least for calcium, is favoured by the observed variation with temperature of the solubility of β-casein. It has been shown (Garnier, 1966) that by increasing the temperature from 5 to 40 °C β-casein goes from a rather relaxed form with an axial ratio of 11–16 (Sullivan *et al.* 1955; Payens & van Markwijk, 1963) to a more compact one which alone is able to polymerize. The temperature of half transition is 23 °C but the solubility curve in the presence of calcium has a midpoint at 18 °C with a steeper slope than the transition curve. Calcium therefore appears to stabilize the compact structure by shifting the equilibrium in its direction.

There is a more remote role for calcium or possibly insoluble calcium phosphate: having an affinity for the subunits, these substances could arrange them spatially so as to promote the earlier stage of micelle formation or nucleation. Although the kinetics of micelle formation cannot yet be explained, the nucleation stage is very probably a critical one.

Electron micrographs of the mammary gland (Wellings & DeOme 1961; Bousquet, Fléchon & Denamur, 1969) show that micelles first form in the Golgi vacuoles (1–3/vacuole) and are then ejected from the cells into the acini; their formation occurs in the cell itself, in small numbers, and separate from other micelles. It would be interesting to observe if a single cell produces micelles of the same size or not. This would permit one to know whether the ratio $\kappa/(\alpha_{s1} + \beta)$ varies with the stage of secretion of a single cell, or with different types of cells indicating a heterogeneous population, or possibly both.

This underlines the possibility that native casein micelles in milk may be in metastable rather than in true (thermodynamic) equilibrium state, i.e. in a local minimum free energy state. We know (Ribadeau Dumas & Garnier, 1970) that the dissociation process is rather slow, and Waugh & Noble (1965) reported path-dependent processes when they reconstituted micelles from isolated casein subunits. The model itself predicts such behaviour: once a subunit is linked with others in the network it binds more strongly than a subunit linked at a free end where there are fewer binding sites to hold it. Possibly only the free ends of the network are able to react with free subunits for the micelle to grow. The first to be formed (the nucleus) can affect the growth and the structure of the micelle, and thus cause distinct differences between native and reconstituted micelles as, for instance, when κ-casein is added to a precipitate of α_{s1}-casein.

The agreement of the model presented in this paper with the known properties of casein micelles in milk is fairly striking, considering the assumptions and various approximations made in order to construct it. The close similarity between dimensions obtained from the model and from the electron micrographs indicates that the treatment of subunits as spheres is not too severe an approximation, at least for the present status of the model. The network with 1–3 branches from each node, as is clearly found in electron micrographs, gives a remarkable functional role to a trimer of κ-casein. The existence of such an assembly of 3 chemically identical polypeptide chains in κ-casein has been suggested from quite different properties of κ-casein: the molecular weight in absence of β-mercaptoethanol (Swaisgood et al. 1964), a proton burst during rennin action (Garnier, 1963), and association with α_{s1}- or β-caseins by the study of the perturbation of a tyrosine residue (Garnier, 1967). Although other models of micelles cannot be completely excluded and this model should, strictly speaking, be considered tentative, the observed agreements provide strong support for it, especially as some interesting predictions can be drawn from it.

One point is the relationship between size and κ-casein content. First, the sponge-like structure of the micelle would produce abnormal sedimentation properties, so that a straightforward relation between size and sedimentation coefficient would not be expected. Secondly, an increased proportion of κ-casein would tend to reduce the length of the copolymers of α_{s1} caseins by offering them more binding sites. This would reduce the size of the micelle both by diminishing the overall bulkiness and by steric hindrance leading to early termination of the growth of the copolymers. The small micelles should be slightly more compact and therefore denser or lower in water content; this would affect in turn their sedimentation behaviour and should add to the effect of a sponge-like structure on the sedimentation coefficient. Conversely, an increase in the proportion of α_{s1}- and β-casein subunits would tend to saturate all the κ-casein trimers and lengthen the copolymer: the size of the micelle would increase because as more α_{s1}-β caseins were bound the size of the mesh would increase. The structure of the micelle is then more open. One can imagine also an upper limit for the size of a micelle when all the valences are saturated and when steric hindrance prevents further binding; this would be a complex function of the size of the mesh, which in turn depends on the ratio of the subunits and on their structure. Hence, we might conclude that the size of this highly organized structure would be regulated by the relative, not the absolute, rate of biosynthesis of the 3 subunits. It will be interesting to know if this type of size regulation is found in more highly organized living systems.

The proposed model suggests an explanation for the clotting action of rennin. It is known that para-κ-casein still binds to α_{s1}- and β-caseins: whole sodium paracaseinate remains clear when para-κ-caseinate alone makes fibrils. Imagine that in an early stage of clotting all the κ-casein nodes have been transformed by rennin into para-κ-casein but the micelle structure is maintained. Through collisions of micelles (highly dependent on temperature) 2 trimers of para-κ-caseins on the exterior surface of the micelle may become linked making the micelles coalesce, as is seen by electron micrography (Hostettler & Imhof, 1951). Any mechanical treatment which disrupts

or distorts the micelle structure (cutting, cheddaring, milling, pressing or even brownian motion) will bring into contact trimers of para-κ-casein within the interior of the micelle, which may also become linked, making a closely packed network of copolymers of α_{s1}-β caseins linked to para-κ-casein, as well as para-κ-casein subunits linked together as shown diagrammatically in Fig. 1(*d*).

The strength of the new network would depend on the number of linked para-κ-casein nodes and therefore on the type of mechanical treatment, its time of application, temperature, etc. Since the new network would be more tightly packed, with new points of contact, it would expel internal aqueous solution or whey; this is the phenomenon called syneresis.

The model offers also an explanation for the loss of β-casein from the micelles at low temperature. The composition and structure of the copolymers allows β-casein subunits to escape slowly without necessarily disrupting the micelle structure. This is due mainly to the possibility of removing one β-casein subunit from the copolymer branches of α_{s1}- and β-casein (see scale model Fig. 3*d* and Fig. 2) but still maintaining a branch of approximately the same length, either by the replacement of one β- by one α_{s1} subunit if a similar function is ascribed to the 2 subunits or by leaving an open space at a β-casein binding site within the copolymer which is maintained in its original conformation by the other interacting casein subunits. It is known (Garnier, 1966) that the β-casein subunit loses its ability to associate at low temperatures.

The model is an example of a quaternary structure with non-covalent binding of subunits but lacking the overall regularity which is found in viruses of known structure. It also, notably, allows variable amounts of the different subunits and an overall comparable quaternary structure with a variable composition. This underlines the difficulty of finding a direct way to analyse its structure. The model emphasizes also the enormous number of subunits, up to a hundred thousand, that can bind together and one might question the use of such a sophisticated structure. This leads also to the question of the role of caseins in nature, apart from that of nutrition. One might think of a possible model of a structural protein which, by the spatial positioning of its subunits, and through the binding of some enzymes to them, might play a role in the mammalian cell analogous to or derived from the organized structures of the cell, e.g. mitochondria. The ability of casein to bind calcium and phosphate is a property similar to that of collagen in bone, and may suggest that casein has evolved from a structural protein. Thus, this complex organization of the casein micelle could exemplify a type of organization of proteins into cell structures.

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EXPLANATION OF PLATE

PLATE 1

Electron micrographs of the rabbit udder during lactation
(through the courtesy of Miss Michèle Bousquet)

Samples have been fixed with glutaraldehyde and osmium tetroxide, embedded in Epon and stained with uranyl acetate and lead citrate. Proteins are stained black. (a) Acina lumen showing casein micelles. $G = 221000$. (b) Casein micelles. $G = 480000$. Circles and arrow point out the Y-shaped polymers.

0·1 μm

(a)

0·1 μm

(b)
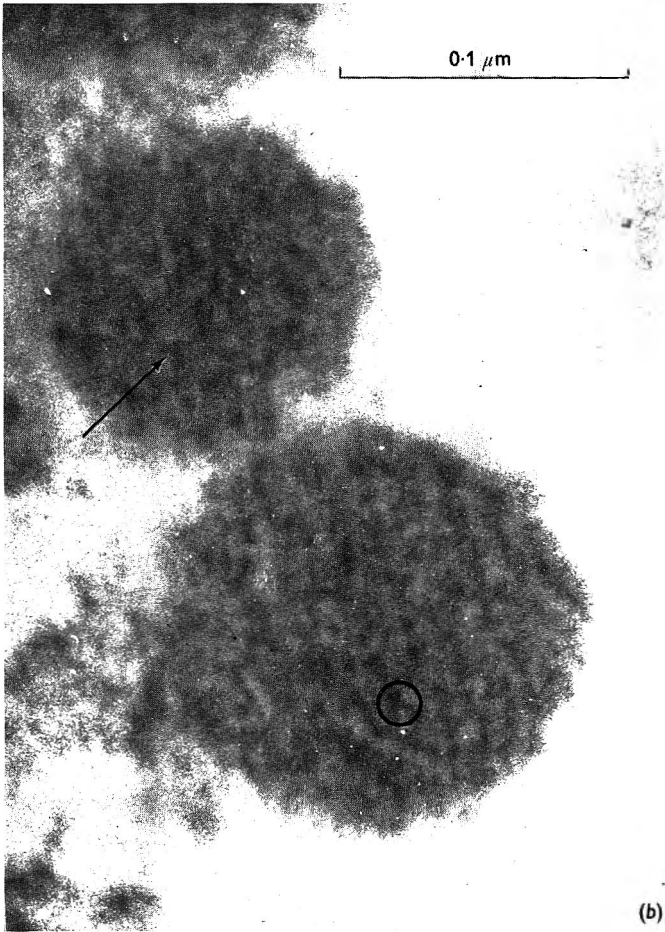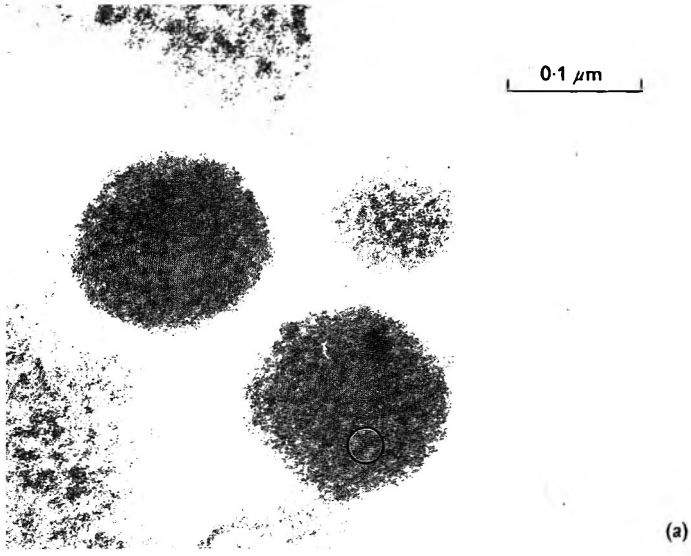
An improved method for canning 3-fold concentrated whole milk

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SUMMARY. A method is described for canning and sterilizing concentrated whole milk containing 36·5 % total solids. The product had acceptable organoleptic properties and did not form sediment or undergo age-thickening during storage. The method involved initially increasing the pH of the raw milk and forewarming. The product was not homogenized nor were polyphosphates added.

Investigations of methods for producing 3-fold concentrated sterile milk have been mainly confined to workers in the U.S.A., who include Leviton, Pallansch & Webb (1962) and Seehafer (1967).

Seehafer (1967) described the commercial process for producing this product. Raw milk is forewarmed for 30 min at about 74 °C and then concentrated under vacuum to about 35 % total solids (TS); after chilling, the concentrate is adjusted to 34·2 % TS and polyphosphates or sodium carbonate are added to improve the physical stability and flavour of the final product. The concentrate is filtered, heated quickly to 66–71 °C in a heat exchanger and sterilized by heating to 146 °C for 2 s. The sterile product is cooled quickly to 66 °C, homogenized at 41·4 MN/m² (6000 lb/in.²), cooled to 10–16 °C, filtered and sealed into presterilized cans, all operations after that of sterilization being done under aseptic conditions. Milk processed in this way commonly coagulates or forms sediment during processing and storage, and gels or age-thickens after prolonged storage. Fat separation is not a problem unless the product is stored at temperatures above 27 °C, when a cream line forms.

Sediment formation is influenced by such factors as the origin and quality of the raw milk, its acidity and the processing method. Forewarming treatments that reduce whey protein nitrogen to low values decrease the tendency of the product to form sediment whereas direct injection of high pressure steam increases this tendency. Age-thickening may be prevented by the addition of polyphosphates, and sodium carbonate is sometimes added to increase the pH from 6·2–6·3 to 6·5–6·6 to reduce the chalky flavour of the sterilized product. Addition of sodium carbonate improves the heat stability of the product but reduces the antigelling effect of the polyphosphates. For products that are to be stored unrefrigerated for long periods Seehafer (1967) advised against pH adjustment because of the risk of age-thickening.

Some steps in the process described by Seehafer (1967) are known to change the structure of fat and protein particles in milk and may thereby reduce the stability of the final product. For example, Knoop, Wortmann & Knoop (1959) showed that the fat globule membrane was modified by simple agitation as well as by homogenization, and Jones, Hall & Trout (1964) showed that direct steam injection has a homogenizing action on oil globules. Mulder (1957) found that the damaged surface of the fat globule adsorbed serum proteins, and Jackson & Brunner (1960) showed that casein was one of the adsorbed proteins. Again, the procedure outlined by Seehafer does not require pH adjustment to compensate for hydrogen ions produced when the milk is heated and it seems likely that the increased acidity may have an adverse effect on the stability of the product. High-temperature, short-time (UHT) processes may sometimes result in incomplete destruction of enzymes which on reactivation could destabilize the product.

Burton (1969) reviewed the many problems encountered in processing milk concentrates by UHT methods. There is clearly a need to develop alternative processing methods that avoid as much as possible the use of elaborate equipment and treatments which induce instability; the present paper gives the results of an investigation of such methods.

<div align="center">EXPERIMENTAL</div>

The experimental work comprised 2 parts; the first involved a canning trial of 2×2 factorial design to determine the effects of pH adjustment and of homogenization on the stability of 36·5 % TS sterile whole milk. The second experiment was a study of the storage stability of canned 36·5 % TS sterile whole milk prepared by the best of the 4 treatments as determined in the first part of the investigation.

<div align="center">Development of the process</div>

Raw milk from the Hunter Valley of New South Wales was standardized to 3·25 % fat, and 8·56 % solids-not-fat and divided into 2 lots. To 1 lot was added 8·8 ml N-NaOH/kg(4 ml/lb) and nothing was added to the other. NaOH was used to adjust the pH because unpublished work carried out in 1955 at the Hunter Valley Co-operative Dairy Co. Ltd, based on the findings of Sommer & Hart (1926), showed that NaOH was superior to phosphates or citrates for stabilizing otherwise unstable concentrated spring milk intended for spray drying. Exploratory experiments in this present investigation also showed that NaOH had a better stabilizing effect than sodium hexametaphosphate with 28 % TS and 36 % TS sterile concentrates. Maximum stability for processing was obtained when the pH values of the sterilized concentrates were 6·85 (28 % TS) and 6·65 (36 % TS) and these pH values were obtained by adding 8·8 ml N-NaOH/kg (4 ml/lb) raw milk. This addition gave an average pH of 7·37 which varied by less than ± 0.05 in 8 samples of raw milk treated during the 4 seasons of the year in the period 1965–9.

After addition of the NaOH to one lot of milk, both lots were forewarmed to 97 °C in 20 min, cooled and each divided into 2 sublots, one of which was homogenized at 71 °C at 13·8 MN/m² (2000 lb/in.²), while the other was not homogenized. The treatments were designated 1–4, as follows.

Treatment 1: NaOH added, not homogenized.

Treatment 2: NaOH added, homogenized.

Treatment 3: NaOH not added, not homogenized.

Treatment 4: NaOH not added, homogenized.

The samples from the 4 treatments were then vacuum-concentrated and standardized to 36·5 % TS, and stored at 3 °C overnight. Polyphosphates were not added at any stage. The concentrates were then quickly preheated to about 80 °C, filled into plain 301 × 214 (8 oz) cans which were closed and processed in steam at 128 °C for $3\frac{1}{2}$ min while spinning at 150 rev/min with their cylindrical axis horizontal. The cans were then spin-cooled in water. This process causes greater inactivation of enzymes than does the UHT process which has the same sterilizing efficiency towards sporing organisms.

Storage investigation

For the second part of the investigation, raw Hunter Valley milk was processed using treatment 1. The canned concentrate was stored at 5, 20 and 30 °C and examined at intervals up to 210 days.

Examination of processed milk

The pH and apparent viscosity of the samples were measured at 21 °C at each stage during processing and at each examination during storage. A Radiometer pH Meter Type TTT 1 C was used for the pH measurements and the relative viscosities of the samples were observed from the time they took to drain from a 20-ml pipette; the time taken by water to drain from this pipette was 14 s. The reflectance of the concentrated products from the storage experiment was measured in the visible region against a magnesium carbonate standard using a Beckman Model B spectrophotometer, and the concentrations of tin and iron in the concentrates were determined by colorimetric methods after wet oxidation (Board & Elbourne, 1964).

The pH, apparent viscosity and solubility index of the reconstituted single-strength samples were also determined. The solubility index was determined by the method given in an Australian standard based on the method described in Bulletin 916 of the American Dry Milk Institute (Standards Association of Australia, 1965). The organoleptic qualities of the reconstituted samples were compared with those of fresh pasteurized milk from the Sydney domestic supply.

RESULTS

Development of the process

Table 1 gives the results of pH, viscosity and solubility index determinations on the 4 samples in the factorial experiment at different stages during preparation, and at 2 days after processing. These data show that addition of NaOH maintained the pH at least 0·4 units above that of the equivalent unadjusted product through all stages of the process, and that the pH of the final canned product containing added alkali was similar to that of the untreated raw milk.

The viscosity of the concentrated milk in treatment 1 decreased with each preparative step, but increased with each step in the other 3 treatments to such an extent that the canned concentrates in treatments 3 and 4 were soft solids. All the canned concentrates dispersed in water with gentle stirring to give reconstituted milks having

viscosities of about 15s: the samples from treatments 2, 3 and 4 contained large quantities of sediment as shown by the data for solubility index in Table 1. The sediment from treatment 2 appeared fine and chalky, and that from treatments 3 and 4 consisted of large irregular grains. When reconstituted milk from the 4 treatments was held for 1 h at 21 °C in cylinders measuring 9 in. in height and $1\frac{1}{2}$ in. in diameter, the following amounts of sedimentation occurred: treatment 1, none; treatment 2, 0·1 in., treatment 3, 4·7 in. and treatment 4, 5·2 in.

Table 1. *pH, Viscosity and solubility index of samples in the 2 × 2 factorial experiment*

	Treatments			
	NaOH added		NaOH not added	
Stage in process	Not homogenized 1	Homogenized 2	Not homogenized 3	Homogenized 4
		pH		
Before forewarming	7·37	7·37	6·77	6·77
After concentration to 36·5% total solids (TS)	6·90	6·91	6·37	6·37
Preheated before canning	6·91	6·90	6·35	6·35
2 Days after canning	6·67	6·67	6·23	6·22
	Viscosity of concentrated milk, s			
After concentration to 36·5% TS	110	270	41	43
Preheated before canning	78	231	55	57
2 Days after canning	69	427	Solidified	Solidified
	Viscosity of reconstituted milk, s			
Preheated before canning	16	16·4	15·2	15·2
2 Days after canning	15·6	15·6	15·0	15·0
	Solubility index of reconstituted milk			
Preheated before canning	0·05	0·1	0·1	0·1
2 Days after canning	0·1	10·5	13·5	11·5

All the concentrates had a slight caramel colour which was hardly noticeable after they had been reconstituted to single strength. The concentrates also had a caramel flavour which was described as 'pleasant but slightly cooked' in the single-strength milks. Concentrates from treatments 2 to 4 were unacceptable because they contained sediment.

Storage investigation

The results of pH measurements on canned concentrated milk prepared by treatment 1 and stored for periods up to 210 days at 5, 20 and 30 °C are shown in Fig. 1. The pH of the single-strength milk obtained by adding distilled water to the concentrate was 0·2–0·3 units higher than that of the corresponding concentrated sample.

The viscosity of the concentrated milk held at 5 °C increased steadily during 210 days storage, from 70 to 128 s (Table 2). Concentrate stored at 20 and 30 °C showed little or no change in viscosity up to 160 days, but at 210 days all samples showed an increase in viscosity of about 10 s and the first signs of gel formation were seen in the samples stored at 30 °C. Some serum separation occurred in concentrates

stored at 20 and 30 °C but the serum recombined with the product on stirring. There was no evidence of separation of cream or free fat. Despite the changes in viscosity of the concentrates, all the reconstituted samples had viscosities of 15–16 s and there was little or no evidence of sediment or physical instability. For instance, solubility index determinations on reconstituted samples at the 85-day examination gave values of 0·05 for all samples; fresh raw milk gave a value of 0·02.
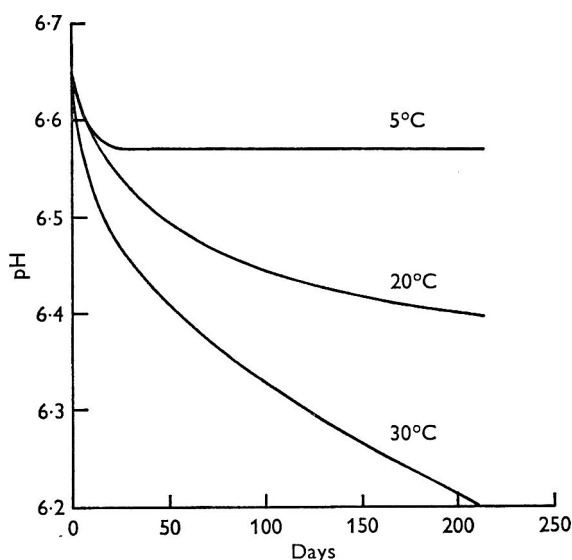
Fig. 1. Change of pH of canned concentrated milk during storage at 5, 20 and 30 °C.

Table 2. *Relative viscosity and reflectance at 450 nm*
of milk concentrate stored at 5, 20 and 30 °C

	Relative viscosity, s			Reflectance, %		
Storage temperature, °C	5	20	30	5	20	30
Storage time, days:						
0	70	70	70	—	—	—
7	85	70	61	30	30	29
85	118	74	65	31	29	23
160	108	72	65	30	30	22
210	128	83	76	31	27	22

The reflectance spectrum of freshly prepared concentrate showed minimum readings near 450 nm rising through an inflection at about 500 nm to a broad maximum near 700 nm. As the samples darkened during storage at the higher temperatures the reflectance decreased over the whole spectrum, the decrease being greater at shorter wavelengths. Reflectance values at 450 nm are recorded in Table 2.

Caramelization was most rapid at 30 °C, slower at 20 °C and apparently absent at 5 °C. Milk reconstituted from concentrate stored at 30 °C for 85 days showed marked caramelization and was probably unacceptable; after storage for 160 days the concentrate gave a single-strength milk with unacceptable colour and flavour. Milk made from concentrates stored at 5 and 20 °C had acceptable quality at all examinations;

the samples had a slight cooked flavour, a full-bodied texture with no dry chalky taste, and a faint caramel colour. In contrast, milk made from commercial samples of canned evaporated milk (29 % TS, pH 6·2) had a watery taste.

Corrosion of the unlacquered electrolytic tinplate cans was by slow detinning and was negligible except in cans stored at 30 °C for 210 days. Many small detinned areas were found on the bodies of these cans, and the side-seam region that had been modified by the heat of soldering during manufacture was also detinned.

DISCUSSION

Evaluation of the 4 procedures used for preparing concentrated whole milk of 36·5 % TS showed that an acceptable product which did not form sediment, undergo age-thickening, or show cream separation during storage for up to 210 days, may be obtained by initially increasing the pH of the raw milk to counteract acid formed during subsequent heat treatments, and by forewarming to denature 90 % of the whey protein. The process involving addition of 8·8 ml N-NaOH/kg raw milk (4 ml/lb) (treatment 1) gave stable canned concentrates on 8 occasions during the 4 seasons of the year. Variations of $\pm 0·05$ pH unit from the average of 7·37 for the NaOH-treated raw milks had no observable effect on the stability or quality of the 8 lots of canned concentrate. The repeatability of the process probably depends in part on the fact that seasonal variations in the properties of Hunter Valley milk are minimized by the current practice of calving throughout the year. It is possible that milk from other areas may have different pH and buffering capacities and may require different amounts of NaOH to give stable concentrates. It seems likely, however, that the procedure of increasing pH of raw milk to about 7·37 to improve heat stability may have general application. Morrissey (1969) showed that there are two pH regions in which milk is highly heat stable, one at about pH 6·7 and another at pH > 7·1. Exploratory experiments in the present investigation covered pH values up to 7·8 and maximum stability was found to be at about pH 7·4. At pH values above 7·4 increased browning and peptization of casein caused quality deterioration. It is interesting that Jenness & Patton (1959) suggested that sodium ions have a specific stabilizing effect on milk.

Homogenization, which is widely used commercially, gave products that formed sediment and thickened with age. It appears that improved stability could be imparted to the current commercial product if the pH of the raw milk was increased and if treatments such as high velocity pumping, steam injection, flash cooling, and homogenization itself were eliminated or minimized. Under such circumstances it may not be necessary to add polyphosphates to improve physical stability and the chalky taste may not then be a problem.

Although the rate of browning during processing increases with increase in pH, the use of a rapid in-can heating process reduced this quality defect to the extent that it was not commercially significant.

The importance of storing concentrated milk at low temperatures to retain quality was shown by the results of the storage investigation, which were consistent with those reported by Patton (1952) and by Loney, Bassette & Claydon (1968). Although the stored concentrate was physically stable for long periods at elevated

temperatures, the colour and flavour deteriorated rapidly. These adverse changes are commonly found in concentrated foods and are generally classified as browning reactions. There is no method known, other than cold storage, that will prevent such changes in products of this type.

The changes in fine structure that the protein bodies and fat globules undergo during the preparation and processing of canned concentrated milk are complex and are described in a paper by Board, Bain, Gove & Mullett (1970).

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Structural changes in whole milk during the production of sterile concentrates: an electron microscope study

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SUMMARY. Electron microscopy was used to study the effect of addition of NaOH, forewarming, homogenization, concentration, and heat sterilization on the structure of whole milk during the production of 4 heat-sterilized concentrates that showed different degrees of physical stability. The samples were prepared for electron microscopy by adding fixative either to liquid or freeze-dried material, and then embedding for sectioning.

Noticeable structural changes occurred during forewarming of samples containing added NaOH, during homogenization, and during heat sterilization. Addition of NaOH and forewarming decreased the closeness of packing of the subunits in the casein micelles. Homogenization reduced the average size of the fat globules, and protein became attached to their surfaces. Heat sterilization caused coalescence of protein. In non-homogenized sterilized concentrate with added NaOH, protein bodies of about 100 times the volume of the original casein micelles were formed; these were free-floating and the concentrate was stable. In non-homogenized sterilized concentrate with no added NaOH the protein bodies were about 15 times the size of the casein micelles and bridged to each other, thereby forming a sediment consisting of large irregular particles. Very large protein bodies containing fat globules formed during heat sterilization of the homogenized samples, both in the presence and in the absence of NaOH, and were responsible for the formation of sediment in these 2 products.

Needle crystals observed in most samples were identifed as $CaCO_3 . H_2O$ by selected area diffraction; KCl crystals were also detected.

Board & Mullett (1970) prepared heat-sterilized whole-milk concentrate (36·5% total solids) using 4 different processes (see Table 1). All 4 products were stable after they were concentrated, but sediment formed in 3 samples during the final heat sterilization process. These differences in stability depended on whether or not the pH was increased at the initial stage of processing and on whether or not the milk was homogenized before concentration. A physically stable product was obtained when the initial pH was increased and the milk was not homogenized; a chalky sediment formed when the pH was increased and the milk was homogenized; sediment composed of

large irregular grains formed in non-homogenized and homogenized milk when the pH was not increased.

The present electron microscope study was carried out to examine the structure of raw milk and to observe the changes brought about by increased pH, forewarming, homogenization, concentration, and heat sterilization.

Several previous workers have investigated the fine structure of milk, examining either the structure of fat globules (Knoop, Wortmann & Knoop, 1958) or of casein micelles in fat-free material (e.g. Shimmin & Hill, 1964, 1965; Calapaj, 1968; Carroll, Thompson & Nutting, 1968; Schmidt, 1968; Schmidt & Buchheim, 1968). Although Schmidt (1968) claimed that fat globules seriously interfered in electron microscope studies of casein particles, we examined whole milk because both fat and protein could be important in determining the physical stability of whole-milk concentrates. Eggmann (1969) has investigated the structure of whole milk using the freeze-etching technique.

METHODS

Processing

The processing treatments applied to the milk are outlined in Table 1 and were described in detail by Board & Mullett (1970). Samples taken from each treatment during processing are designated by letters (a)–(n).

Table 1. *Processing treatments applied to the milks*
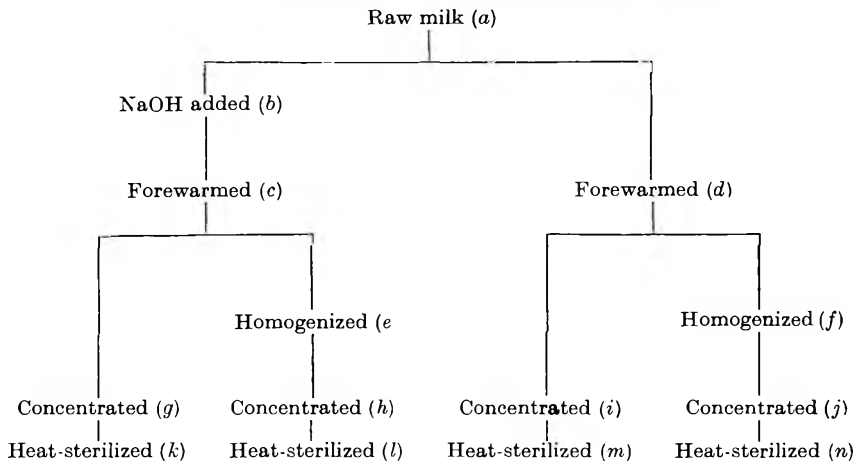
Preparation for electron microscopy

Attempts were made to examine the samples directly in the microscope. Material diluted 1 in 50 with distilled water was applied to the grid as a drop and allowed to dry. It was examined after no further treatment and after fixing in osmium vapour for 30 min. Diluted material was also fixed in glutaraldehyde, but osmium fixation gave the best results. Although the overall structure of the milk examined in this way was consistent with the structure shown later in sectioned material and with that observed by optical microscopy after staining with crystal violet, the method of examination was not satisfactory because of the damage caused by the electron beam (Plate 1a)

and because of the considerable contamination of the microscope. Carbon, evaporated on to the sample, gave some protection of the fat globules from beam damage, but focusing was difficult at magnifications greater than 5000. Shadowing of the samples with platinum–palladium masked the structure of the casein micelles and other protein bodies.

Henceforth, material was taken at each stage in processing and fixed either in the liquid or in the freeze-dried state.

Liquid samples. One ml of milk was fixed in 9 ml of 1 % OsO_4 in cacodylate buffer (pH 6·8) for $2\frac{1}{2}$ h. The black pellet that formed during fixation was washed with buffer solution and dehydrated in an alcohol series, the sample being centrifuged at each change of solution; staining with uranyl acetate (1 % in 70 % alcohol) was carried out for 1 h during dehydration. Pieces of the pellet were then embedded in Araldite, and sectioned using a Porter–Blum MT 2 ultramicrotome. The sections were stained with lead citrate on the grid (Reynolds, 1963) and examined in a Siemens Elmiskop 1 electron microscope.

Freeze-dried samples. Samples of milk, each of 5 ml, were frozen on dry ice or in liquid nitrogen and then freeze-dried. Pieces of dried material (*c.* 1 mm³) were fixed in 1 % buffered OsO_4 for $2\frac{1}{2}$ h and then treated as above, except that the material was not centrifuged.

Both methods gave satisfactory results, but because fat tended to separate during centrifugation of material prepared from liquid samples, the method based on freeze-dried material was preferred and was generally used throughout the investigation.

<center>RESULTS</center>

Structure of raw milk

Electron micrographs of raw milk showed fat globules and casein micelles (up to $0·2\,\mu$m diam.) and diffuse material which probably was serum protein; needle crystals (*c.* $0·1\,\mu$m long) were frequently associated with the protein fraction (Plate 1*b*). The fat globules were surrounded by a double membrane (Prentice, 1969) and the casein micelles appeared to be granular with a rough surface (Shimmin & Hill, 1965; Rose, 1969); some casein micelles appeared to be associated with the fat globules (Plate 1*c*).

Effect of increased pH

The addition of 8·8 ml N-NaOH/kg milk (4 ml/lb) as recommended by Board & Mullett (1970) had no effect on the apparent closeness of packing of the subunits in casein micelles. Larger additions of NaOH appeared to reduce the density of the individual micelles. With additions of 17 ml or more, micelles disintegrated and the opacity of the milk decreased.

Effect of forewarming

Forewarming causes interaction of casein and serum proteins (Sawyer, 1969), so from this stage in the processing, the large micelle structures are referred to as protein bodies rather than as casein micelles.

Forewarming of the samples with 8·8 ml NaOH added (sample *c*) reduced the apparent closeness of packing of the subunits in the protein bodies and may have

caused partial disintegration of some former casein micelles; the amount of electron-dense material in the serum appeared to have increased. Forewarming of the milk containing no added NaOH (sample (d)) resulted in the formation of diffuse particles of about 0·02 μm diam. in the serum; many of these particles were attached to the surface of the dense protein bodies. The double membrane around the fat globule was no longer obvious after forewarming.

Effect of homogenization

Homogenization of forewarmed milk increased the number of fat globules, reduced their average size and increased their total surface area. It appeared that serum proteins and occasionally protein bodies were attached to the fat globules. Eggmann (1969) also observed protein associated with the fat globules in whole milk powder from homogenized milk.

Effect of concentration

The structures of the 4 samples at this stage in processing are shown in Plate 2. The protein bodies in all the samples at this magnification appeared to be similar in size and structure to the casein micelles of raw milk. The homogenized samples (h) and (j) contained more diffuse material in the serum and the average size of the fat globules was smaller than in the non-homogenized samples (g) and (i). At higher magnification, the subunits of the protein bodies of the samples with added NaOH (samples (g) and (h)) appeared to be less closely packed than in casein micelles of raw milk or in the protein bodies of samples (i) and (j).

Effect of heat sterilization

Heat sterilization caused the most marked changes in the structure of the milk concentrates. These changes involved fusion of the protein bodies, and sometimes fat globules and protein bodies, to form entirely new structures. In sample (k) (Plate 3a) these structures were physically stable, but in sample (l) (Plate 3b) the new structures formed a chalky sediment and in samples (m) and (n) (Plate 3c, d) the structures formed large, soft, irregularly shaped particles of sediment. The areas shown in Plate 3 (b–d) are only a small part of the individual sediment particles in samples (l), (m) and (n), and as such do not adequately represent the gross morphology of the samples.

Sample (k) (Plate 3a). The protein bodies in this non-homogenized concentrate containing NaOH increased more than 100-fold in volume during heat sterilization. The protein bodies (diam. 1·0 μm) were now approximately 5 times the diameter of the casein micelles in raw milk. Most of the protein bodies were free-floating. Fat globules were either free or attached to single protein bodies. Diffuse background material was present as in raw milk. The absence of sediment in this sample was associated with the free-floating nature of the protein bodies, fat globules, and protein–fat complexes.

Sample (l) (Plate 3b). The particles in this fine chalky sediment were made up of enlarged irregularly shaped protein bodies which were several μm across and which contained numerous fat globules. The addition of NaOH was again responsible for enlargement of the protein bodies in the milk and homogenization caused the fat

globules to be embedded in them. The diffuse background material was present as in raw milk.

Sample (m) (Plate 3c). The sediment particles in this non-homogenized sample with no NaOH added were much larger and softer than those in sample (*l*). The protein bodies were about 15 times greater in volume than the casein micelles in raw milk, but were smaller (0·5 μm diam.) than any other protein bodies in the concentrated and heat-sterilized samples. Their surface was covered with strands of protein which appeared to bridge with neighbouring protein bodies to form the sediment particles in the product. Fat globules usually had one or more protein bodies attached to their surface, but the globules were not deeply embedded as in the homogenized product (samples (*l*) and (*n*)).

Sample (n) (Plate 3d). The effect of homogenization in this sample was similar to that in sample (*l*) (Plate 3*b*), but there was not so much fusion of the protein bodies nor embedding of the fat globules in the absence of NaOH. At higher magnification, there was evidence that the protein bodies were joined to each other by protein bridges as in sample (*m*), which would have helped stabilize the sediment particles.

Crystalline deposits

Many samples of raw milk and processed milk contained needle crystals about 0·1 μm long, which were usually associated with the protein fraction (e.g. Plates 1*b*, 2*a*, 3*a*). Electron diffraction measurements made on these preparations indicated interference by the fixative or staining materials, so selected area diffraction measurements were made on unfixed and unstained samples applied directly to the grid. The electron diffraction patterns for the needle crystals corresponded to those published for $CaCO_3.H_2O$; an additional pattern, obtained from the serum region, corresponded to that of KCl, though no crystals were visible (Table 2).

Table 2. *Electron diffraction parameters for $CaCO_3.H_2O$ and KCl, compared with those for crystalline components of milk preparations*

Values for needle crystals	$CaCO_3.H_2O$*	Values from serum region	KCl*
2·37	2·37	3·14	3·15
2·17	2·16	2·22	2·22
1·93	1·93	1·81	1·82
1·76	1·76	1·58	1·57
1·34	1·37	1·42	1·41
		1·28	1·28

* X-ray powder data file index of American Society for Testing and Materials (1962)

DISCUSSION

The electron micrographs of milk at various stages in the production of the unsterilized concentrates showed that addition of NaOH and/or homogenization caused changes in the structure of the protein bodies and fat globules, and these changes influenced the way in which the concentrates reacted during heat sterilization.

The decrease in apparent packing density of the subunits in the casein micelles on addition of NaOH and forewarming was probably caused by the breaking of apatite

bridges (Rose, 1969). It may, therefore, be significant that samples containing added NaOH generally contained more needle crystals having a diffraction pattern corresponding to that of the $CaCO_3 . H_2O$ than did samples which contained no added NaOH. It seems likely that the open-structured casein micelles and free subunits reacted with whey proteins during forewarming (Burton, 1969) and that during heat sterilization about 100 of the resultant protein bodies coalesced to form each of the large free-floating spherical protein bodies found in sample (k). A similar sequence of reactions probably led to coalescence of protein in sample (l), but the form of the final protein bodies was also influenced by earlier homogenization. Coalescence of protein bodies also took place in the heat-sterilized concentrate with no NaOH added (samples (m) and (n)), but to a lesser extent. The difference in the amount of protein coalescence, in samples with and without added NaOH, may indicate differences in the distribution of whey proteins in the surface of the protein bodies in the concentrates immediately before heat sterilization.

Two consecutive steps seem to be involved in the formation of sediment in the heat-sterilized samples of homogenized milk (samples (l) and (n)). The first step occurred during homogenization and involved deposition of serum proteins and occasionally protein bodies on the newly formed fat–serum interfaces. The deposited proteins effectively embedded most of the fat globules even though up to the stage before sterilization (samples (h) and (j)) there was no evidence of sediment. The second step in sediment formation involved coalescence of neighbouring protein bodies as occurred in the non-homogenized samples (k) and (m) during heat sterilization. Fusion of these bodies gave the irregularly shaped particles of sediment containing embedded fat.

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EXPLANATION OF PLATES

All plates, except Plate 1 (a), are electron micrographs of sections of freeze-dried milk which was fixed in 1 % OsO_4 in cacodylate buffer and stained with uranyl acetate during dehydration. Sections were stained with lead citrate on the grid.

PLATE 1

RAW WHOLE MILK

(a) Electron micrograph of material fixed on the grid by exposure to osmium vapour for 30 min. Casein micelles (C) are in close contact with a fat globule (F) which has suffered damage in the electron beam. × 40 000.

P. W. BOARD, JOAN M. BAIN, D. W. GOVE AND J. T. MULLETT

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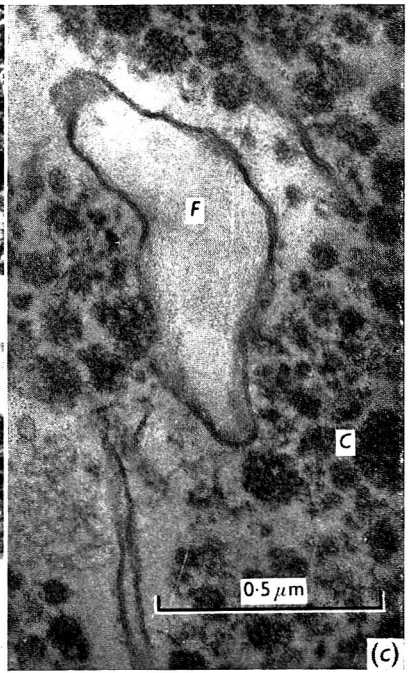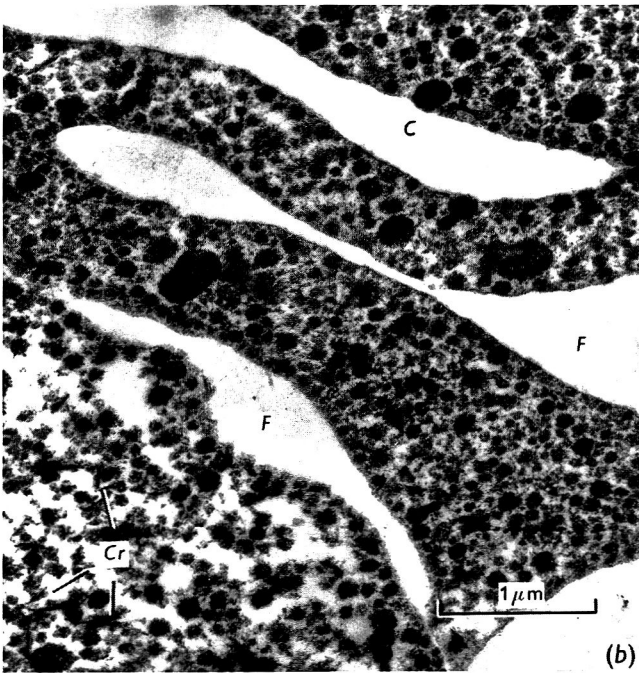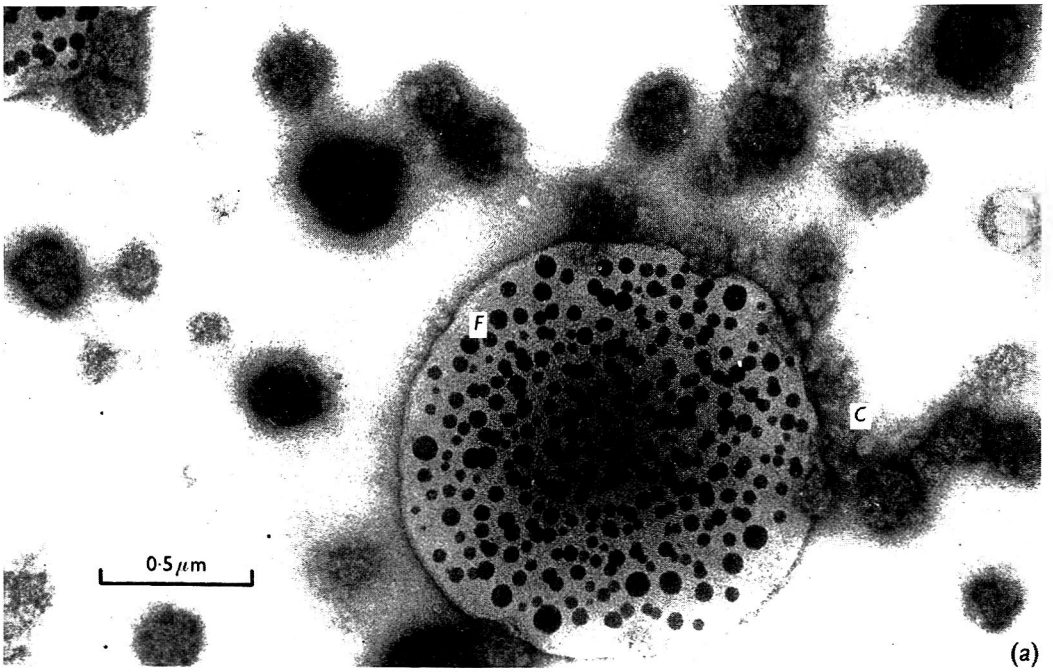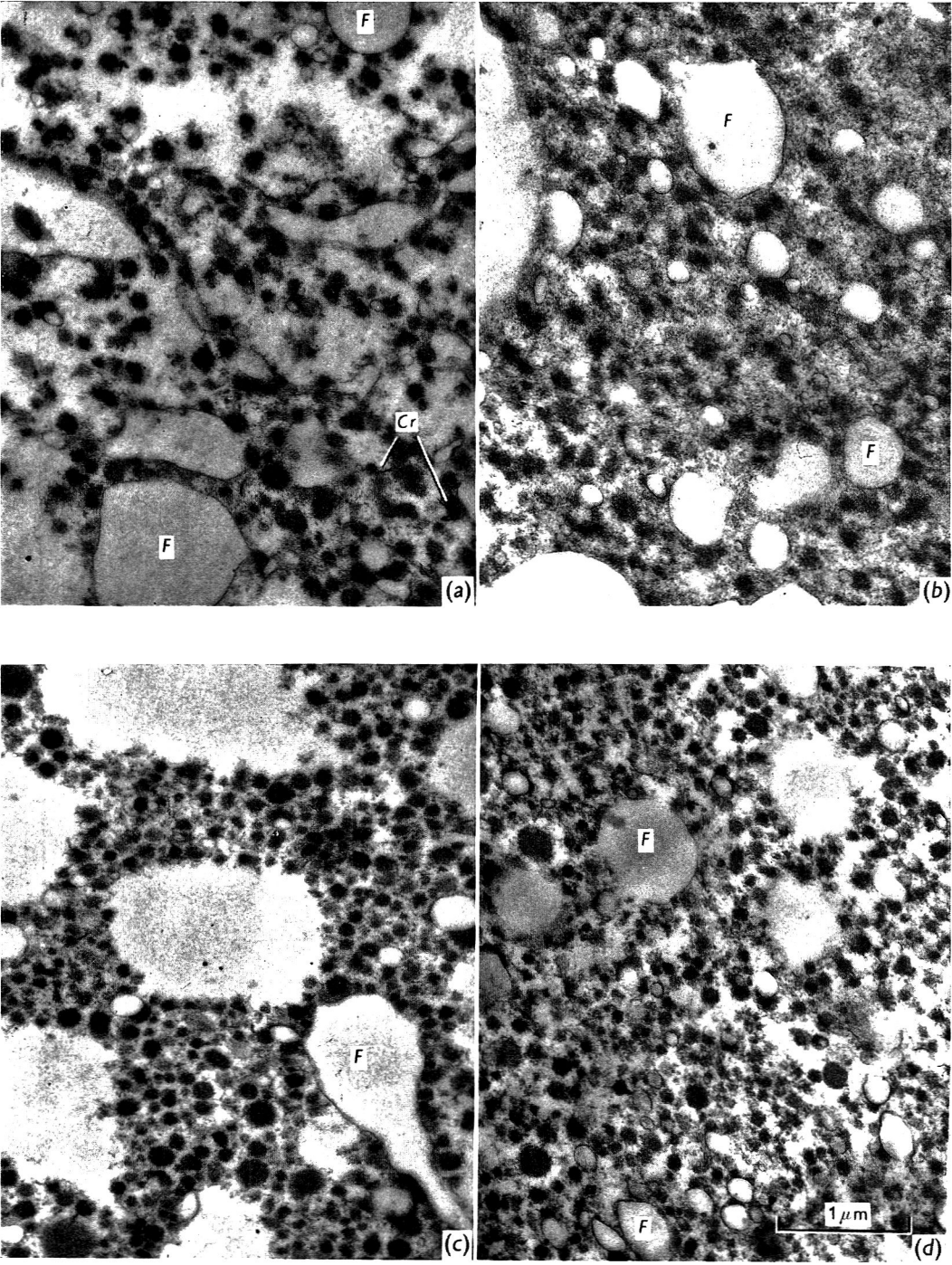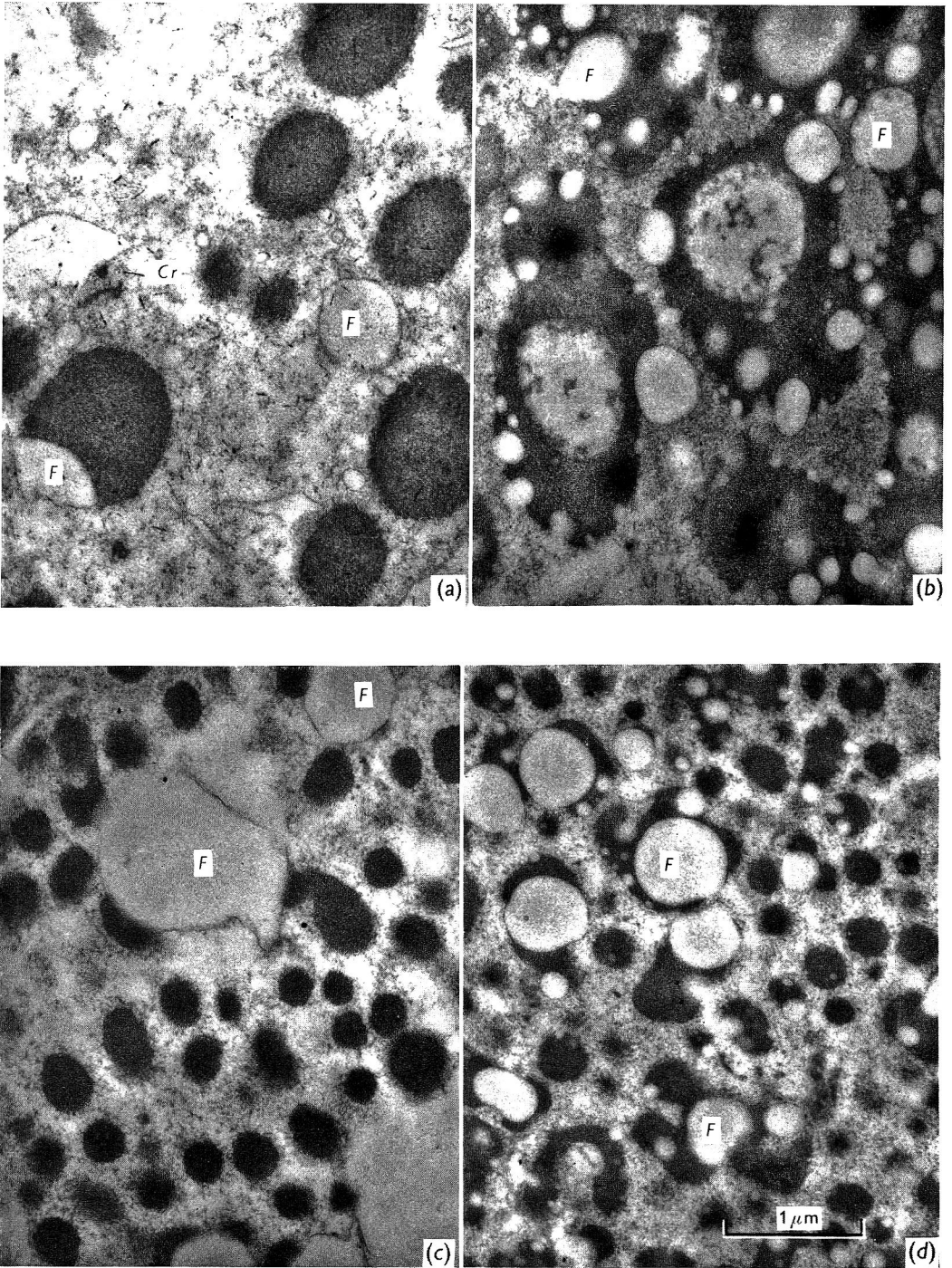
(*b*) Distribution of casein micelles (*C*) and fat globules (*F*). Needle-like crystals (*Cr*) are indicated. × 20000. (*c*) Detail of casein micelles (*C*) and of a fat globule (*F*) shown surrounded by a double membrane. × 60000.

<center>PLATE 2</center>

<center>CONCENTRATED WHOLE MILK</center>

Protein bodies in all 4 samples appear similar to those in raw milk (Plate 1*b*). Fat globules (*F*) are smaller in the homogenized samples. Needle crystals are indicated by (*Cr*). × 20000. (*a*) Sample (*g*). NaOH added, forewarmed, not homogenized. (*b*) Sample (*h*). NaOH added, forewarmed, homogenized. (*c*) Sample (*i*). NaOH not added, forewarmed, not homogenized. (*d*) Sample (*j*). NaOH not added, forewarmed, homogenized.

<center>PLATE 3</center>

<center>HEAT-STERILIZED CONCENTRATED WHOLE MILK</center>

(*a*) Sample (*k*). NaOH added, forewarmed, not homogenized. Showing enlarged, roughly spherical protein bodies. Fat globules (*F*) are free or in contact with a single protein body. Needle crystals (*Cr*) are associated with the protein. × 20000. (*b*) Sample (*l*). NaOH added, forewarmed, homogenized. Numerous small fat globules (*F*) are embedded in large areas of protein. × 20000. (*c*) Sample (*m*). No NaOH added, forewarmed, not homogenized. Protein bodies have enlarged, but to a lesser extent than in sample (*k*). They appear bridged to one another by protein strands. Some protein bodies are associated with the fat globules (*F*). × 20 000. (*d*) Sample (*n*). No NaOH added, forewarmed, homogenized. Numerous small fat globules (*F*) are embedded in protein, as in sample (*l*), but the protein bodies have not coalesced to the same extent. × 20000.

<center>*Printed in Great Britain*</center>

The determination of vitamin C in evaporated and fortified sterilized milks

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SUMMARY. A study has been made of the use of 2,6-dichlorophenolindophenol (DCP) and also of 2,4-dinitrophenylhydrazine (DNPH) with and without chromatography of the DNPH derivatives, for the determination of ascorbic acid (AA) and dehydro-ascorbic acid (DHA) in evaporated milks, and of AA in AA-fortified sterilized milks.

In the DNPH method, when interfering compounds were removed by the chromato-graphic separation of the DNPH derivatives, absorption curves typical of the pure DHA derivative were obtained.

The DCP method gave erroneously high values for the AA and DHA content, and the lower values obtained by the DNPH method with chromatography of the DNPH derivatives were judged correct. Thus for an evaporated milk, the values for vitamin C content (AA + DHA) were, in mg/100 ml reconstituted milk, 0·88 (DCP method), 0·66 (DNPH method without chromatography), and 0·48 (DNPH method combined with chromatography). The AA contents (mg/100 ml) of the 2 fortified sterilized milks were 3·63 and 5·78 (DCP method); 3·01 and 4·28 (DNPH method combined with chromatography).

The AA contents of the 5 different evaporated milks, determined by chromatography of the DNPH derivatives, ranged from 0·07 to 0·63 mg/100 ml reconstituted milk. The DHA content was negligible.

As judged by the shape of the absorption curves after chromatography of the DNPH derivatives, it is concluded that this method is the most reliable of those studied.

The use of 2,6-dichlorophenolindophenol (DCP) and of 2,4-dinitrophenylhydrazine (DNPH) for the determination of vitamin C [ascorbic acid (AA) + dehydroascorbic acid (DHA)] in milks given various heat treatments has been studied by Toothill, Thompson & Edwards-Webb (1970). The intensive heat treatment used in the pre-paration of evaporated and sterilized milks may lead to the formation of compounds with free sulphydryl groups and of reductones, which may interfere with the deter-mination of vitamin C by simple oxidation–reduction titration procedures, as for example with DCP, causing the results to be erroneously high. Although the speci-ficity of the DCP procedure can be improved to some extent, this reagent is essentially unspecific. DNPH, which reacts with carbonyl compounds, is more specific and forms an osazone with DHA after the transformation of the DHA to 2,3-dioxogulonic acid (DOA). Treatment of the osazone with sulphuric acid (Roe & Kuether, 1943) gives a red coloured solution with an absorbance peak at 522–526 nm. Other carbonyl com-

pounds, including reductones, may interfere but the specificity of the method can be greatly improved by the chromatographic separation of the DOA osazone from other DNPH derivatives (Gordon & Noble, 1959; Mapson, 1961; Vuilleumier & Nobile, 1962; Stroheeker & Henning, 1965; Association of Vitamin Chemists, 1966). AA reacts with DNPH only after oxidation to DHA, and with the inclusion of a reduction step the method can be used to determine AA, DHA and DOA (Roe, Mills, Oesterling & Damron, 1948).

The method of Roe *et al.* (1948), which does not include chromatography, was used in a modified form by Toothill *et al.* (1970) and was found to be unsatisfactory for strongly heated milks, since although the calculated results indicated the presence of moderate amounts of AA, the absorption curves of the DNPH derivatives showed no evidence of this. Because Toothill *et al.* (1970) found the method of Roe *et al.* (1948) to be specific for the determination of vitamin C in in-bottle sterilized milk only after the chromatographic separation of the DNPH derivatives, it was felt that the investigation should be extended to include evaporated and AA-fortified sterilized milks. The results of this study are now reported. For brevity the DNPH derivatives are referred to as hydrazones.

EXPERIMENTAL

Milks

Evaporated milks (unsweetened). Five different proprietary brands (Evap. A–E) purchased from local shops were kept in a cool room and opened within a few days, except for Evap. Bb, which was stored unopened at about 3 °C for 6 weeks. Evap. Ba and Bb had the same batch number.

Sterilized milks. These were a proprietary full-cream (Ster. A) and half-cream (Ster. B) baby-food formulation, supplied by the manufacturer immediately after processing. These milks contained added vitamin C, vitamin D and ferrous iron. The sugar content had been raised to about 7 % with sucrose (Ster. A) or lactose (Ster. B). The heat treatment, to which they had been subjected, included in-bottle sterilization at 116 °C for 10 min followed by incubation at 32 °C for several days. Before analysis the milks, which were in clear glass bottles, were kept for 64 days in the laboratory at ambient temperature and in the light, about 4 m from a shaded, south-facing window. The oxygen content of samples opened on the first day of storage was measured and found to be < 1 ppm (J. E. Ford, personal communication).

Analysis

Details of the methods used are given by Toothill *et al.* (1970). The evaporated milks were reconstituted with distilled water and samples were taken immediately for analysis.

DCP method. The titrations were carried out on metaphosphoric acid extracts of the milks. Bromine was used as the oxidizing agent and hydrogen sulphide as the reducing agent.

DNPH methods. Diluting Solution (Toothill *et al.* 1970) was added to the extracts of the sterilized milks to give solutions with a suitable concentration of AA.

For determinations without chromatography, aliquots of standards and of untreated, oxidized and reduced milk extracts were coupled with DNPH for 3 h at 37 °C. With Evap. C, additional aliquots were coupled for about $19\frac{1}{2}$ h at room temperature.

The absorbance was determined at 524 and 540 nm with 42 % sulphuric acid solution in the reference cell, 40 min after the addition of 85 % sulphuric acid solution to the hydrazones for colour development. Blanks were also determined. Immediately afterwards, the absorption spectrum was determined, first with 42 % sulphuric acid solution and then with the appropriate blank in the reference cell, using a recording spectrophotometer over the range 400–600 nm. At the same time as aliquots were taken for the 3-h coupling period, additional aliquots were removed for the chromatographic procedure and, for convenience, were coupled at room temperature (about 20 °C) overnight for a total of about $19\frac{1}{2}$ h. Because of the long time required for the analysis, the column eluates were stored overnight at -30 °C and the final separation of the DNPH derivatives by thin layer chromatography was carried out on the next day. The absorption spectrum of the osazone was determined over the range 300–600 nm with 42 % sulphuric acid solution in the reference cell, 40 min after the addition of 42 % sulphuric acid solution for colour development.

Rubber gloves were worn to avoid contact with DNPH.
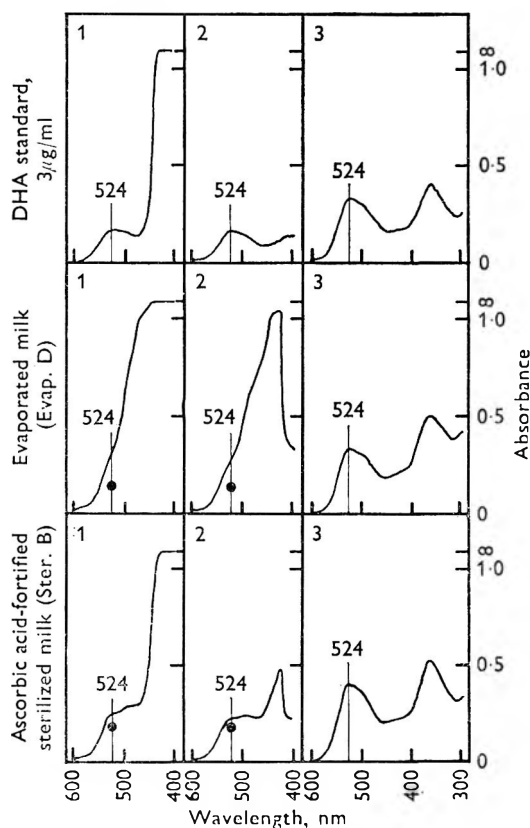
Fig. 1. The absorption spectra of the 2,4-dinitrophenylhydrazones of dehydroascorbic acid (DHA) and of the oxidized extracts (ascorbic acid (AA) + DHA + dioxogulonic acid + interfering substances) of evaporated and AA-fortified sterilized milks, 40 min after development of the colour with 85 % sulphuric acid. (1 and 2), before chromatography, 42 % sulphuric acid (1) or the corresponding blank (2) in the reference cell; (3), after chromatography, 42 % sulphuric acid in the reference cell. The correct absorbance due to AA content as calculated by the chromatographic procedure is shown (●). Because of the larger volumes of test solution used, the chromatographic procedure results in higher absorbance values.

RESULTS

Absorption spectra of the hydrazones

For all the milks, the typical absorption curve of DHA with a peak at about 524 nm (Fig. 1) was obtained only after chromatography of the hydrazones.

Evaporated milks. Without chromatography, all the curves fell steeply in the 524 nm wavelength region, whether 42 % sulphuric acid solution or the corresponding blank was in the reference cell. Fig. 1 shows the absorption curves obtained for the oxidized extract of Evap. D before and after chromatography.

Sterilized milks. Before chromatography, the curves of the hydrazones of the untreated extracts showed a very small shoulder, but this was in the region of 500 nm. Those of the oxidized extracts indicated that DHA might be present, but there was also a component with a peak at about 495 nm which was not eliminated when the corresponding blank, instead of sulphuric acid solution, was in the reference cell. Fig. 1 shows the absorption curves obtained for the oxidized extract of Ster. B, before and after chromatography. The reduced extracts of these milks were not studied since only AA was being determined.

Table 1. *Mean values for vitamin C content (mg/100 ml) of reconstituted evaporated milks and of fortified sterilized milks,* determined by titration with 2,6-dichlorophenolindophenol (DCP) and from the absorbance values of the 2,4-dinitrophenylhydrazine (DNPH) derivatives at 524 and 540 nm*

	Ascorbic acid				Dehydroascorbic acid			
		DNPH method				DNPH method		
		No chromatography†		With chromatography‡		No chromatography†		With chromatography‡
Milk	DCP method	524 nm	540 nm	524 nm	DCP method	524 nm	540 nm	524 nm
Evaporated								
A	0·80	0·60	0·57	0·46	0·08	0·11	0·09	0·02
Ba	—	0·25	0·21	0·08	—	0·10	0·09	0·01
Bb	0·37	0·18	0·15	0·07	0·23	0·09	0·10	0·00
C	—	{0·50 / 0·49‡	0·50 / 0·50‡	0·39	—	{0·04 / 0·08‡	0·07 / 0·08‡	0·01
D	—	0·87	0·84	0·63	—	0·04	0·10	0·03
E	—	0·53	0·49	0·49	—	0·11	0·12	0·02
Fortified sterilized								
A	3·63	3·00	3·03	3·01	—	—	—	—
B	5·78	4·77	4·81	4·28	—	—	—	—

* A proprietary fortified and sterilized liquid baby food. Details given in text.
† Coupled at 37 °C for 3 h.
‡ Coupled at room temperature (about 20 °C) for about 19 h 30 min.

AA content

Table 1 shows that the DCP method gave higher AA values for the 2 evaporated milks studied (Evap. A, Bb), and for the sterilized milks (Ster. A, B), than were obtained by the DNPH methods.

Without chromatography of the hydrazones and using the absorbance values at 524 nm, the AA content of the 5 evaporated milks ranged from 0·18 to 0·87 mg/100 ml. Slightly lower values for AA content were generally obtained using the absorbance values at 540 nm. Coupling with DNPH for 19½ h at room temperature, as was used in the chromatographic procedure, gave with Evap. C the same AA values as coupling for 3 h at 37 °C. The values of 3·0 and 4·8 mg AA/100 ml for Ster. A and B, respectively, indicated that even after 64 days of storage in the laboratory at ambient temperature and exposed to light, these samples apparently still contained a considerable amount of AA.

After chromatography of the hydrazones, the AA content of the evaporated milks was generally 0·08–0·21 mg/100 ml less than the values obtained from the absorbances determined at 540 nm without chromatography. The value for Evap. E remained unchanged, however. Whereas almost identical and very low values for the AA content of Evap. Ba and Bb were obtained by the chromatographic procedure, there was a discrepancy in the results obtained without the chromatography step on account of the high absorbance of the blank of the oxidized extract of Evap. Bb. This may have been due to the instability of the absorbance readings which is typical of strongly heated milks. Of the 2 sterilized milks, only the value for Ster. B showed any appreciable decrease after chromatography of the hydrazones, and the high level of AA in these milks was confirmed.

DHA content

Of the 2 milks analysed using the DCP method, the value of 0·08 mg DHA/100 ml for Evap. A was similar to that found by the DNPH method without chromatography, whilst that of 0·23 mg/100 ml for Evap. Bb was considerably higher.

Using the absorbances at 524 and 540 nm before chromatography of the hydrazones, the values found for the DHA content of the 5 evaporated milks were very low and ranged from 0·04 to 0·12 mg/100 ml. Using the chromatographic procedure virtually zero values were obtained.

DISCUSSION

The DCP method gave higher values for vitamin C content (AA + DHA) than were obtained by the DNPH methods. For Evap. Bb, which contained only a trace of vitamin C (0·07 mg/100 ml) as measured by the DNPH method with chromatography of the hydrazones, the DCP method gave a value of 0·60 mg/100 ml. The corresponding values for Evap. A were 0·48 and 0·88 mg/100 ml. Higher values for AA were obtained also in the sterilized milks by the DCP method.

In the DNPH method it was necessary, for both types of milk, to carry out the chromatographic separation of the hydrazones to ascertain that AA was present, although the error was small when this step was omitted. The AA contents of the various samples of evaporated milks differed, and after chromatography of the hydrazones the highest level, 0·63 mg/100 ml in Evap. D, was almost a third of the AA content found in freshly secreted cow's milk (Kon & Watson, 1936) whereas only a trace was present in Evap. B. It is likely that the AA remaining in the evaporated milks after processing is reasonably stable and that the tinned surface of the can may assist in this stability by the removal of oxygen, as reported by Kohman (1923) for canned

fruit. The low level of DHA in the evaporated milks was consistent with the known heat lability of DHA (Hartman & Dryden, 1965). The high level of AA found in the fortified sterilized milks even after storage for a long period in the light could be accounted for by their low initial oxygen content (Ford, Porter, Thompson, Toothill & Edwards-Webb, 1969).

That the absorbance of other DNPH derivatives besides that of DHA was measured by the method of Roe et al. (1948) was evident from the shape of the absorption curves (Fig. 1) and also from the instability of the absorbance readings (Toothill et al. 1970). The determination of the absorbance values at 540 nm, as suggested by Mills & Roe (1947), rather than at the wavelength maximum of the DHA derivative, did not, however, overcome the effect of interfering substances. The inclusion of the chromatographic step generally resulted in lower values for AA and DHA and this decrease was due to the removal of interfering substances since mean recoveries of 97 and 100 % were obtained when 0·5 mg AA was added to 100 ml of reconstituted milk (Evap. B). Unfortunately, the chromatographic procedure is very time-consuming, but for products containing low levels of DHA and DOA, such as evaporated milks, it could be shortened by using only the oxidized extracts, which gives a combined value for AA + DHA + DOA. For the present series of evaporated milks this procedure would lead to a maximum error of 0·05 mg/100 ml reconstituted milk compared with a maximum error of 0·21 (Evap. D) if chromatography were omitted. For Ster. A and B, the error would be 0·27 and 0·42 mg/100 ml, respectively.

An isomer of AA, namely D-isoascorbic acid (IAA) also known as erythorbic or D-araboascorbic acid, is allowed in some countries, but not in the United Kingdom, as an antioxidant in foods. It has only about one-twentieth the antiscorbutic activity of AA (Smith, 1946). IAA has been reported to prevent the loss of AA in fruit juices (Esselen, Powers & Woodward, 1945) and to reduce the headspace oxygen in cans of apples (Hope, 1961). We have found that the chromatographic procedure used in the present work fails to separate the osazone of IAA from that of the naturally occurring form of AA and, in the unlikely event of IAA being added to milk, the DNPH method would require modification. Methods are available for separating the less stable free acids (Weeks & Deutsch, 1967).

The present work shows that of the 3 methods used for the determination of vitamin C in evaporated and fortified sterilized milk, that involving the chromatographic separation of the DNPH derivatives was the most specific.

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Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization

IV. The vitamin composition of milks sterilized by different processes

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SUMMARY. A comparison was made of the effects of direct and of indirect ultra-high-temperature (UHT) processing of milk, under standardized operating conditions giving equal sporicidal effects, on some of the more labile water-soluble vitamins and on vitamin A and carotene. The effects of processing *per se* were negligibly small, and the method of processing was important only in so far as the presence of residual oxygen in the sterilized milk has been found to cause losses of folic acid and ascorbic acid during storage subsequent to sterilization. The incorporation of a de-aerator vessel, to reduce the oxygen level in the indirectly heated milk and so eliminate the adverse effects of oxygen during storage, had no effect on the vitamin loss occurring during heat treatment. It is concluded that milk produced by indirect heating plant incorporating a de-aerator should be similar in vitamin content to milk produced on a direct heating plant, both immediately after processing and after storage.

We have already described the effects of ultra-high-temperature (UHT) processing and of subsequent storage on the vitamin composition of milk (Ford, Porter, Thompson, Toothill & Edwards-Webb, 1969). However, a true comparison of the effects of indirect and direct heating could not be made, as the milk samples were obtained from different commercial sources using non-standardized processing conditions, and manufactured from different bulk milk supplies. In the present experiments, these uncertainties have been avoided by the use of milk from a common bulk, and of a single plant operated alternately as a direct and as an indirect heater under conditions standardized to give equivalent heat treatments in terms of sporicidal efficiency. We now report a comparison of these 2 processes on some of the more labile water-soluble vitamins—namely, vitamin C, thiamin, folic acid, vitamin B_6 and vitamin B_{12}. In addition, vitamin A and carotene were measured because, in contrast to our earlier finding (Ford *et al.* 1969) that vitamin A is quite stable during UHT processing, Lembke, Frahm & Wegener (1968) reported appreciable losses.

The presence of dissolved oxygen has a determining influence on the stability of vitamin C, folic acid and vitamin B_{12} during in-bottle sterilization (Ford, 1957, 1967), and we have therefore examined the effects of the oxygen level in the milk during the indirect processing.

Table 1. *Comparison of the effects of direct and indirect processing on some of the water-soluble vitamins in milk*

Figures in parentheses indicate percentage loss

| Expt | Heat treatment | Vitamin C | | Thiamin μg/ml | Folic acid, μg/ml | Vitamin B_6, μg/ml | Vitamin B_{12}, μg/ml | Vitamin A, μg/g fat | Carotene μg/g fat |
		Ascorbic acid, mg/100 ml	Dehydro-ascorbic acid, mg/100 ml						
(1) 4. iii. 69	None	1·42	0·37	0·33	0·048	0·46	0·0033	6·4	2·8
	Indirect	1·20 (15)	0·07 (81)	0·33 (0)	0·039 (19)	0·43 (7)	0·0031 (6)	6·5 (0)	2·9 (0)
	Direct	1·28 (10)	0·00 (100)	0·34 (0)	0·047 (2)	0·43 (7)	0·0029 (12)	6·8 (0)	2·8 (0)
(2) 18. iii. 69	None	1·31	0·57	0·33	0·055	0·51	0·0037	6·5	2·8
	Indirect	1·26 (4)	0·08 (86)	0·33 (0)	0·053 (4)	0·42 (18)	0·0035 (5)	6·6 (0)	2·8 (0)
	Direct	1·21 (8)	0·15 (74)	0·30 (9)	0·056 (0)	0·42 (18)	0·0036 (3)	6·6 (0)	2·8 (0)
(3) 6. v. 69	None	1·47	0·46	0·27	0·047	0·53	0·0028	10·1	8·4
	Indirect	1·38 (6)	0·10 (78)	0·26 (4)	0·043 (9)	0·51 (4)	0·0028 (0)	10·3 (0)	8·4 (0)
	Direct	1·42 (3)	0·04 (91)	0·29 (0)	0·041 (13)	0·55 (0)	0·0020 (29)	10·3 (0)	8·4 (0)
Means	None	1·40	0·47	0·31	0·050	0·50	0·0033	7·7	4·7
	Indirect	1·28 (9)	0·08 (82)	0·31 (0)	0·045 (10)	0·45 (10)	0·0031 (4)	7·8 (0)	4·7 (0)
	Direct	1·30 (7)	0·06 (86)	0·31 (0)	0·048 (4)	0·47 (6)	0·0028 (13)	7·9 (0)	4·7 (0)

Milk supplies. For each experiment approximately 8000 l bulk milk collected from refrigerated farm tanks was delivered direct to the Institute and stored at 5–7 °C for 24 h. The milk was then mechanically stirred for at least 5 min, and a 2400-l portion transferred to an insulated tank from which the UHT plant was supplied. For comparisons of direct and indirect processing, 1200 l were processed during the morning by the direct method, and 1200 l during the afternoon of the same day by the indirect method.

Operation of the plant. A full description of the plant has been given by Burton & Perkin (1970). Before each run it was sterilized as described by Burton & Perkin (1970) and the required operating conditions were established. From the results of Franklin, Underwood, Perkin & Burton (1970) it was known that, for this plant, processing temperatures of 144 °C for direct heating and 141 °C for indirect heating gave equal sporicidal effects, and these temperatures were used throughout the experiment.

For the first 3 experiments (see Table 1) the plant was operated without the de-aerator vessel (Burton & Perkin, 1970), and the oxygen content of the milk during indirect processing was about 9 ppm. In a further 2 experiments to determine the effects of oxygen level during indirect processing (see Table 2) the plant was operated both with and without the de-aerator vessel which, when used, was operated at an absolute pressure of 52·4 kN/m² (0·517 atm absolute pressure) and reduced the oxygen content of the milk during indirect processing to less than 1 ppm.

Sampling. Milk samples were taken at about 45 °C into sterile clear glass milk bottles which were filled brim-full, tightly stoppered with rubber bungs, shielded from light, cooled and tested as quickly as possible for oxygen and vitamin C content. Subsamples for B-vitamin assay were taken and stored at −30 °C until required for assay. All the vitamin assays were completed within 2 weeks of processing the milk.

Vitamin assays. The methods of vitamin assay were all as described by Ford *et al.* (1969).

Measurement of dissolved oxygen. Oxygen tension was determined with a Clark-type oxygen cathode (Clark, 1956). The electrode and its water jacket were manufactured by Radiometer A/S (Copenhagen) and were used in conjunction with a Radiometer pH meter fitted with a gas monitor, model 27 GM. The scale was calibrated using water at 25 °C in equilibrium with atmospheric oxygen, and the zero was set with an oxygen-free solution prepared by dissolving 1 g sodium sulphite heptahydrate in 50 ml freshly boiled 0·01 M-borax solution. All measurements were made at 25 °C.

RESULTS

The results of the first 3 experiments are set out in Table 1. Processing, both direct and indirect, reduced the ascorbic acid content of the milk by an average of about 8 %, and largely destroyed the dehydroascorbic acid which contributed about 25 % of the vitamin C activity in the raw milk at the time of sampling. The thiamin content of the milk was unchanged. With folic acid the losses averaged 10 % in the indirect process and 4 % in the direct, but there were clear differences between experiments in the extent of the losses, which ranged from 4 to 19 % in the indirect process, and

Table 2. *Influence of the dissolved oxygen in milk on the stability of some vitamins during UHT processing by the indirect method*

Figures in parentheses indicate percentage loss

Expt	Milk	Oxygen ppm	Vitamin C Ascorbic acid, mg/100 ml	Vitamin C Dehydro-ascorbic acid, mg/100 ml	Thiamin, μg/ml	Folic acid, μg/ml	Vitamin B_6, μg/ml	Vitamin B_{12}, μg/ml	Vitamin A, μg/g fat	Carotene μg/g fat
(4) 13 v. 69	Control		1·45	0·71	0·33	0·033	0·53	0·0027	10·0	8·7
	Heated	9	1·35 (7)	0·06 (92)	0·34 (0)	0·030 (9)	0·50 (6)	0·0026 (4)	10·1 (0)	8·7 (0)
	Heated	< 1	1·41 (3)	0·08 (89)	0·30 (9)	0·030 (9)	0·40 (25)	0·0026 (4)	10·1 (0)	8·7 (0)
(5) 24 vi. 69	Control		1·52	0·24	0·32	0·040	0·46	0·0031	9·6	9·3
	Heated	9	1·31 (14)	0·02 (92)	0·33 (0)	0·039 (2)	0·46 (0)	0·0028 (10)	9·6 (0)	9·3 (0)
	Heated	< 1	1·52 (0)	0·04 (83)	0·33 (0)	0·041 (0)	0·46 (0)	0·0031 (0)	9·6 (0)	9·4 (0)

from 0 to 13 % in the direct. Similarly with vitamin B_6, there were differences between experiments in the extent of the losses, which averaged 10 % in the indirect process and 6 % in the direct. With vitamin B_{12} the indirect process caused an average loss of 4 %, as against the somewhat higher figure of 13 % in the direct process.

Table 2 shows the results of the 2 further experiments in which the influence of the dissolved oxygen was examined. The removal of oxygen further reduced the small loss of ascorbic acid but had otherwise no significant effect.

CONCLUSIONS

In general, the present results confirm our earlier conclusions (Ford *et al.* 1969) concerning the negligibly small effects of UHT processing on the vitamins in milk; such differences as were detected between directly and indirectly sterilized milks were of only marginal significance. It is probable that larger differences reported by earlier workers (cf. Burton, 1969; Rossikhina, Mastakov & Seleznev, 1969) were more apparent than real, and were in fact attributable to the use of a comparatively more severe heat treatment in the indirect process, or to the effects of storage. We conclude that the method of processing is of nutritional importance only in so far as it affects the losses of certain vitamins during storage subsequent to sterilization. There may be considerable losses of vitamin C, folic acid, vitamin B_6 and vitamin B_{12} during storage (Ford *et al.* 1969); vitamin C and folic acid may indeed disappear within a few days after processing. The stability of these last 2 vitamins is wholly determined by the level of residual oxygen in the sterilized milk and not by the method of processing *per se*. In milk processed by direct heating followed by expansion cooling, the oxygen content is very low and both vitamins are stable indefinitely. The indirect process, when employed without a de-aerator, gives a product that may be near-saturated in oxygen, and in which loss of vitamin C and folate proceeds rapidly.

However, it is known to be technically possible on a commercial scale to produce milk of very low oxygen content by the indirect process, by the use of a de-aerator, and such milk should be closely similar in its nutritional properties to that produced by the direct process.

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A comparison of the milking characteristics of transparent and conventional teatcup liners

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SUMMARY. The milking characteristics of 4 transparent and 8 conventional rubber teatcup liners in new condition were compared for peak flow rate, machine time and strip yield, using 30 cows in two 15×15 Latin squares. The milking characteristics of transparent liners were similar to those of normal liners at the same cluster weight. The 4 transparent liners were then compared with 4 of the conventional liners using 16 cows in two 8×8 Latin squares, to observe the movement of teats within the liners throughout milking. The various analyses indicated significant differences between liners but the ranked liner mean values showed the transparent liners widely distributed in the range. It was concluded that findings from studies of teatcup action using transparent liners could be expected to apply also to conventional liners.

The conventional liners were selected to form groups differing mainly in one physical characteristic only (stiffness of the mouthpiece, wall thickness, bore and rubber hardness). Within groups, there were no significant differences in milking properties of practical importance, but between groups there were small differences in machine time and strip yield. Two properties of the liners which appeared important in controlling movement of the teat into the liner were bore of the barrel and friction, bore having the greater influence at the beginning and friction being the dominant influence at the end of milking. With the transparent liners it could be seen that the end of the teat was frequently bathed in milk. Both with transparent and with conventional liners it was surprising how often the teat penetrated so deeply that complete collapse of the liner in each pulsation cycle was prevented.

Radiography (Pier, Schalm & Hage, 1956) and cine radiography (Ardran, Kemp, Clough & Dodd, 1958) have been used very effectively to study the action of the teatcup liner and its effect on the teat. Now that transparent liners are more readily available, a simpler alternative for some studies may be cine photography using transparent shells and liners.

The main object of the present experiments was to study the variation in milking characteristics of transparent and conventional rubber liners to find out whether conclusions obtained with transparent liners are applicable to rubber liners. Only 4 types of liners sufficiently transparent to be used in conjunction with cine photo-

graphy were found. They are shown in Plate 1, together with the 8 opaque rubber liners used for comparison. The selection of the conventional liners was made to obtain more information on liner design in relation to milking characteristics.

The transparent liners were compared with the rubber liners in 2 experiments: (1) the peak milk flow rates, machine times (from the start of milk flow to the time when milk flow rate fell below (i) 0·25 kg/min and (ii) 0·05 kg/min), and strip yields (after milk flow rate had fallen below (i) 0·25 kg/min and (ii) 0·05 kg/min) were measured for all liners; and (2) the mean depth of teat penetration into each liner at the beginning of milking and subseqent changes in position of the teat were observed during milking in each of the 4 transparent liners and 4 of the normal liners.

METHODS AND RESULTS

The cows were milked in a 5-unit, 10-stall herring-bone parlour equipped with a recorder type of milking machine in which the vacuum level was maintained at 380 mmHg in the vacuum supply line near the sanitary trap (for terminology see Thiel, Claesson & Rabold, 1969). Pulsation rate was 58 ± 2 c/min. Alternate pulsation

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EXPLANATION OF PLATE

Transparent and conventional liners used. All liners were used at a cluster weight of 2·7 kg (6·0 lb) and given the treatment letters A–L. In addition, the first 3 transparent liners were used at a lighter cluster weight of 2·0 kg (4·5 lb) and designated. A1, B1 and C1.

Treatment letter		Liner	Supplier
Top left:	Group 1	Four transparent liners. (a) 3 of them (A1, B1, C1) used at a light cluster weight; (b) all 4 at the common cluster weight.	
(a)	(b)		
A1	A	'Transflow' (transparent PVC)	Norton International Inc., Akron, Ohio, U.S.A.
B1	B	'Zero' (transparent PVC)	Zero Manufacturing Co., Washington, Missouri, U.S.A.
C1	C	'Surge' (translucent silicone rubber)	Babson Bros., Co., Oakbrook, Illinois, U.S.A.
	D	Un-named (transparent silicone rubber)	Takeda Chemical Industries, Doshomachi, Higashiku, Osaka, Japan.
Top right:	Group 2	Two moulded rubber liners differing mainly in stiffness of the mouthpiece.	
	E	Milkrite-Skellerup MM 10/11	J. A. C. Kingston and Sons Ltd,
	F	Milkrite-Skellerup MM 10	Reading, Berks., U.K.
Bottom left:	Group 3	Three moulded rubber liners differing mainly in wall thickness of the barrel.	
	G	Alfa–Laval 7271330	Alfa–Laval Co., Ltd, Cwmbran, Mon., U.K.
	H	Milkrite–Skellerup MM 65	
	I	Milkrite–Skellerup unmarked (a thicker-walled version of H)	J. A. C. Kingston and Sons Ltd.
Bottom right:	Group 4	Two extruded rubber liners (J, K) differing mainly in bore.	
	Group 5	Two rubber liners (K, L) differing mainly in rubber hardness.	
	J	Alfa–Laval 22048B	
	K	Alfa–Laval 20003B	Alfa–Laval Co., Ltd
	L	Milkrite–Skellerup MM 98	J. A. C. Kingston and Sons Ltd.

Plate 1

G. A. MEIN, P. A. CLOUGH, D. R. WESTGARTH AND C. C. THIEL

was used. The pulsator ratio was 75 % giving a pulsation ratio of 65 % (liner more than half open for 65 % of each pulsation cycle measured with the liners stoppered). During milking, the outlet of the claw was about 910 mm below the level of the milk inlet to the recorder jar. The claws (Alfa-Laval No. 24933) were fitted with plain rubber bungs. An air admission hole, drilled through the metal wall of each claw, admitted 5 l/min free air. Each shell (Alfa-Laval No. 24932) was cut radially into 2 sections and modified to accept a screwed brass sleeve to enable liner tension to be adjusted (Fig. 1). The same proprietary short milk tube was fitted to all liners. This meant that the effective length of the barrels of some liners was reduced by up to 10 mm. The bore of the short milk tube contracted from 9 to 7 mm where it passed through the shell.
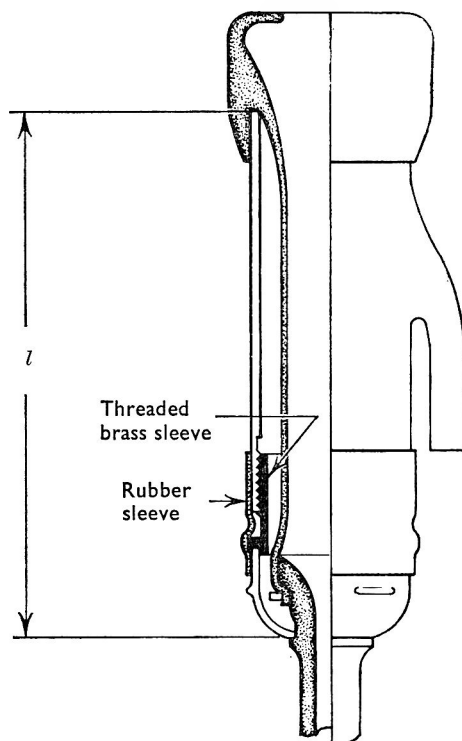
Fig. 1. The split shell in which all liners were assembled for the first experiment. The effective length l, could be adjusted by means of the threaded brass sleeve. The thin rubber sleeve formed an airtight seal.

Milking performance of transparent and conventional liners

Some of the physical characteristics of the liners are given in Table 1. The transparent liners differed in several respects, their main common characteristic simply being optical transparency. The opaque liners E and F differed by 23 % in thickness (and hence stiffness) of the mouthpiece. Liners G, H and I differed mainly in wall thickness of the barrel. The extruded liners J and K differed mainly in bore (16 %). They were assembled so that the size and shape of their mouthpieces were the same. The remaining liner L, similar in dimensions to K, was made from a less hard rubber.

Liner tension. There was no obvious method of unifying liner tension as some of the

Table 1. *Some physical characteristics of the 12 liners*

(Within each of the 4 groups of conventional liners: | represents a 5–10% difference between liners in a property; || represents 10–20% difference; ||| represents a difference greater than 20%.)

Treatment letter	Liner	Bore of barrel at midpoint mm (in.)		Wall thickness of barrel mm (in.)		Tension at 10% stretch kg (lb)		Bore of barrel at 10% stretch mm (in.)		Diameter of mouthpiece lip mm (in.)		Coeff. of friction measured on teats
					Group 1 (*a, b*)—Transparent liners							
A1 A	Transflow	18·3	(0·72)	2·62	(0·103)	2·7	(6·0)	17·3	(0·68)	20·3	(0·80)	0·35
B1 B	Zero	18·8	(0·74)	1·70	(0·067)	3·2	(7·0)	17·8	(0·70)	19·1	(0·75)	0·33
C1 C	Surge	19·8	(0·78)	2·16	(0·085)	1·8	(4·0)	19·1	(0·75)	21·3	(0·84)	0·53
D	Japanese	25·9	(1·02)	2·54	(0·100)	2·9	(6·5)	24·9	(0·98)	26·4	(1·04)	0·35
				Group 2—Moulded rubber liners differing mainly in stiffness of mouthpiece								
E	M-R MM 10/11	21·8	(0·86)	2·54	(0·100)	4·1	(9·0)	21·1	(0·83)	19·3	(0·76)	0·42
F	M-R MM 10	21·8	(0·86)	2·54	(0·100)	3·6	(8·0)	20·6	(0·81)	19·3	(0·76)	—
				Group 3—Moulded rubber liners differing mainly in wall thickness								
G	A-L 7271330	23·9	(0·94)	2·42	(0·095)	4·5	(10·0)	23·1	(0·91)	23·9	(0·94)	0·35
H	M-R MM 65	24·6	(0·97)	1·98	(0·078)	4·0	(8·8)	23·6	(0·93)	23·6	(0·93)	—
I	M-R unmarked	23·4	(0·92)	2·80	(0·110)	5·4	(11·8)	22·6	(0·89)	23·6	(0·93)	—
				Group 4 (J, K)—Extruded rubber liners differing mainly in bore								
				Group 5 (K, L)—Liners differing mainly in rubber hardness								
J	A-L 22048B	18·6	(0·73)	2·80	(0·110)	3·6	(8·0)	17·8	(0·70)	21·3	(0·84)	0·64
K	A-L 20003B	21·3	(0·84)	2·64	(0·104)	3·6	(8·0)	20·6	(0·81)	21·3	(0·84)	0·62
L	M-R MM 98	20·6	(0·81)	3·05	(0·120)	1·8	(4·0)	20·1	(0·79)	21·3	(0·84)	—

liners were designed to be used under tension and others slack. The variation in wall thickness between liners G, H and I, and the difference in rubber properties between liners K and L, were reflected almost entirely in their variation in tension at constant stretch. It was felt that a comparison of these liners at a standard tension could mask any influence of their main physical differences on milking performance. As a compromise, all liners were used at a constant 10 % stretch. Accordingly, all liners were assembled in split shells (see Fig. 1), the lengths of which were adjustable. Length of individual liners was measured between the points at which the liner was restrained in the shell. Liner tension at 10 % stretch was measured (to plus or minus 0·1 kg) using a spring balance to determine the pull required to separate the 2 halves of the split-cup assembly. Two or three days after they were first assembled, the tension of all liners had declined to the steady values shown in Table 1. Tension was checked and maintained at these levels throughout the experiment. Very little adjustment was required because each liner milked only 2 cows/day and clusters were washed in a lukewarm non-caustic alkaline detergent and rinsed in cold hypochlorite solution.

Cluster weights. With conventional liners, the main effect of increasing cluster weight is to reduce strip yields (Dodd & Henriques, 1949; Clough, 1964; Report, 1965). No information was available on the effect of cluster weight on strip yields for the transparent liners. Because they were supplied with light-weight transparent plastics shells, it was decided to operate them at 2 cluster weights, 2·0 and 2·7 kg (4·5, 6·0 lb). It was not possible to include liner D at both cluster weights, however, as only 4 of these liners could be obtained prior to the experiment. The 8 types of opaque liners were assembled in clusters having an effective weight of 2·7 kg. To measure effective cluster weight, the 4 teatcups of each cluster were suspended at average udder height in a wire frame attached to a spring balance. Hence, the effective cluster weight included part of the weight of the long-milk and long-air tubes.

Experimental design. Fifteen cows in their first or second lactation and 15 mature cows were chosen from a herd of 75 Friesian cows as having consistent milk yields and a wide range of peak milking rates (2·0–6·4 kg/min). To enable the selection to be made, cumulative milk yields were recorded every 0·5 min during 4 milkings for each animal.

The 15 treatments (8 opaque rubber liners and 4 transparent liners in clusters at 2·7 kg, plus 3 of the transparent liners in clusters at 2·0 kg) for the 30 cows were randomized in two 15 × 15 Latin squares, one being for the group of young cows and the other for mature cows. Each cow was milked once with each cluster during 15 consecutive morning milkings, the cows being milked in a similar order at each milking. At the evening milking they were milked with the clusters normally used in the parlour, to reduce possible residual effects between the experimental clusters. Measurements of peak flow, machine time, strip yield and total yield were made by observing the amount of milk in each of the recorder jars at intervals of 0·5 min. Machine stripping was started only after the milk flow rate had fallen below 0·05 kg/min and was continued until the flow rate had again fallen below this level. A similar experimental design devised by P. A. Clough has been used previously at this Institute (Report, 1965).

Results. Data for both groups of cows were pooled because there were no significant interactions of liners and their milking characteristics between squares. The ranking

Table 2. *Ranked liner mean values of milking characteristics*

Total yield (kg/milking) — S.E. of a single liner mean (364 D.F.): 0·165

G	J	L	A	B	Bl	K	E	D	A1	F	I	H	C	Cl
11·37	11·36	11·34	11·25	11·25	11·24	11·17	11·17	11·06	11·05	11·04	11·01	10·99	10·90	10·85

Peak flow rate (kg/min) — S.E. 0·075

G	Cl	E	H	F	I	B	C	K	A1	L	D	Bl	J	A
3·72	3·71	3·70	3·69	3·66	3·65	3·64	3·64	3·63	3·62	3·62	3·59	3·59	3·55	3·47

Machine time to 0·25 kg/min (min) — S.E. 0·180

J	F	A1	Bl	B	G	K	D	A	E	Cl	L	C	H	I
6·03	5·65	5·63	5·57	5·55	5·45	5·43	5·43	5·40	5·38	5·33	5·30	5·23	5·07	5·07

Machine time to 0·05 kg/min (min) — S.E. 0·204

J	A1	K	F	A	L	Bl	D	B	G	Cl	C	E	H	I
6·85	6·60	6·43	6·38	6·35	6·35	6·33	6·32	6·25	6·20	6·17	6·17	6·17	5·82	5·77

Strip yield below 0·25 kg/min (kg/milking) — S.E. 0·046

Cl	Bl	A1	A	B	E	C	F	D	K	L	I	H	J	G
0·62	0·59	0·56	0·49	0·46	0·45	0·44	0·41	0·40	0·39	0·38	0·38	0·35	0·32	0·32

Strip yield below 0·05 kg/min (kg/milking) — S.E. 0·042

Cl	Bl	A1	B	A	E	C	F	D	I	K	L	H	G	J
0·51	0·49	0·44	0·37	0·35	0·34	0·33	0·31	0·29	0·28	0·27	0·26	0·24	0·23	0·21

Liner mean values not underscored by the same line differ significantly ($P < 0.05$).

orders of the liners for each characteristic are given in Table 2. No significant differences between liners for either total yield or for peak milk flow rate were detected. The mean peak flow rates for the transparent liners were spread throughout the remarkably limited range of means of all liners, with no evidence of a significant effect due to cluster weight. Significant differences between liners for milk yield were not expected in this short-term experiment.

There was a difference of 19 % in machine time between the slowest and the fastest milking liners. Machine time was measured only to the nearest 0·5 min, however, and the sole significant difference to a stripping point of 0·25 kg/min was between the slowest liner J and the 10 fastest liners. For machine time to 0·05 kg/min, only the 2 slowest milking liners J and A 1 differed significantly from the 2 fastest liners H and I. In both analyses for machine time, the means for the transparent liners were again spread throughout the range of means for all liners, with no evidence of a significant effect due to cluster weight.

Table 3. *Comparisons between groups of liners for milking characteristics*

Group 1a, transparent liners A1, B1 and C1, at lower cluster weight; group 1b, transparent liners A, B, C and D; group 2, moulded liners E and F; group 3, moulded liners G, H and I; group 4, extruded liners J and K; group 5, liners K and L

| Total yield, kg/milking | | Peak flow rate, kg/min | | Machine time, min | | | | Strip yield, kg/milking | | | |
				to 0·25 kg/min		to 0·05 kg/min		Below 0·25 kg/min		Below 0·05 kg/min	
4	11·27	3	3·69	4	5·73	4	6·64	1a	0·59	1a	0·48
5	11·26	2	3·68	2	5·52	5	6·39	1b	0·45	1b	0·34
3	11·12	1a	3·64	1a	5·51	1a	6·37	2	0·43	2	0·33
1b	11·11	5	3·63	1b	5·40	1b	6·28	5	0·39	5	0·26
2	11·10	4	3·59	5	5·37	2	6·27	4	0·36	3	0·25
1a	11·05	1b	3·58	3	5·19	3	5·93	3	0·34	4	0·24

s.e. of a single liner mean (364 D.F.)

| ±0·165 | ±0·075 | ±0·180 | ±0·204 | ±0·046 | ±0·042 |

Mean values are averaged over all cows.
Those not scored by the same vertical line differ significantly ($P \leqslant 0.05$).

In both analyses for strip yield the 3 transparent liners used at the lower cluster weight (A 1, B 1, C 1) left 0·13 kg more strippings on average than the same 3 liners (A, B, C) used at the higher cluster weight ($P < 0.05$). Of all liners tested at the higher cluster weight, the same 3 liners A, B and C were grouped together near the upper end of both ranking orders of strip yield.

In all the analyses of milking characteristics, the means for the transparent liner D were near the centre of the ranking orders of all the conventional liners. Within each of the groups of liners, the only significant difference was in machine time to 0·25 kg/min for the extruded liners K and J. Comparing groups of liners (Table 3), liners G, H and I milked cows in the shortest time and left the least strippings. The extruded liners also left very little milk behind, but they had a significantly longer machine time. As a group, the transparent liners A, B, C and D left a significantly higher amount of strippings than either the extruded liners or the moulded liners, G, H and I. The numerical differences in strip yield between the transparent liners and normal liners at the same cluster weight were small, however. When clusters of the same weight

were left unattended on cows until milk ceased to flow, the liner leaving the least strippings obtained 98 % of the available milk, and that leaving the most obtained nearly 97 %.

Movement of teats in transparent and conventional liners during milking

The 4 transparent liners were used at the higher cluster weight only (A, B, C, D). Four conventional liners (E, G, J, K) were selected from the 8 used in the first experiment. One moulded rubber liner from each of groups 2 and 3 was included, since there were no significant differences in milking performance between liners within each of these groups. Both extruded rubber liners in group 4 were included, as there was a significant difference in milking time between them. There were no significant differences between the rubber liners in group 5 and one of these 2 liners had already been selected, being common to 2 groups.

Sixteen cows in two 8×8 Latin squares were selected from those used in the first experiment, to include cows having a wide range of strip yields and a wide variation in teat size. Cows in their first or second lactation were again allocated to one group and mature cows to the other. All cows were milked once with each of the 8 experimental clusters during 8 consecutive p.m. milkings.

The machine conditions, including liner tension, were the same as those of the first experiment except that transparent plastics shells, suitably weighted, were substituted for the pair of metal shells nearest to the long milk tube in each cluster. They were marked with thin rings of self-adhesive coloured tape at intervals of 10 mm so that the depth of penetration of the teat into the liner could be estimated, for the 2 teats of each cow nearest to the observer, at 1-min intervals throughout milking. Observations for all experimental clusters were made on the same half of each cow's udder. The depth of teat penetration was estimated by observing the shape of the liner when collapsed. Comparing the results with visual observation of teats in the transparent liners, it was apparent that estimates with the opaque liners could be made to within 5 mm. Preliminary observations showed that the repeatability of depth of penetration of the same teat into the same liner was good. In a total of 56 pairs of such observations at consecutive p.m. milkings, 35 pairs were identical, 19 differed by 10 mm, and 2 pairs differed by 20 mm. In measuring depth of penetration, allowance was made for the height of the mouthpiece lip of each liner above the top of the shell during milking. Usually, this height declined throughout milking as the mouthpiece lip became more depressed.

Other measurements made besides depth of teat penetration at 1-min intervals were: movement of the liner mouthpiece along the teat, by marking the teat at its point of contact with the mouthpiece immediately after the first observation of depth of teat penetration and then just before the cluster was removed; extent of collapse of the liner each time that depth of teat penetration was estimated; and, for the transparent liners, the occasions when the barrel of the liner below the teat was filled with milk so that the tip of the teat remained continuously bathed in milk, i.e. flooding of the liner.

Frictional properties of liners. The coefficient of static friction between cows' teats and each of the liners was measured under a variety of conditions. By means of a spring of known tension, teats were squeezed between 2 flat plates to which pieces of

liners were glued. The force necessary to overcome the static friction between the teat and each pair of liner sections was measured, using a small spring balance to pull the plates along the teat.

Measurements were made with teats wetted with water and liners wetted with milk as this was felt to be most closely related to conditions during milking. Results were rather variable. The range of values for coefficient of static friction of the most slippery liner B was 0·20–0·45 (mean 0·33), and for the least slippery liner J, 0·50–0·77 (mean 0·64). The coefficient was also measured between a weighted part of each liner and human skin (the palmar surface of a finger). The liner sections were dipped in milk and the finger dipped in water. Results were highly correlated to the mean values obtained with teats ($r = 0.99$). The regression of frictional values obtained with teats (y) and with a finger (x) was $y = 0.27 + 0.35x$.

Table 4. *Ranked liner mean values for depth of teat penetration into the liners* A–E, G, J and K

									S.E. of a single liner mean (42 D.F.)
Teat penetration at start of milking, mm									
Young cows	J	K	A	E	B	C	G	D	
	60·6	61·9	63·8	63·8	65·6	67·5	69·4	73·1	1·57
Mature cows	J	A	E	K	B	C	G	D	
	53·1	64·4	64·4	69·4	70·0	71·9	72·5	76·9	2·10
Teat penetration after 4 min of milking, mm									
Young cows	J	C	K	A	E	D	G	B	
	73·1	78·1	80·0	82·5	83·8	86·9	87·5	88·1	1·25
Mature cows	J	E	A	K	C	G	B	D	
	70·6	86·3	87·5	87·5	88·8	93·1	94·4	97·5	1·71
Increase in teat penetration during milking, mm									
Young cows	C	J	D	G	K	A	E	B	
	10·6	12·5	15·0	18·1	18·1	18·8	19·4	22·5	1·81
Mature cows	J	K	C	D	E	G	A	B	
	17·5	18·1	18·8	20·6	21·9	21·9	23·1	23·1	2·34

Liner mean values not underscored by the same line differ significantly ($P < 0.05$).

Results. The ranking orders of all liners for teat penetration are given in Table 4. In all of these analyses, the means for the transparent liners were spread uniformly amongst the means for the conventional liners. The differences between liners for mean depth of teat penetration, whether measured at the beginning or at the end of milking, were highly significant for both groups of cows. There were also significant differences between liners for the increase in teat length in the liner during milking, but with the young cows only. There was some evidence of interactions between liners and squares for mean depth of teat penetration at the beginning and at the end of milking, but this variation was small compared with the variation between different liners.

Table 5. *Depth of penetration of teats into liners and effect of this on pulsation*

Characteristic	PVC liners		Silicone rubber liners		Moulded rubber liners		Extruded rubber liners	
	A	B	C	D	E	G	J	K
Mean depth of penetration of teat into liner, mm								
Start	64	68	70	75	64	71	57	66
2 min	79	82	80	90	80	85	66	81
4 min	85	90	84	93	85	91	72	84
Increase in teat penetration throughout milking, mm	21	22	14	18	21	20	15	18
Movement of teatcup up teat, mm	14	14	10	11	11	9	10	10
Occasions when increase in teat penetration was 20 mm or more between consecutive observations (total 128/liner)								
Start–1 min	7	7	0	4	5	4	1	2
1–2 min	2	3	1	2	2	0	1	3
2–3 min	2	2	0	0	2	2	2	0
3–4 min	1	0	0	0	0	0	0	2
Number of teats (total 32) on which liner collapsed less than halfway or, in brackets, hardly moved								
Start	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2 min	8 (2)	4 (2)	1 (1)	4 (1)	3 (3)	1 (0)	1 (0)	3 (0)
4 min	10 (8)	9 (9)	1 (1)	4 (3)	4 (3)	1 (0)	3 (0)	3 (2)
Mean minimum penetration of teat into liner when it closed less than halfway, mm	88	92	95	103	95	95	92	95
Length of liner barrel below teat when it closed less than halfway, mm	22	23	20	22	25	30	28	20

Further results of this experiment are summarized in Table 5, in which data for both groups of cows have been pooled.

The depth to which teats penetrated into all of the liners increased throughout milking, the mean increase being 8 mm in the first min, 5 mm in the second, 4 mm in the third and 2 mm in the fourth. The mean depth of teat penetration, averaged over all liners, at the end of milking was 83 mm for the young cows and 88 mm for the mature cows—an increase of almost 30 % in teat penetration throughout milking for both groups of cows. Increases in teat penetration of 20 mm or more/min occurred in less than 6 % of all observations. Such increases were more frequent during the first minute of milking. Teats seldom increased in length after milk flow had ceased.

In all the liners, movement of the teatcup up the teat accounted for at least half the increase in depth of teat penetration throughout milking (Table 5). This measurement was made on the unstressed teats as soon as clusters were removed and therefore it could be underestimated by up to perhaps 50 %. The range of relative movement was 0–30 mm for most liners.

Table 6. *Percentage variation in mean depth of teat penetration accounted for by 2 physical properties of the liners A–E, G, J and K*

| | Variation in depth of penetration accounted for, % | | |
Characteristic	At start of milking	After 2 min	After 4 min
Bore	40	43	26
Friction	20	36	59
Bore and friction	48	65	73
Bore/friction	46	60	72

Differences in mean depth of teat penetration into the different liners may be partly explained by bore and coefficient of friction. Simple linear regressions relating teat penetration to bore, coefficient of friction, and the ratio of bore to coefficient of friction were examined, as well as the multiple regression on bore and friction. Table 6 gives the percentage variation in teat penetration between liners associated with each form of regression for the start, middle and end of milking.

At the beginning of milking the more important single characteristic was bore (accounting for 40 % of the variation); the multiple regression was only slightly better (48 %) and the improvement arising from the inclusion of coefficient of friction was not significant. At the end of milking the position was reversed with coefficient of friction dominant (59 %), the addition of bore giving a non-significant improvement for the multiple regression (73 %). At mid-stage, the influences of bore and coefficient of friction were similar and the multiple regression accounted for significantly more of the variation in teat penetration than either characteristic alone.

A simpler description of variation in teat penetration of different liners was obtained by choosing the ratio of bore to coefficient of friction as the independent variate. At each of the 3 stages of milking the simple regression of teat penetration on this ratio was significant (Fig. 2) and the ratio was very nearly as effective as the multiple regression in accounting for variation in penetration (Table 6).

Liner J was the only one in which the mean length occupied by teats of the mature cows was less than that of the young cows. With its small bore and high coefficient of

friction it seemed unable to accept much more than the tip of the larger teats. If liner J is excluded, then 80 % of the total variation in mean depth of teat penetration into the liners can be accounted for by the simple relationship of bore/coefficient of friction.
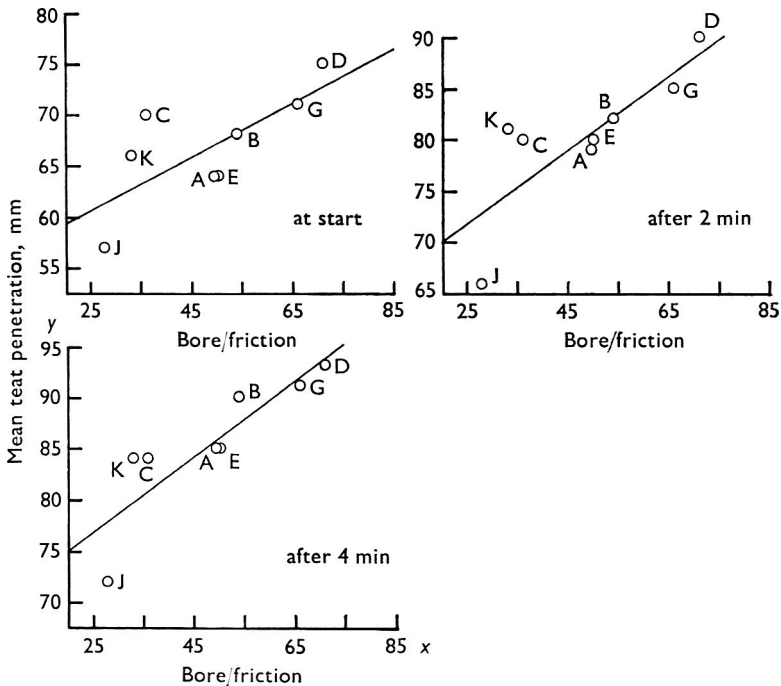
Fig. 2. Relationship between mean depth of teat penetration into the liner and the ratio of bore of liner (mm) to coefficient of friction measured between teats and the various liners, at the start and after 2- and 4-min milking. Regressions: start, $y = 54 + 0.26^*x$; after 2 min, $y = 63 + 0.36^*x$; after 4 min, $y = 68 + 0.36^{**}x$. Levels of significance: $^*(P < 0.05)$; $^{**}(P < 0.01)$. For identification of liners from the code letters see Plate 1.

All liners failed to collapse completely on one or more of the 32 teats on which they were observed, because of obstruction by the teat (Table 5). On 5 of the 8 mature cows and 3 of the 8 young cows the liners were unable to collapse more than halfway in at least one of the experimental clusters for this reason. On one cow the liners in 6 of the 8 clusters failed to collapse completely, and on 2 others 5 of the 8 clusters were similarly affected. The fact that some liners had to be shortened (by up to 10 mm) no doubt increased the incidence of such failure. The shortened PVC liners A and B were unable to collapse on nearly one-third of all teats. For liners assembled under lower tension (see Table 1), the liner first failed to collapse more than halfway when the teat was about 20 mm from the bottom. Liners under higher tension first failed to collapse when the teats were about 30 mm from the bottom.

In the transparent liners, it was easy to see that the tips of more than half of the 128 teats observed were continuously bathed in milk at some stage during milking. The incidence of flooding in the transparent liners (37 % of all observations at the start of milking, 58 % after 1 min, 19 % after 2 min, and 5 % after 3 min) was greatly increased by the use of the proprietary short-milk tube which was fitted to all experi-

mental liners. Although the bore of this tube was much smaller than that of each of the integral short-milk tubes removed from the transparent liners, it is a size commonly used with conventional liners.

These results suggest that conclusions from studies of teatcup action using transparent liners may reasonably be expected to apply also to conventional liners. At least for liners in new condition, the milking characteristics of the 2 types and the behaviour of the teat in the liners were similar. The results do not prove that the dynamics of the action of the 2 are identical but there was no indication that the mode of action of transparent liners is strikingly different from that of conventional opaque liners.

As a group, the transparent liners occupied the middle of the range of machine times of the 4 groups of conventional opaque liners, but tended to have higher strip yields. However, one of the transparent liners, D, was near the middle of the ranking orders of the conventional liners for strip yield so that this tendency can hardly be said to be a general property of the transparent liners used. It is also interesting that the main effect of operating 3 of the transparent liners at 2 cluster weights was to give increased strippings at the lower weight, as is the case with conventional liners. Again this tends to emphasize the similarity of action of transparent and conventional liners.

Because of the different materials of construction of transparent and conventional liners, it was felt that they might differ in their frictional properties. The figures for coefficient of static friction in Table 1 are too few to indicate whether there is a class difference. However, the depth to which teats penetrated into liners for all causes, including differences in frictional properties, showed no relationship with the property of transparency (Table 4).

Turning to the properties of the whole range of liners used, the absence of any significant differences between liners for peak milk flow rate was surprising, considering the range of physical characteristics involved. Peak flow was a sensitive and repeatable measurement, this comparison being precise enough to claim as significant a difference of less than 0·25 kg/min. This is equivalent to a difference of about 6 % between 2 liners for peak flow rate. In fact, the first experiment provided data with a notable absence of significant differences between liners although, between groups of liners, there were small differences in machine time and strip yield. The range of mean values for all milking characteristics was small and, within groups, no differences were detected for stiffness of the mouthpiece, wall thickness of the barrel, and rubber hardness. Within all groups of liners, the sole significantly different milking characteristic was in machine time for the 2 extruded liners J and K, which differed mainly in their bore. The small-bore liner J was intended for milking small-teated breeds of cows and the fact that it milked this group of Friesians more slowly than liner K was not unexpected.

Two properties of the liners which appeared important in controlling movement of the teat into the liner were bore of the barrel and frictional properties. After the teatcup is applied at the beginning of milking, the teat moves into the liner in response to the force due to the reduced pressure within the barrel of the liner. The

teat will continue to move into the liner until there is an equilibrium of all the forces acting on it. As Thiel (1969) suggests, frictional forces must be present since the teat comes to rest in relation to the liner even if some of the parallel-sided teat is visible above the mouth of the liner. Therefore, it is not surprising that teats penetrate further into the more slippery liners, i.e. those having a low coefficient of friction.

The figures in Table 6 suggest that bore of the liner has a greater influence than friction in determining depth of teat penetration into the liner at the start of milking, but that their relative importance is reversed by the end of milking. It was anticipated that friction would rapidly become the dominant factor influencing teat movement in a given liner, but these results suggest that the change is gradual, the effect of bore being relatively unchanged after 2 min of milking. It is difficult to see why the change occurs. Perhaps it is related to changes in milk ejection pressure although teat movement in the liner was greater and more frequent during the first part of milking when the milk ejection pressure would have been relatively constant.

Failure of pulsation, in the sense that the liner was partially or completely prevented from closing because the teat had penetrated almost to the bottom of the barrel, was surprisingly common. Teats, which were only 70 mm in length when measured after milk ejection and just before the teatcup was applied, usually penetrated deeply enough to prevent collapse of the shorter liners. For this particular group of liners, the length of the barrel was reduced slightly but, even so, their lengths were similar to those of liners in current use. Although little information is available defining effective pulsation, one might reasonably expect that the liner should at least close fully below the teat throughout milking.

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Contents

No. 1 (February 1970)

No. 2 (June 1970)

No. 3 (October 1970)

ORIGINAL ARTICLES

Index of Subjects

Index of Authors

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CONTENTS